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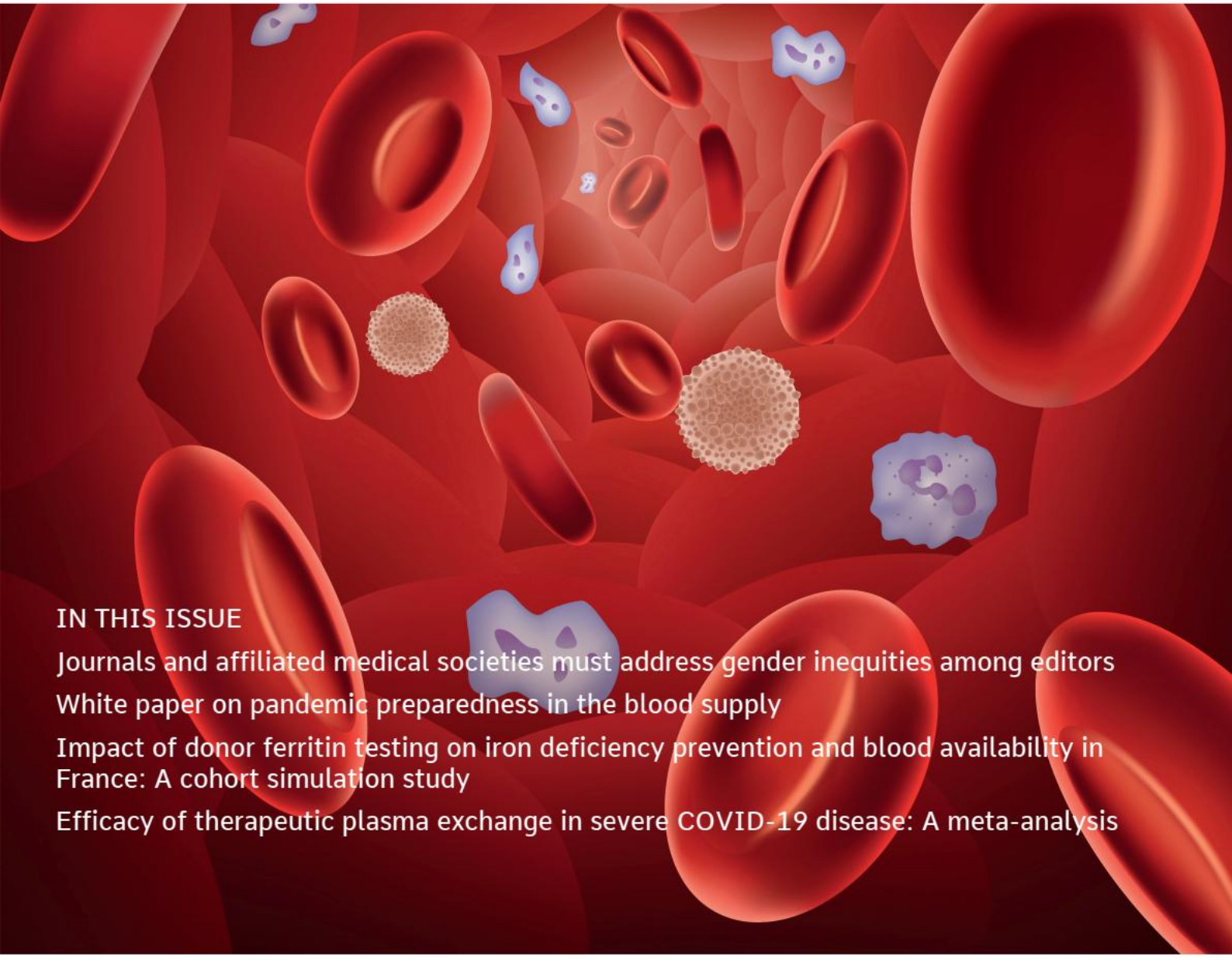

IN THIS ISSUE

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International Journal of Blood Transfusion

Official Journal of the International Society of Blood Transfusion

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Vox Sanguinis

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 10 main sections:

1. Blood Component Collection and Production: Blood collection methods and devices (including apheresis); blood component preparation; inventory management; collection and storage of tissues; quality management and good manufacturing practice; automation and information technology; plasma fractionation techniques and plasma derivatives;
2. Cellular Therapy: Cell-based therapies; CAR T-cell therapies; genetically modified cell therapies; cellular therapy (sources; products; processing and storage); stem cells; cell-based regenerative medicine; cellular immunotherapy; molecular therapy;
3. Donors and Donations: Donor recruitment and retention; donor selection; donor health
4. Haemovigliance: Adverse events in blood and blood component donors and transfusion recipients; corrective and preventive measures of complications; near-misses and errors in the transfusion chain; evaluation and outcomes of adverse events
5. Immunohaematology and Immunogenetics: autoimmunity in haematology; alloimmunity of blood; pre-transfusion testing; complement in immunohaematology; blood phenotyping and genotyping; genetic markers of blood cells and serum proteins: polymorphisms and function; parentage testing and forensic immunohaematology;
6. International Forum: Section in which topics related to any aspects of the transfusion chain (from technical to scientific, including socioeconomic and ethical facets) are discussed by invited participants and summarized by guest editors
7. Patient Blood Management: Bloodless surgery; preoperative anaemia; minimizing blood loss during surgery; alternatives to blood transfusion;
8. Review: Comprehensive reviews or short comments of any aspect related to transfusion medicine;
9. Transfusion Medicine: Transfusion indication, transfusion practice, thresholds and audits; transfusion efficacy assessment, clinical trials; therapeutic apheresis;
10. 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;

This comprehensive coverage has made the journal essential reading for a wide range of specialists interested in the present state of transfusion research and practice.

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REVIEW

Vox Sanguinis ISBT International Society of Blood Transfusion

What influences decisions to donate plasma? A rapid review of the literature

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Abstract

Background and Objectives: Plasma has become an essential ingredient for various medical treatments. Many blood collection agencies rely on voluntary non-remunerated donation when collecting plasma, but at present many do not collect sufficient plasma to meet domestic demands. This rapid review sought to explore the factors that have been found to influence people's decisions to donate plasma to inform future research.

Methods: Searches were conducted in PubMed, PsycINFO, Social Sciences Citation Index and CINAHL for peer-reviewed journal articles that discussed plasma donation and the factors associated with donor behaviour. Pertinent information from included articles was extracted and arranged in themes.

Results: In total, 33 articles were included in this review. Three main themes were identified by the authors. The first focused on site-level factors related to blood collection agencies' engagement with plasma donors and their influence on plasma donation experiences. The second theme considered how individual characteristics and experiences influence willingness to donate plasma. The third theme examined social and cultural-level factors, such as how social networks and community shape perceptions and experiences with donation.

Conclusion: Our findings suggest that the current understanding of plasma donation is focused mainly on converting whole blood donors and also centres on individual-level factors to donation. Further research must examine what factors attract non-whole blood donors to become plasma donors, focusing on broader social-level influences. This review will inform policies and interventions for blood collection agencies to increase plasma donors.

Keywords

donor motivation, donor retention, donors, plasma

Highlights

- This rapid review explores what influences people's decision to donate plasma. Most studies focus on existing whole blood donors, examining individual-level factors such as intention, altruism and fear surrounding the donation process.

- There is little literature on non-whole blood donors or exploring social and cultural factors such as community influences and accessibility for diverse groups.
- Given that the findings show a lack of research on the influences of ethnicity, socioeconomic status and gender on plasma donation, specifically with non-donors, future studies should explore these factors in more depth.

INTRODUCTION

Plasma protein therapies are vital for patient recovery in clinical settings [1]. Plasma forms the treatment for patients with autoimmune disorders, immune deficiencies, rare blood disorders, various cancers, tetanus infections, nervous system disorders and so forth [1]. It can also be transfused directly into patients in hospitals or manufactured into specialized medicines called plasma protein products [2]. Due to the increasing use of these plasma protein products, the demand for plasma collection has increased globally [3].

Many blood collection agencies worldwide rely on voluntary, non-remunerated donations when collecting plasma. However, most fail to collect sufficient plasma to meet demand. Therefore, many countries purchase plasma from the international market, which often includes plasma from countries where people are paid to donate. While international purchasing allows countries to provide care, it raises concerns about the security of the supply. These concerns have been exacerbated during the COVID-19 pandemic, which has led to increasing costs of plasma products and disruptions to the supply chain [4].

It is important to understand the factors that influence people to either donate or not donate plasma to increase domestic plasma donation. Since whole blood donation is dominant in donation literature, recognizing the work done in plasma donation literature is a timely contribution. As discussed below, most plasma studies focus on converting whole blood donors to plasma. Addressing plasma donations' unique barriers and facilitators is vital to increase the plasma donor pool with new, inexperienced donors to ensure the whole blood donor pool remains stable. Thus, this review aims to summarize current plasma literature to provide insight for future research on improving plasma donor recruitment.

MATERIALS AND METHODS

A rapid review of plasma donation literature was done from February to April 2022. A rapid review follows a similar strategy to a systematic review but is more efficient due to limiting the search parameters and aspects of the synthesis. By applying a more streamlined approach to data collection and analysis, rapid reviews are best utilized in areas where information needs are immediate. This is relevant for plasma research, as the need for increasing supply is ever-growing; therefore, we chose a rapid review to quickly disseminate knowledge on how this demand can be met in a time of dire need. While there is no formal rapid review guideline, we used reviews that discussed the various approaches other authors have used [5–7], as well as supplemented the preliminary findings of the unpublished PRISMA-RR protocol [8].

Information sources

The search was completed by one reviewer (M.B.). The databases that we searched included PubMed, PsycINFO, CINAHL and Social Sciences Citation Index. We searched for peer-reviewed journal articles that discussed plasma donation and the factors associated with intention, motivation, facilitators and deterrents. The search term used was 'plasma don*' and was further specified using either [title/abstract] or depending on the database. The search began on 25 February 2022 and was completed on 19 April 2022. Test searches were performed before the official search using more specific terms alongside plasma don* (e.g., willingness, intent, barriers). However, the search results were too specific and did not provide a sufficient number of articles necessary for the review's aim. We searched Social Sciences Citation Index using the search terms and parameters; however, no relevant or new articles emerged. Therefore, we chose not to include any articles found in this database.

Inclusion criteria

We included journal articles that focused on factors of plasma donation that impact donors' willingness to, or not to, donate. These factors included motivation, intent, self-efficacy, barriers, facilitators, donor identity and willingness. In addition, eligible studies included those discussing factors that impacted the amount people donated, recruitment of new plasma donors, experiences of people who donated and perceptions surrounding plasma donation. More succinctly, we focused on the donor experience and how their backgrounds influence their perceptions and attitude towards donating plasma.

Exclusion criteria

Articles were excluded if not written in English or based outside North America, Europe or Australia. Articles outside of this geographic region examine healthcare systems that are structurally different from the Canadian system. Even with included countries varying in their reliance on remunerated donations, they historically have collected and distributed blood and plasma donations through a not-for-profit Blood Collection Agency (BCA) or an entity separate from the public health authority. Therefore, we examine countries with similarities in procurement, as the collection agencies often determine the type of people who donate [9]. We did not review any medical articles or letters to the editor. We chose not to include articles that described donor demographics (e.g., sex, age) alone as the review's focus is on

how to increase donations, and descriptions of donor demographics are insufficient for this understanding. We also chose not to include op-ed articles or letters to the editor that discussed the ethical implications of paying donors (Figure 1).

Synthesis

Articles were organized in an Excel spreadsheet using descriptions of the studies, including methodology, sampling, study aims and findings. The authors kept notes on the emergent themes. The wider study team developed final themes by identifying each study's main goals and outcomes and grouping similar articles. In these groups, the authors highlighted how key findings fit together to create common trends across the articles, allowing for the development of broader themes. Moreover, grouping articles in such a way allowed the authors to find potential knowledge gaps explored in the discussion. Preliminary themes were developed by one author (M.B.), and then verified by another author (A.E.), with any disagreements resolved by a third author (N.B.).

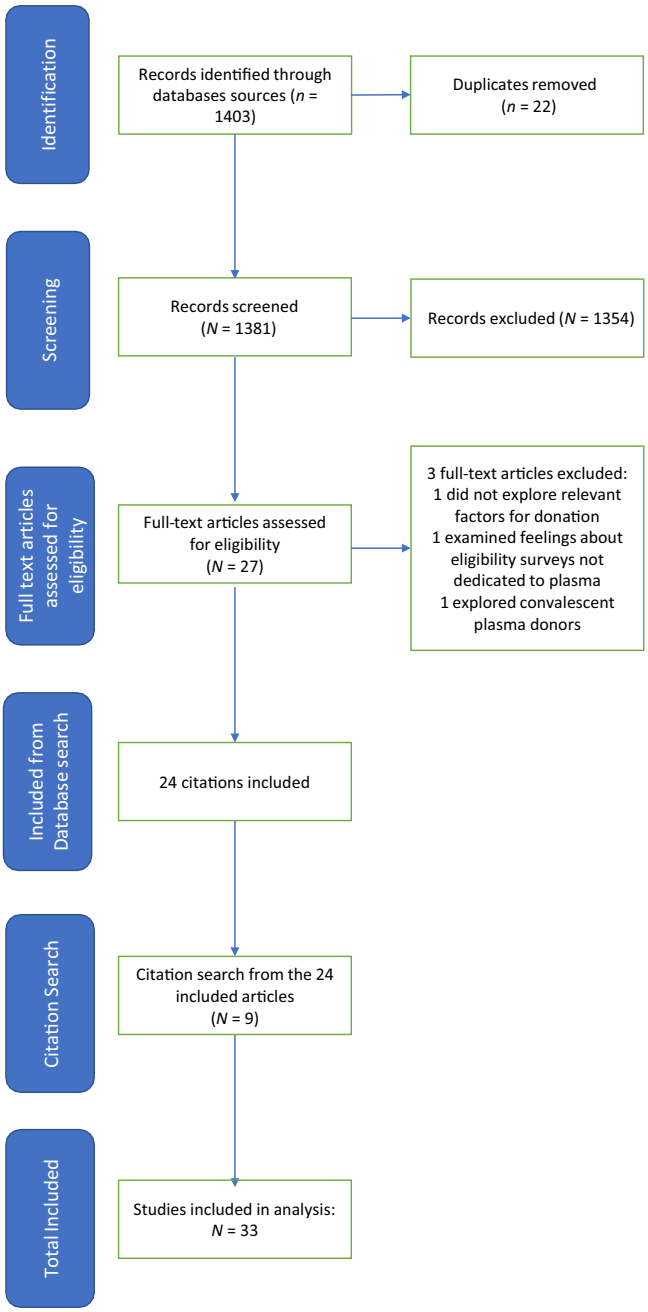

FIGURE 1 Flow chart of the selection process.

RESULTS

The electronic databases yielded 1403 articles. From these, 51 met our inclusion and exclusion criteria. After eliminating repeat articles, 24 unique studies met the inclusion/exclusion criteria to review fully. An additional nine articles were added from citation searches for a total of 33 articles (Table S1). The year range is from 1999 to 2022, as no articles appeared before 1999 in our search. Ten articles were qualitative and nine were quantitative. Additionally, three were mixed methods, and seven were experimental studies (e.g., randomized control trials). One article was a systematic review of whole blood donor willingness to convert to another substance of human origin [10]. Three were narrative reviews of motivations and deterrents towards plasma donation [11], motivations and compensation for plasma donation [12], and a summary of the current literature to provide strategies for BCAs [13].

We grouped our findings into three broad themes (Figure 2). The first theme focused on site factors, which include processes related to accessing plasma donation sites. The second theme was on individual-level factors, which focus on the motivations and deterrents of people who are approached to donate or already donate plasma. The third theme examined social/cultural-level factors, which explore the broader influences communities and social institutions have on individuals' perceptions of donating plasma.

Site and blood collection agency factors

Influence of BCA staff

To increase plasma donation, BCAs have explored both the recruitment of new donors and the conversion of whole blood donors to increase plasma donation [14–16]. The study by Thorpe et al. [16] aimed to increase the retention of first-time donors by comparing four strategies: calling donors, e-mailing donors, approaching donors to make advanced appointments and a simple 'thank you' message (control group). Compared with the control group, all intervention groups reported higher return rates and increased frequency of plasma donation. The most impactful strategy for getting donors to return included a call from the BCA asking about their experiences. This intervention demonstrated the importance of post-donation communication since

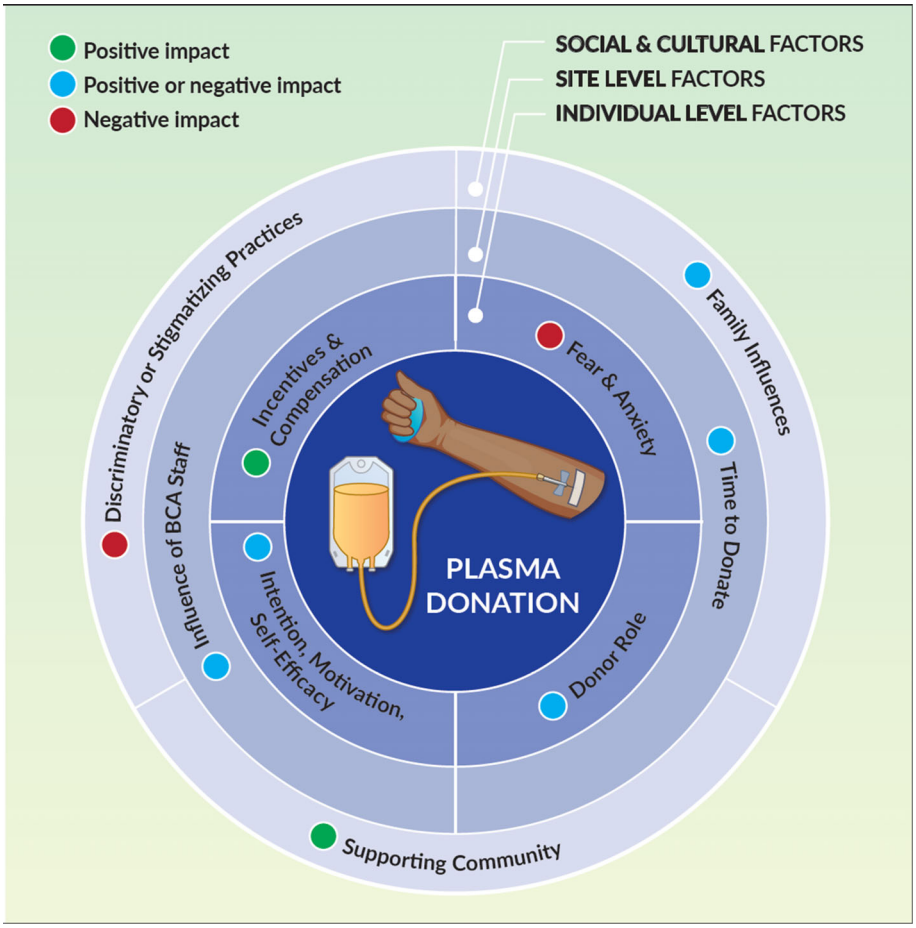

FIGURE 2 An illustration of the themes that emerged from the review, beginning with the individual-level factors in the innermost circle, and moving out to the site-level factors, and finally the larger social- and cultural-level factors that influence donation.

retention and recruitment improved when staff took the time to explain plasma donation and how to book an appointment.

Similarly, other studies [15, 17] compared the efficacy of different strategies for converting whole blood donors to plasma donation and found that in-person conversations with centre staff were more effective than call-centre phone conversations. Moreover, the relationships donors create with staff strongly influence return donations. Bove et al. [22] interviewed plasma donors on what triggered them to donate and what made them continue. For many, their relationship with the staff prompted them to switch from whole blood to plasma and kept them returning.

Time to donate plasma

Plasma donation takes approximately 1 h due to the separation and the return of the red blood cells, which is four times the length of blood donation and is the primary barrier preventing people from donating [11, 13, 18–21]. Bagot et al. [20] interviewed donors who declined to convert to plasma from whole blood donation and found that across all groups, the amount of time it took to donate plasma was the most cited reason for not wanting to donate plasma. Thorpe et al. [19] found that most plasma donors who lapsed did so because of changes in life circumstances, such as changes to work schedules, shifting family obligations and the ebb and flow of everyday life, which causes plasma donation to get sidelined.

Individual-level factors

Incentives and compensation

There has been substantial research into financial compensation's impact on people's willingness to donate plasma [12]; however, findings remain mixed and contradictory. Two experimental studies found that incentives did not influence willingness to donate [23] and return rates [24]. Gyuris et al. [23] found that donor willingness did not differ between the group given a monetary reward and the group that had the social significance of plasma donation emphasized to them. However, those who were given the option to pay their monetary reward forward to a non-governmental organization reported higher willingness to donate. Chell et al. [24] provided participants with a voucher for a local cafe and found that while nearly 70% of the sample took the voucher, only 18% redeemed it. Moreover, the return rate

between the control group that received no voucher and the experimental group with a voucher was not significantly different.

Two non-experimental studies explored the motivations of paid-plasma donors in the United States [25] and Hungary [26]. Anderson et al. [25] found that university students who were paid to donate their plasma were motivated by monetary incentives, not altruism. These students used the funds on activities they otherwise could not afford. Similarly, Trimmel et al. [26] surveyed paid plasma donors and voluntary whole blood donors in Hungary and found that paid plasma donors were motivated by monetary incentives and would not continue to donate if payment ceased. Moreover, paid donors had lower altruistic thinking levels than voluntary whole blood donors. More recently, France and France [27] surveyed university students on their preference to donate plasma or whole blood. They found that students' willingness to donate plasma rose from 3.9% to 47.5% after being informed that plasma donors are paid in the United States, where the study was based.

Anxiety and fear about donating plasma

Anxiety is often higher for new plasma donors due to navigating the apheresis machine and the unpleasant cold feeling experienced when blood is returned [28]. The medical environment in which plasma donation takes place appears to cause some unease and anxiety, especially for those new to the process.

A common factor for donor lapse is adverse events when donating, such as fainting. Vasovagal reactions (VVRs) are common for novice plasma donors [18, 28] and occur when individuals experience a sudden drop in blood pressure due to an extreme emotional response to a trigger, like seeing blood, and faint. Thijsen et al. [29] found that those plasma donors who experienced a VVR took significantly longer to return to donate and were more likely to experience another adverse event. Similarly, Thijsen et al. [30] explored the impact of a new policy in Australia, which allowed individuals to donate plasma without a previous whole blood donation. Compared to experienced donors, first-time plasma donors were the most likely to experience an adverse event and were not as likely to return.

Attempts have been made to address the anxiety and fears of new plasma donors. Gilchrist et al. [31] conducted an experimental study with two treatment groups for new donors. They gave the first group an informative brochure that normalized donor fear and provided stress reduction strategies—the second met with a trained research assistant who discussed concerns about with process with each donor. Both groups had fewer adverse events and higher positive support levels than the control.

Individual intention, motivation and self-efficacy

The most common framework for understanding individual intention and motivation towards plasma donation is the Theory of Planned Behaviour (TPB) [21, 32, 33]. The theory suggests that the best predictor of behaviour is intention. Three factors determine the strength of intention: perceived behavioural control, which is an individual's perception of how easily or not a behaviour can be performed; attitude, which is the evaluation of the behaviour as a positive or negative act; subjective norm, which is the perception of others approval towards the enacted behaviour. Godin and Germain [33] used TPB to evaluate which factors were the most impactful when determining the intention of plasma donors. They found that perceived behavioural control and attitude were the most significant determinants of intention, as well as age (i.e., older adults) and donor experience. Based on these findings, an individual's intention to donate plasma hinges on whether they believe they can do so successfully.

In response to these findings, studies have begun to supplement TPB with Bandura's concept of self-efficacy [34]. In this context, self-efficacy describes an individual's belief in their ability to carry out a behaviour well. Veldhuizen and van Dongen [32] used a modified TPB to identify the most significant predictor of conversion to plasma donation or the onset of a plasma donor career. Plasma donors had stronger positive attitudes than whole blood donors.

However, the most significant difference was the higher levels of self-efficacy reported in plasma donors. This result was mirrored in Bagot et al. [21]. This study incorporated additional factors such as moral norms and anticipated regret. Moral norms assess the individual's perceptions of whether the behaviour is right or wrong. Anticipated regret drives people to perform a behaviour to avoid feeling guilty if they fail to do so. Using these additional factors, Bagot et al. [21] found that moral norms were a significant predictor of intention to donate plasma; however, anticipated regret was only significant if the individual reported high moral norms.

Donor role

Bagot et al. [21] found that those blood donors with a strong tie to their 'blood donor identity' were less likely to convert to plasma donation. However, Thorpe et al. [35] found that converted plasma donors who felt whole blood donation was similar to plasma donation believed their donor identity remained stable. For many, their donor identity was also tied to the motivation to help as much as possible. Thus, when informed of the dire need for plasma, those who switched to plasma donation did not experience tension in the donor identity since it was seen as helpful and did not conflict with their blood donor identity [35].

Social and cultural-level factors

Donation frequency and supporting community

Several studies explored why people converted to plasma donation and what made them stay [22, 36–40]. The increased frequency at which donors could donate was a draw to plasma donation, as several

researchers found that donors often wanted to donate as much as possible to address the need for plasma and benefit the broader community [22, 36, 38]. Bagot et al. [39] examined underlying beliefs that promote the intention to donate plasma and found that individuals who made a second plasma donation reported a strong desire to help others.

Family influences

A strong emotional connection to whole blood or plasma increased donation likelihood. Charbonneau et al. [40] found that many plasma donors had family members who had previously donated, knew of someone close to them who needed whole blood or plasma, or had someone close to them get ill or pass away. Thus, learning first-hand the impact of plasma donation on people, whether it was a favourable report of the experience from a loved one or witnessing its lifesaving capabilities, is a powerful motivator for many plasma donors.

Discriminatory or stigmatizing practices

Programmes have been developed to mitigate the exclusion of LGBTQ+ donors by Hema-Quebec in Canada. They proposed that men who have sex with men (MSM) could donate plasma, but their donation would be quarantined for 3 months and would require them to return to be tested for blood-borne infections such as HIV [41]. Several studies explored how members of the community, specifically MSM, felt about the plasma programme [41–43]. Using a TPB lens, Veillette-Bourbeau et al. [42] found that study participants reported a moderate level of perceived behavioural control, suggesting that the MSM programme was something they could see themselves doing. While some participants were keen to join the programme, a significant number of people still felt that the programme reinforced donation as an exclusionary practice that perpetrated an 'us vs. them' mentality Grace et al. [43]. Therefore, while the study by Veillette-Bourbeau et al. [42] found that MSM may feel they *could* donate, both Grace et al. [43] and Caruso et al. [41] found that the programme perpetuates the notion that the blood of MSM is 'impure' and reinforces the stigmatizing and discriminatory practices of BCAs. Participants in both studies did feel that the programmes were a step in the right direction and were hopeful that they would aid in the reduction of harmful prejudices held against them.

DISCUSSION

This rapid review of the plasma donor literature examined the various factors influencing plasma donor behaviour. A primary finding is that much of this research has focused on converting whole blood donors. Given the shortages in the supply of whole blood donors, exacerbated due to COVID-19 [44], this group will not sufficiently resolve plasma supply issues meaningfully. Historically, recruiting whole blood donors has been an effective way of collecting plasma, which was considered secondary to whole blood donation, until the 1990s when demand for plasma-derived products dramatically increased [45]. More research is needed on people not currently donating blood, including diverse populations who tend to be underrepresented amongst whole blood donors [46, 47].

There was a gap in the literature surrounding how knowledge of plasma donation and its uses influenced donor motivations. This was surprising, considering in whole blood donation literature, knowledge is discussed as an important factor [47–51]. Further research should be implemented to understand knowledge's influence on individuals when deciding whether they will donate plasma.

We suggest a more thorough discussion of altruism as a motivator within the context of blood plasma donation. In whole blood donation literature, attempts to broaden definitions of altruism have included influences like self-worth (warm glow) [52] and moral obligations (reluctant altruism) [53]. However, these expanded definitions are not found within plasma donation literature. Furthermore, because the discussion of altruism is lacking in blood plasma literature, it is examined using individual-level factors that do not capture the nuance of this socially driven behaviour. Based on this, we argue for having a more nuanced exploration of altruism in blood plasma literature, which may allow for more effective targeting strategies for specific groups of people instead of general marketing approaches.

Exploring macro-level social factors would greatly increase the understanding of the pushes and pulls people experience when deciding whether or not to donate plasma. Although some studies have examined how ethnicity and cultural background influence whole blood [46, 54, 55] and organ [56] donation, this literature remains scant and other influences, such as gender, socio-economic status, disability status and language, remain largely under-explored. Greater attention to larger social influences such as immigration status and socio-economic status should be explored to improve understanding of the pushes and pulls towards plasma donation.

It is important to note the strengths and limitations of this rapid review. The strengths of this article include a strict inclusion/exclusion criterion created before the initial search. Moreover, throughout the search and writing this review, the primary reviewer (M.B.) collaborated with other authors to negotiate and determine the best way to organize the data and reinforce the rigour of the review. The limitations are the nature of the rapid review and the method's efficiency, which means the review's scope is limited. For example, we omitted scholarship on COVID-19 convalescent plasma donation, as this donor base has different motivations that were irrelevant for future non-convalescent donation. However, this area would likely inform future plasma collection strategies and should be examined with non-convalescent donation [57]. As such, limiting the search terms and the databases may have resulted in publications being missed. Further, excluding grey literature, op-ed articles and publications in languages other than English may have created a potential publication bias.

Overall, the main message across the literature is that the need for plasma donors is dire, and the plasma supply must be increased to ensure people get the treatment they need. Future research should

look at how to recruit new donors from a diverse population and the larger structural- and cultural-level factors that influence people's desire to donate plasma or not.

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CONFLICT OF INTEREST STATEMENT

The authors declared no potential conflicts of interest concerning the authorship, and/or publication of this article.

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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 added value of ferritin levels and genetic markers for the prediction of haemoglobin deferral

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Abstract

Background and Objectives: On-site haemoglobin deferral for blood donors is sometimes necessary for donor health but demotivating for donors and inefficient for the blood bank. Deferral rates could be reduced by accurately predicting donors' haemoglobin status before they visit the blood bank. Although such predictive models have been published, there is ample room for improvement in predictive performance. We aim to assess the added value of ferritin levels or genetic markers as predictor variables in haemoglobin deferral prediction models.

Materials and Methods: Support vector machines with and without this information (the full and reduced model, respectively) are compared in Finland and the Netherlands. Genetic markers are available in the Finnish data and ferritin levels in the Dutch data.

Results: Although there is a clear association between haemoglobin deferral and both ferritin levels and several genetic markers, predictive performance increases only marginally with their inclusion as predictors. The recall of deferrals increases from 68.6% to 69.9% with genetic markers and from 79.7% to 80.0% with ferritin levels included. Subgroup analyses show that the added value of these predictors is higher in specific subgroups, for example, for donors with minor alleles on single-nucleotide polymorphism 17:58358769, recall of deferral increases from 73.3% to 93.3%.

Conclusion: Including ferritin levels or genetic markers in haemoglobin deferral prediction models improves predictive performance. The increase in overall performance is small but may be substantial for specific subgroups. We recommend including this information as predictor variables when available, but not to collect it for this purpose only.

Keywords

donor deferral, ferritin, genetic markers, hemoglobin

Highlights

- Ferritin is routinely measured in some blood services, and genetic information for several iron-related single-nucleotide polymorphisms (SNPs) are collected by some others.

- Inclusion of ferritin levels or genetic markers in haemoglobin deferral prediction models only marginally increases the predictability of blood donor deferrals.
- For donors with minor alleles on SNP 17:58358769, or donors with ferritin levels between 30 and 50 μg/L, the predictability of donor deferral increases considerably.

INTRODUCTION

Deferral of blood donors with low haemoglobin levels is necessary to prevent iron depletion. Currently, in Finland and the Netherlands, haemoglobin is measured before donation, which leads to on-site deferral if haemoglobin is below the donation threshold of 7.8 mmol/L (125 g/L) for women or 8.4 mmol/L (135 g/L) for men. On-site deferral is demotivating for donors and can be a reason to drop out of the donor pool permanently [1]. Haemoglobin deferral prediction models can help reduce the on-site deferral rate: for invitation-based donations, predictions can be included in the decision-making process of which donors to invite; for walk-in donations, the prediction could be communicated to the donor (e.g., shown on a donor dashboard or app that many blood banks offer), who can use this information to decide when to visit the blood bank.

Currently, haemoglobin deferral prediction models are not very accurate at predicting deferral on the specific day a donor may visit the blood bank. Although it is possible to correctly predict most deferrals as such (and therefore prevent them), this comes at the cost of incorrectly predicting some non-deferrals to be deferrals, which results in a large net loss of donations if these donors are then not invited to the blood bank based on this incorrect prediction. However, in a previous study we showed that predicting haemoglobin deferral at different time points, and inviting a donor once the predicted outcome is 'non-deferral', results in non-deferred donors to be invited earlier and deferred donors to be invited later, thereby eliminating the loss of successful donations [2]. This tells us that haemoglobin deferral prediction models are useful, and it is worth the effort of trying to improve the predictions.

Multiple studies [3–5] have shown previous haemoglobin levels to be the most important predictor of future haemoglobin deferral. Researchers from blood services in different countries have investigated many different potential predictors of haemoglobin deferral, to assess whether the inclusion of these predictors improves prediction performance. Most of these predictors were found to not substantially improve the models: information on menstruation, diet, ethnicity and smoking all only slightly improve model performance, even though they are known to be associated with iron stores [4]. One small-scale study on 261 donors did show that ferritin, soluble transferrin receptor and hepcidin were associated with subsequent anaemia [5].

In this study, we investigate the added value of including ferritin levels and genetic information in haemoglobin deferral prediction models. Ferritin is routinely measured at Sanquin, the Dutch national blood service, and therefore available for all donors. Genetic information for several iron-related single-nucleotide polymorphisms (SNPs) is collected for many donors by the Finnish Red Cross blood service. Because the information in both countries is collected without targeting specific donors, our results provide a realistic indication of how much predictions would be improved if the prediction model was to be used in practice. Our results will therefore be useful for blood services that would like to collect additional donor information to improve haemoglobin deferral predictions.

METHODS

Data

Data on blood donation attempts by whole-blood donors from (almost) five recent years were extracted from the eProgesa database (MAK-SYSTEM, Paris, France) in Finland and the Netherlands. Only data from donors who explicitly provided informed consent for the use of their data for scientific research were used. This consent is given by more than 99% of all Dutch donors. All Finnish blood donors studied provided informed consent for biobank research in accordance with the Finnish Biobank Act and the study was approved by the Blood Service Biobank (project 004_2019). In Finland, ~23% of active blood donors have given this consent since the founding of the Blood Service Biobank in 2017.

Finnish data reflect data entries from January 2016 through April 2020 and Dutch data from January 2017 through December 2021. For each visit, the following information was collected in both countries: donor sex, donor age, donation date and haemoglobin level. Additionally, ferritin level is measured at every new donor intake and upon every fifth donation in repeat donors in the Netherlands.

In Finland, only donors participating in the Blood Service Biobank are included, as only for these donors, genetic information related to iron metabolism is available [6]. Four SNPs were identified as significantly associated with higher prevalence of iron deficiency anaemia in an iron deficiency anaemia meta-analysis on Finnish and UK data. Polygenic risk scores were derived for three related endpoints: iron deficiency anaemia, ferritin and haemoglobin [7].

In total, complete information on the predictor variables (see Table 1) was available for 172,508 donation attempts by 42,255 donors in Finland and 456,384 donation attempts by 157,423 donors in the Netherlands.

The variable of interest is 'HbOK', a dichotomous variable that indicates whether the result of the donation attempt was deferral (i.e., haemoglobin [Hb] level below the eligibility threshold for donation) or non-deferral (i.e., Hb level equal to or above the threshold).

Donor deferral due to low haemoglobin is similar in Finland and the Netherlands. Haemoglobin is measured using a capillary skin-prick device before each donation, and eligibility thresholds for donation are 7.8 mmol/L for women and 8.4 mmol/L for men. However, in case

TABLE 1 Predictor variables used in each country.

Variable used	Unit or values	Description	Country/Countries where data are available
Sex	{male, female}	Biological sex of the donor; separate models are trained for men and women	Both
Age	Years	Donor age at time of donation	Both
Month	{1–12}	Month of the year that the visit took place	Both
NumDon	Donations	Number of successful (collected volume >250 mL) whole-blood donations in the last 24 months	Both
DaysSinceFirstDon	Days	The number of days since the donor visited the blood bank for the first time	Both
HbPrev*i*	mmol/L	Haemoglobin level at *i*th previous visit, for *i* between 1 and 5	Both
DaysSinceHb*i*	Days	Time since related Hb measurement at *i*th previous visit, for *i* between 1 and 5	Both
FerritinPrev	μg/L	Most recent ferritin level measured in this donor	The Netherlands
SNP 1:169549811	{0, 1, 2}	Number of minor alleles in SNP rs6025	Finland
SNP 6:32617727	{0, 1, 2}	Number of minor alleles in SNP rs3129761	Finland
SNP 15:45095352	{0, 1, 2}	Number of minor alleles in SNP rs199138	Finland
SNP 17:58358769	{0, 1, 2}	Number of minor alleles in SNP rs199598395	Finland
PRS_anaemia	Standard deviations	Standardized polygenic risk score for anaemia	Finland
PRS_ferritin	Standard deviations	Standardized polygenic risk score for ferritin	Finland
PRS_haemoglobin	Standard deviations	Standardized polygenic risk score for haemoglobin	Finland

Abbreviation: SNP, single-nucleotide polymorphisms.

the measurement is below the eligibility threshold in Finland, haemoglobin is measured again (using the same device) in a venous sample, and this measurement is used for the deferral decision. In the Netherlands, two additional capillary haemoglobin measurements are taken when the first measurement outcome is below the eligibility threshold, and the donor is allowed to donate if any of the three measurement outcomes is above the eligibility threshold.

Analyses

For both countries, two models were fitted for each sex: one with all predictor variables available (the full model), and one with only those predictor variables that are available in both countries (the reduced model). By comparing the full model with the reduced model in both countries, the added value of the extra predictor variables (i.e., genetic information in Finland and ferritin information in the Netherlands) can be assessed.

The prediction models used were based on models developed for an earlier study considering Dutch data only [2]. All models are based on support vector machines (SVMs), supervised machine learning models that learn a separation between outcome classes from a *training set*, after which the model can be used to predict donor deferral for observations in an unseen *test set*. Here, the training set consists of blood bank visits in the first 4 years of data, whereas the test set consists of data collected in the final year.

Given a dataset and a set of predictor variables, a model consists of 10 SVM sub-models. The sub-models are named SVM-*sex*-*n*, where *sex* indicates donor sex (m for male, f for female donors) and *n* indicates the number of previous blood bank visits that are used for prediction. That is, each sub-model includes HbPrev*i* and DaysSinceHb*i* for *i* ranging from 1 to *n* as predictor variables. If *sex* is omitted in the sub-model name, it refers to the combination of two sex-specific sub-models. The number of blood bank visits (*n*) considered in this study varies from 1 to 5, and so five sub-models per sex are created. Donors can be included in the SVM-*sex*-*n* sub-model only if they have at least *n* previous visits; therefore, the sizes of the datasets used for both training and testing decrease from SVM-1 to SVM-5. Hyperparameters were optimized separately for each sub-model using stratified (on the outcome variable) fivefold cross-validation within the training set data only. Hyperparameters were optimized using grid search, using the balanced accuracy (defined as the weighted average of recall in both classes) as scoring method, which is suitable for datasets with imbalanced outcome sizes, as mistakes in the minority class are penalized more than those in the majority class.

During model training, the classification threshold is chosen again by optimizing the balanced accuracy. The predictive performance of the models is assessed using precision (also known as positive predictive value) and recall (also known as sensitivity) at this classification threshold. For non-deferral prediction, precision is defined as the proportion of true non-deferrals out of all predicted non-deferrals; recall is defined as the proportion of predicted non-deferrals out of all true non-deferrals. In this context, the complement of the precision is the hypothetical new deferral rate if the model would be used to choose which donors to invite, and the complement of the recall is the

TABLE 2 Number of donation attempts available per model for both countries; number of deferrals and deferral rates are given in brackets.

	Women		Men	
Model	Finland	Netherlands	Finland	Netherlands
SVM-1	83,628 (3216; 3.85%)	236,994 (7724; 3.26%)	88,880 (1480; 1.67%)	219,390 (2411; 1.10%)
SVM-2	68,718 (2494; 3.63%)	166,640 (5875; 3.53%)	78,268 (1264; 1.61%)	179,465 (2114; 1.18%)
SVM-3	55,011 (1859; 3.38%)	123,171 (4370; 3.55%)	68,225 (1054; 1.54%)	150,396 (1889; 1.26%)
SVM-4	43,164 (1307; 3.03%)	93,868 (3149; 3.35%)	58,951 (896; 1.52%)	127,807 (1667; 1.40%)
SVM-5	33,179 (868; 2.62%)	72,165 (2112; 2.93%)	50,540 (749; 1.48%)	108,832 (1424; 1.31%)

Abbreviation: SVM, support vector machines.

proportion of successful donations that would be missed by the model because the donors are incorrectly predicted to have a low haemoglobin level. Precision and recall can be calculated for both outcome classes ('deferral' and 'non-deferral').

The precision–recall (PR) curve is a graph in which the recall and the precision of a prediction model at varying classification thresholds are shown. The area under this curve (AUPR), is a number between 0 and 1, where 1 would indicate a perfect classifier. By subtracting the deferral rate from the AUPR, we get an adjusted AUPR, which reflects the improvement by the model over a strategy that would always predict non-deferral. Without this correction, the improvement made by the model would be biased by the difference in deferral rate. AUPR represents the ability of the model to distinguish between two classes at differing classification thresholds. It is possible for model A to have a higher AUPR than model B even if precision and recall at the optimal classification threshold are the same in both models.

Model explanations

Because SVMs do not provide model coefficients that can be directly interpreted, we use Shapley Additive exPlanations (SHAP) values to investigate the importance of different predictor variables [8]. SHAP is a model agnostic explainer that shows the contribution of each predictor variable to the predicted outcome. This contribution is calculated for each individual observation separately (in a subsample of the test set) and is therefore very informative.

Subgroup analysis

To further investigate the value of including ferritin and genetic information in the models, we perform additional analyses in which donors are placed in groups defined by ferritin level or genotype. Deferral rate, model performance and the difference between reduced and full model performance are calculated and compared to assess whether there are subgroups of donors for whom including the extra variables results in better predictions.

Software

All analyses were performed in Python 3.10 using packages *numpy* and *pandas* for data processing, *scikit-learn* for model training and predictions, *shap* for calculating SHAP values and *matplotlib* for creating graphs. All code is available on GitHub and is indexed on Zenodo at https://doi.org/10.5281/zenodo.7780718.

RESULTS

Table 2 shows the number of donation attempts used for each model in both countries. Deferral counts and rates are given in brackets. Sample sizes are much larger in the Netherlands than in Finland. This is because the total number of blood donations is much higher in the Netherlands than in Finland, which is due to a larger population (17.4 million vs. 5.5 million in 2020), but also because genetic information is available in Finland in only a subgroup of donors, whereas ferritin measurements are available for all Dutch donors.

Deferral rates are very similar in both countries, around 3% for women and 1% for men. The biggest difference in deferral rates is found in men with at least one previous haemoglobin measurement, where the deferral rate is 0.57 percentage points higher in Finland. In most cases, deferral rates go down whenever more previous visits are included; this is most likely the result of self-selection, where donors with lower haemoglobin levels are less likely to return for subsequent donations than donors with higher haemoglobin levels. Surprisingly, for Dutch men this pattern seems to some extent to be reversed as their deferral rate goes up with an increasing number of donations.

Table S1 shows the marginal distribution of the predictor variables, combined for all sub-models. Donors in Finland are older than

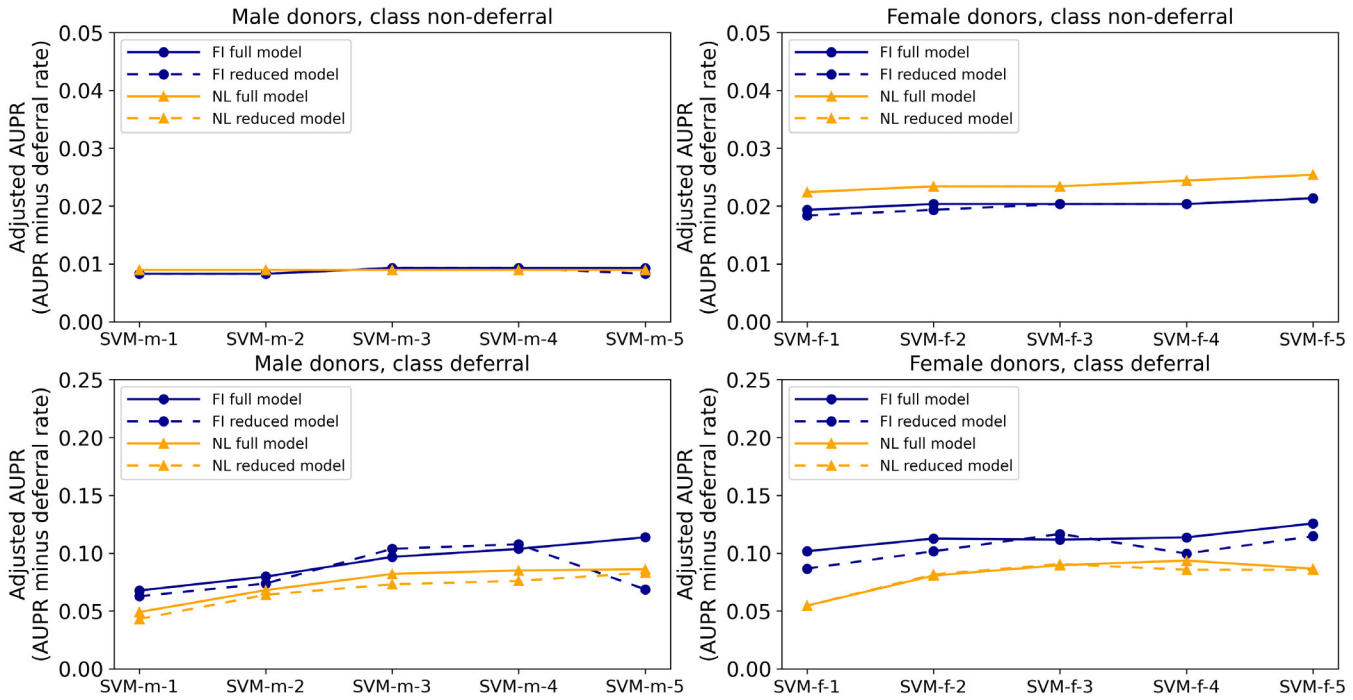

FIGURE 1 Adjusted area under precision–recall curve (AUPR) by sub-model for the Netherlands (NL) and Finland (FI) for both sets of predictor variables. SVM, support vector machines.

donors in the Netherlands (median age 46 vs. 30 years in women, 52 vs. 34 years in men) and the number of donations in the past 2 years ('NumDon') is also higher, with a difference in median donations of 2 for both sexes. This difference can be explained by the sample composition: the Finnish dataset consists of participants of the Blood Service Biobank, who have given consent for medical research and are typically regular, committed blood donors. Genetic information is available only for these donors.

Haemoglobin levels are slightly higher in Finland for both sexes for all variables HbPrev*i*, by 0.1–0.3 mmol/L. The time between subsequent donation attempts (variables DaysSinceHb*i*) is slightly shorter for Finnish women than for Dutch women, but almost identical for men. This difference can be partly explained by a difference in minimum donation interval between blood donations: for women, 91 days in Finland versus 122 days in the Netherlands; for men, 61 days in Finland versus 57 days in the Netherlands.

Predictive performance

Predictive performance can be assessed for individual sub-models, or for all sub-models combined, by using the most complex sub-model possible to predict each outcome. When more previous blood bank visits are taken into consideration, more predictor variables are used, and we expect the performance of the sub-model to increase. Figure 1 shows that this is the case for both the full and reduced model in both countries. The adjusted AUPR increases from SVM-1 to SVM-5 almost everywhere. An exception is the AUPR for class deferral in SVM-m-5, where the reduced model for Finnish donors shows an unexpected drop in the adjusted AUPR. For male donors, class non-deferral, the adjusted AUPR does not seem to change from SVM-m-1 to SVM-m-5.

Overall model performance and the difference in model performance between the full and reduced models are assessed by PR curves and adjusted AUPR values as described in Section 2. Figure 2 shows the PR curves for various models (SVM-1 through SVM-5, using the model with the most predictor variables possible for each donation attempt) by sex and true outcome class. In general, models are better at identifying non-deferrals (the most common outcome) than deferrals, even with scoring methods that weigh mistakes in both outcome classes proportionally. However, all curves are well above the baseline, indicating a structural improvement as compared to random guessing.

When comparing the reduced models with each other, one can observe that the performance is very similar in both countries. For women the AUPR is higher in Finland than in the Netherlands for the class deferral, but lower for the class non-deferral. This indicates that deferrals are more likely to be predicted correctly, but at the cost of more inaccuracies when predicting non-deferrals.

Moving from the reduced to the full model has virtually no effect on the AUPR for the class non-deferral: the AUPR of the full model is almost identical to that of the reduced model for both countries and sexes. For the class deferral, however, there is a difference: in Finland, AUPR increases by 58% (from 0.066 to 0.104) for men and by 8.5% (from 0.106 to 0.115) for women. In the Netherlands, AUPR remains the same for women (0.086 for both) but increases by 8.3% (from 0.072 to 0.078) for men.

Table 3 provides the confusion matrices of model predictions by the reduced and full models for both countries. In the Finnish data, going from the reduced to the full model causes 7 (1.9%) more

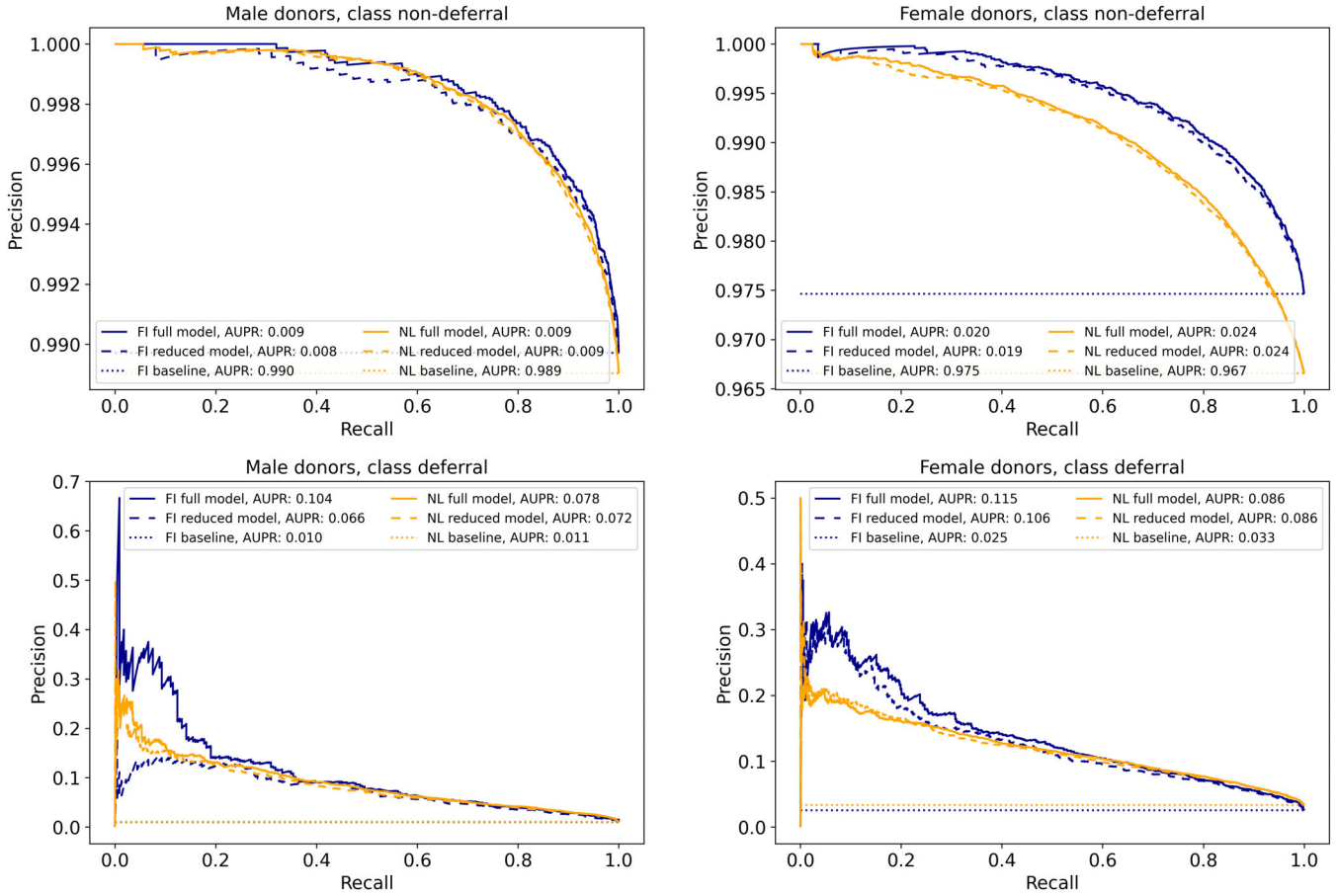

FIGURE 2 Precision–recall curves for the prediction models. For the Netherlands (NL) and Finland (FI), the curve is shown for the reduced and full prediction models. The baseline (proportion of observations belonging to this outcome class, i.e., for class deferral, the deferral rate) is shown as a dotted horizontal line. AUPR, adjusted area under precision–recall.

TABLE 3 Confusion matrices of predictions by the reduced and full models.

Finnish donors: Reduced-model			**Finnish donors: Full model**		
	Predicted deferral	**Predicted non-deferral**		**Predicted deferral**	**Predicted non-deferral**
True deferral	363	166	True deferral	370 (+7)	159 (−7)
True non-deferral	4573	18,713	True non-deferral	4662 (−59)	18,624 (+59)
Dutch donors: Reduced model			**Dutch donors: Full model**		
	Predicted deferral	**Predicted non-deferral**		**Predicted deferral**	**Predicted non-deferral**
True deferral	3762	957	True deferral	3775 (+13)	944 (−13)
True non-deferral	56,676	145,549	True non-deferral	55,203 (−1473)	147,022 (+1473)

Note: Numbers are summed over both sexes and over all sub-models SVM-1 through SVM-5. Observations that can be predicted with multiple sub-models are included in the most complex sub-model.
Abbreviation: SVM, support vector machines.

deferrals to be predicted correctly, while 59 (0.3%) more non-deferrals are predicted correctly. These improvements were all for female donors; at the chosen threshold values, no net changes in the confusion matrix were seen for male donors. In the Dutch data, 13 (0.3%) more deferrals, as well as 1473 (1.0%) more non-deferrals, are predicted correctly by the full model as compared with the reduced model.

Note that the large increase in AUPR for Finnish male donors, class deferral, is not reflected in the confusion matrices. The PR curve in Figure 2 shows that the AUPR increase is due to higher precision in the full model between a recall of 0 and 0.2. However, the optimal classification threshold that is used by the models corresponds to a recall of 0.7, at which point precision in the full model is exactly equal to precision in the reduced model.

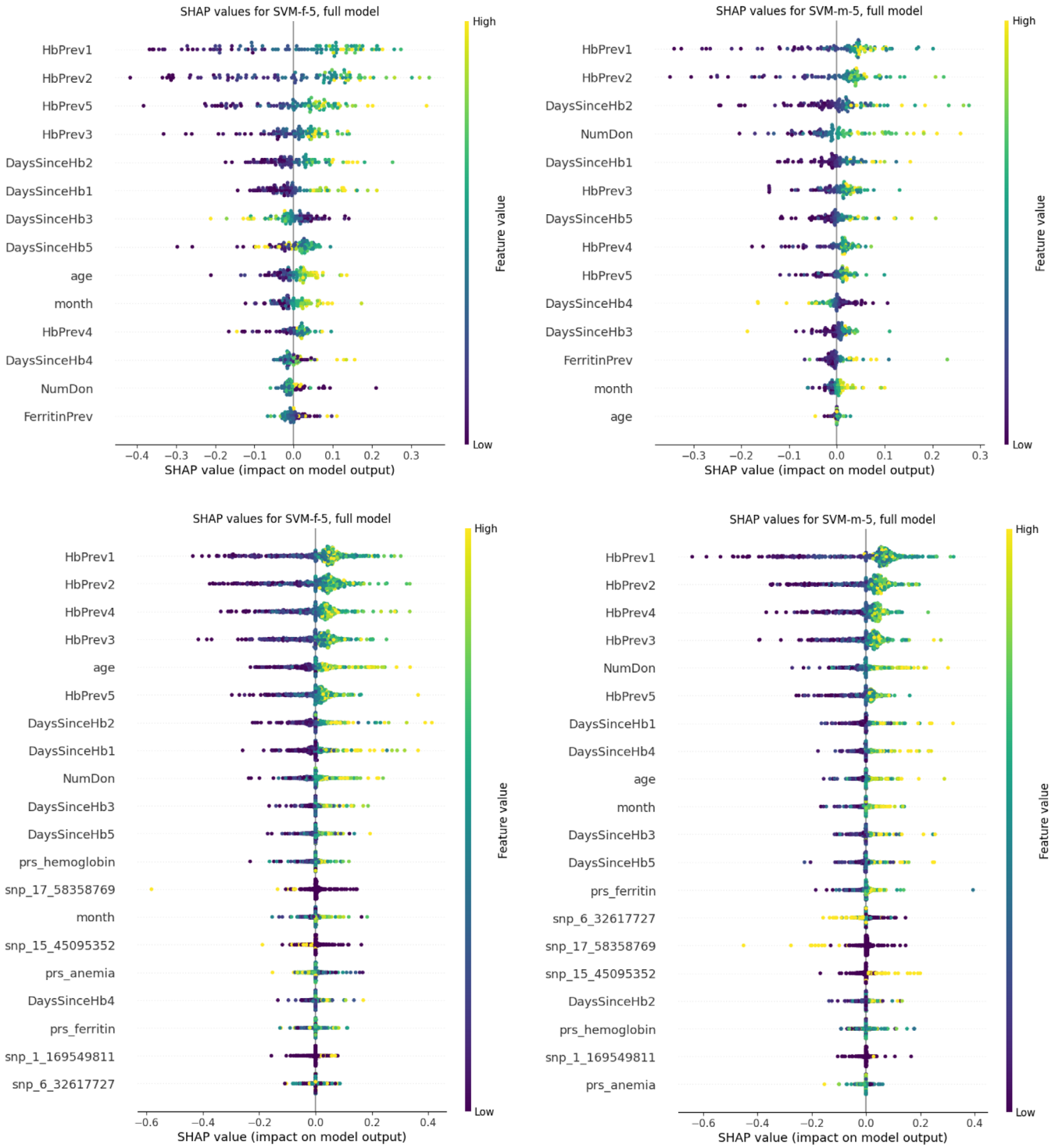

FIGURE 3 Shapley Additive exPlanations (SHAP) plots for the full models on Dutch (top row) and Finnish (bottom row) data, for women (left column) and men (right column) separately. SVM, support vector machines.

Variable importance

For all sub-models, SHAP values show the importance of the different predictor variables on the predicted outcome. Figure 3 shows SHAP plots of the sub-model SVM-5 of the full model, separately for both sexes and countries.

These plots show that in both countries and for both sexes, the most important predictor variable is HbPrev1, that is, the most recent haemoglobin measurement. The direction of the association between the impact on the model output and the feature value for all HbPrev*i* variables is sensible: a lower haemoglobin measurement is predictive of deferral. Age is a more important predictor variable for women than

TABLE 4 Sample sizes, deferral rates and precision and recall of outcome class deferral for subsets of donors based on values for four SNPs (single-nucleotide polymorphisms).

SNP	Minor alleles	*N*	Deferral rate	Precision (class deferral)		Recall (class deferral)	
				Reduced model	Full model	Reduced model	Full model
SNP 1:169549811	0	22,810	0.022	0.073	0.073	0.686	0.702
SNP 6:32617727	1 or 2	1005	0.026	0.087	0.095	0.692	0.692
	0	7268	0.021	0.063	0.067	0.573	0.587
	1	11,908	0.022	0.072	0.074	0.704	0.742
	2	4639	0.026	0.092	0.081	0.790	0.756
SNP 15:45095352	0	20,831	0.022	0.073	0.073	0.676	0.691
	1 or 2	2984	0.022	0.080	0.080	0.758	0.773
SNP 17:58358769	0	23,427	0.021	0.071	0.071	0.683	0.687
	1 or 2	388	0.077	0.156	0.129	0.733	0.933
Total	-	23,815	0.022	0.074	0.074	0.686	0.701

for men in both countries, which is known from previous studies: young women have the highest probability of being deferred because of low haemoglobin, due to monthly iron loss with menstruation.

The additional genetic and ferritin variables for either country end up rather low in the variable importance ranking. The importance of all polygenic risk score and SNP variables in the Finnish models is very low. However, having the minor allele present in either SNP 6:32617727, SNP 15:45095354 or SNP 17:58358769 impacts the model output negatively. This effect is more pronounced in male than female donors.

Subgroup analysis in Finnish data

To further investigate the effect of the SNPs on deferral prediction, model performance was calculated for groups of donors with the same value for one SNP at a time. Donors with values 1 and 2 are grouped together, as the proportion of donors with value 2 is extremely low, except for the SNP on chromosome 6.

Table 4 shows that for the SNPs on chromosomes 1, 6 and 17, deferral rates are higher among donors with one or two minor alleles than in donors with only major alleles. As these SNPs are selected because of their association with iron deficiency or anaemia, this is to be expected. Additionally, precision and recall of class deferral are generally higher for donors with minor alleles than for those without, for both the reduced and full models. The SNP 17:58358769 shows this same trend, but the difference between donors with and without minor alleles is much larger. Precision in this subgroup is about twice as high as the overall precision in both the reduced and full model. The increase in recall between the full and reduced model (which changes from 0.733 to 0.933) is the highest of all subgroups.

An additional analysis on the distribution of haemoglobin measurement per donor showed that the higher deferral rate among donors with minor alleles on SNP 17:58358769 can be explained through a combination of a slightly lower average haemoglobin level and a slightly higher variance. This causes these donors to have a slightly higher deferral probability (median 32.6% for donors without minor alleles, median 36.6% for those with minor alleles). This difference was not observed for the other SNPs.

Subset analysis in Dutch data

Similar to the subset analysis in Finnish data, model performance was calculated for groups of donors with similar ferritin levels: <15, 15–30, 30–50, 50–100 and >100 μg/L. The first two groups are those that would be deferred for 12 or 6 months, respectively, in accordance with Sanquin's ferritin deferral policy.

Table 5 shows that precision and recall are highest for donors with ferritin levels between 30 and 50 μg/L. This is also the group of donors with the highest deferral rate: 3.2%, versus an overall deferral rate of 2.3%. The fact that this group has the highest deferral rate, and not donors with lower ferritin levels, can be explained by the fact that donors with ferritin levels below 30 μg/L were deferred for 6 months (12 months for ferritin levels below 15 μg/L) in accordance with Sanquin's ferritin deferral policy. This delay for the next donation provides the donors with sufficient time to replenish their iron stores and therefore reduces the deferral probability. Hence, donors with ferritin levels just above the ferritin-deferral threshold will have the highest haemoglobin-deferral rate, as they have neither the advantage of the donation break nor that of a very high ferritin level, which also protects against low haemoglobin levels.

DISCUSSION

Predicting deferral for low haemoglobin levels is a topic of interest to many blood banks, as accurate predictions could aid in decreasing deferral rates. This study investigates the added value of including

TABLE 5 Sample sizes, deferral rates and precision and recall of outcome class deferral for various subsets of donors based on their ferritin level.

Ferritin level	*N*	Deferral rate	Precision (class deferral)		Recall (class deferral)	
			Hb only model	All variables	Hb only model	All variables
<15 μg/L	7172	0.022	0.054	0.054	0.700	0.681
15–30 μg/L	19,903	0.022	0.058	0.056	0.744	0.783
30–50 μg/L	62,140	0.032	0.082	0.079	0.815	0.833
50–100 μg/L	65,141	0.024	0.064	0.063	0.798	0.799
>100 μg/L	52,588	0.010	0.033	0.040	0.801	0.730
Total	206,944	0.023	0.062	0.064	0.797	0.800

Abbreviation: Hb, haemoglobin.

information on the donor's ferritin level or iron-related genetic information to improve haemoglobin deferral prediction. This is done by comparing prediction models with and without information on genetic markers and ferritin levels for the Finnish and Dutch blood bank, respectively. The reduced models (i.e., without the additional information) use the exact same predictor variables in both countries. The increase in AUPR is larger for adding genetic markers than it is for adding ferritin levels. Especially for the Finnish male donors, including genetic markers in the prediction model improves the ability of the model to distinguish between the two outcome classes, although at the optimal classification threshold precision and recall do not increase from the reduced model. The SHAP values of the predictions by the full models in both countries show that both genetic markers and ferritin levels have a much smaller impact on the prediction than the variables included in the reduced models, as confirmed by the modest increase in AUPR between the reduced and full models.

Overall, including either genetic or ferritin information has little effect on the predictions made by the models. Both increase the proportion of deferrals that are predicted correctly: 1.9% and 0.3% more deferrals are correctly identified in the Finnish and Dutch setting, respectively, when the full model is used rather than the reduced model. However, we found that in both countries, there is a subgroup of donors for which the full model performs substantially better than the reduced model. These are Finnish donors with minor alleles on SNP 17:58358769 and Dutch donors with ferritin levels between 30 and 50 μg/L. In both cases, these are subgroups of donors with a higher than average deferral rate. Performance for these subgroups is already higher than average in the reduced model, but when using the full model this difference increases even further.

Other studies have shown that previous haemoglobin measurements are the most influential predictors for haemoglobin deferral. Including lifestyle behaviour, smoking, ethnicity or menstruation in prediction models also improves performance, but only marginally [4]. A Finnish study showed that genetic information does not improve the predictive performance of haemoglobin levels (as opposed to haemoglobin deferral) [9]. This study confirms that the performance of prediction models increases slightly when either ferritin or genetic information is added. Still, considering the large number of donation visits blood banks receive yearly, even a small increase could potentially prevent hundreds of deferrals. It should be noted that the Finnish population is more genetically homogenous than in other countries and that they are also genetically distinct from other countries due to several historic population bottlenecks and geographical isolation [10]. According to the Genome Aggregation Database (gnomAD) [11], the SNP 17:58358769 minor allele frequency in the Finnish population is 0.0147 but only 0.0007 in the European (non-Finnish) population. It is not found in any other populations and was discovered by an iron deficiency GWAS in the FinnGen project [7]. This means that findings on Finnish genetic data may not be representative for other countries, but analyses in other populations may discover similar population-specific variations that may make the use of genetic data more beneficial.

The main limitation of this study is that the effect of including ferritin and genetic information is studied in two different countries, rather than in a single population. By comparing against the reduced model and reporting the relative increase in performance, we attempt to mitigate this limitation. The very similar adjusted AUPRs of the reduced models and the similarity in SHAP values of the models indicate that the countries are rather comparable. A second limitation is that all Dutch donors could be included in this study but only Finnish donors from the Blood Service Biobank, as genetic information is not available for other donors.

In general, we again confirm that accurately distinguishing deferrals from non-deferrals by predictive modelling is a complex task that comes at the cost of losing a substantial number of successful donations by incorrectly predicting them to be deferrals. A major reason for the low performance of our prediction models is the measurement variability, partly caused by the (pre-) analytical variability of the capillary haemoglobin measurements [12]. As long as we try to predict an outcome that is highly variable, the performance of any prediction model will remain unsatisfactory, regardless of the number of predictor variables included.

However, in the absence of a better measurement or decision strategy, it is worthwhile investigating which information would lead to better haemoglobin deferral predictions, as it still leads to a better understanding of the underlying process(es). Based on our results, we would recommend including ferritin and genetic information in prediction models in case these are readily available. Compared

with the reduced model, including genetic information would have resulted in 7 fewer deferrals and 59 more donations in 1 year, at a cost of genotyping approximately 24,000 donors. Including ferritin levels results in 13 fewer deferrals and 1473 more donations in 1 year, and although measuring ferritin levels is less expensive than genotyping, this measurement must be repeated regularly whereas genotyping has to be performed only once for each donor. We would therefore not recommend collecting this information explicitly for the use in haemoglobin deferral prediction, as the marginal increase in performance is not likely to be worth the investment of both time and money.

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All authors contributed to the study design; M.V. and J.T. developed the software and analysed the data; M.V. aggregated the results and wrote the paper and all authors reviewed and edited the paper.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

As the data used for this study contains personal information of blood donors, the data will not be shared outside the organisations.

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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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Association of blood donor characteristics and in vitro haemolysis of packed red blood cell concentrates during storage

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Abstract

Background and Objectives: Blood donor variability can affect the storage properties of packed red blood cells (PRBCs). This study aimed to determine the association of donor characteristics with in vitro storage haemolysis of PRBCs.

Materials and Methods: In the prospective observational study, a total of 109 whole blood donors were enrolled using the purposive sampling method. A pre-donation sample was collected for haemoglobin (Hb) and serum uric acid (UA) levels. PRBC aliquots were tested for potassium, lactate dehydrogenase (LDH), Hb, haematocrit, plasma Hb and haemolysis on days 1, 21 and 35 of storage. The association of these parameters with donor age, sex, donation status, dietary pattern and body mass index was determined.

Results: Mean haemolysis was significantly higher in PRBCs from donors with UA levels ≤6 mg/dL than donors with UA levels >6 mg/dL on day 35 of storage (0.22 ± 0.11 vs. 0.18 ± 0.07, $p = 0.03$). Median plasma Hb (mg/L) was significantly higher in PRBCs from first-time donors on day 21 (586 vs. 509, $p = 0.05$) and day 35 (1507 vs. 1358, $p = 0.02$) of storage in comparison to frequent donors. Significantly higher mean potassium ($p = 0.04$ day 1; $p = 0.02$ day 21) and median LDH values ($p = 0.02$ day 1, $p = 0.05$ day 21) were observed in PRBCs from male donors. A statistically significant positive association was observed between donor UA and LDH levels of PRBCs on day 35 of storage (β coefficient: 715.52, p-value: 0.003) on multiple regression analysis.

Conclusion: In vitro haemolysis of PRBCs is affected by blood donor characteristics.

Keywords

donor characteristics, packed red blood cells, storage haemolysis

Highlights

- A significantly higher rate of haemolysis was observed in packed red blood cells (PRBCs) from donors with low uric acid (UA) levels during the storage period.
- The median plasma haemoglobin level was significantly higher in PRBCs from first-time donors on days 21 and 35 of storage.
- A statistically significant positive association was observed between donor UA levels and lactate dehydrogenase levels in PRBCs on day 35 of storage on multiple regression analysis.

INTRODUCTION

Packed red blood cells (PRBCs) are the most frequently transfused blood product in foetal and neonatal medicine, high-risk obstetric care, trauma, surgery, cancer, haematological diseases and degenerative conditions [1]. During storage, red blood cells (RBCs) undergo numerous time-dependent structural and biochemical changes that can adversely affect their post-transfusion recovery and also increase the risk of adverse reactions in the recipients. These time-dependent modifications and deterioration in a PRBC unit are collectively known as RBC storage lesions. These RBC storage lesions can lead to haemolysis, the release of potassium and micro-particle accumulation, resulting in reduced post-transfusion survival [2]. The exact mechanisms behind these storage lesions are uncertain, but the donor variation effect that refers to substantial donor-to-donor differences is one of the important contributing factors [3].

Inherent inter-donor variability plays an important role in determining the quality of PRBCs. Studies have found that PRBC products prepared from male blood donors consistently have higher haemolysis compared with their female counterparts, due to higher testosterone levels, which increases RBCs' susceptibility to haemolysis [3, 4]. In addition, a protective effect of progesterone has also been demonstrated on the red cell membrane [5]. The rate of PRBC haemolysis has been observed to differ with age in males and females [6, 7]. The frequency of blood donation has also emerged as one of the possible factors affecting red cell susceptibility to storage and stress-induced haemolysis [8]. Dietary habits can also affect RBC quality by affecting donor haemoglobin (Hb) and plasma antioxidant levels [9, 10]. A study was conducted to compare the effect of vegetarian and non-vegetarian diets on lipid peroxidation and antioxidant status. The authors observed a significant decrease in antioxidant glutathione peroxidase and non-enzymatic antioxidants in individuals consuming a non-vegetarian diet as compared with those consuming a vegetarian diet ($p < 0.001$) [11]. Blood donor body mass index (BMI) may alter the RBCs' susceptibility to peroxidation and free radical-induced haemolysis [12]. Uric acid (UA) acts as a major in vivo scavenger of hydroxyl/oxygen radicals and other intracellular reactive oxidant species [5]. According to a study, PRBCs donated by regular donors with higher UA levels (7.56 ± 0.59 mg/dL) showed decreased RBC haemolysis than regular donors with lower UA levels (6.01 ± 0.63 mg/dL) [13].

The relationship between blood donor characteristics and RBC haemolysis remains partly investigated. Therefore, the present study aimed to determine the association of blood donor characteristics with in vitro haemolysis in PRBCs during storage.

MATERIALS AND METHODS

The present prospective observational study was conducted in a tertiary care hospital over a period of 12 months from April 2020 to April 2021. The study was approved by the Institute Ethics Committee (No. GMCH/IEC/2019/150 dated 17 December 2019) and written informed consent was obtained from blood donors. Whole blood donors who approached the department either in-house or in outdoor blood donation camps during this period were screened as per the National Regulatory Authority [14]. A total of 109 blood donors were selected using a purposive sampling method. Blood donors not meeting eligibility criteria, where blood was not collected by single venepuncture, under or over-collected bags, blood bags reactive for any transfusion-transmitted infection (TTI), and technical errors during component preparation were excluded from the study. Blood donor characteristics such as age, sex, donation frequency, dietary pattern, height and weight (for BMI calculation) were recorded on pre-structured donor proforma.

Pre-donation blood donor sample

A pre-donation sample was taken from the enrolled study subjects in ethylene diamine tetraacetic acid (EDTA) and plain vial. The EDTA sample was subjected to pre-donation Hb measurement using a cell counter (LH 780, Beckman Coulter India Pvt. Ltd., Mumbai, India). The serum from a plain vial was separated using a tabletop centrifuge at 2000*g* for 3 min (Remi Elektrotechnik Ltd., Mumbai, India). Serum UA levels were estimated using a Chemistry analyser (AU5800, Beckman Coulter India Pvt. Ltd., Mumbai, India).

Blood collection

Whole blood was collected from enrolled blood donors in double blood bags (top and top blood bags with 350 mL capacity) containing 49 mL citrate phosphate dextrose adenine (CPDA-1) anticoagulant (Terumo Penpol, Thiruvananthapuram, India) via single clean venepuncture. The whole blood collected was then transported to the component preparation laboratory in transport boxes maintaining the cold chain. PRBCs and fresh frozen plasma were prepared on the same day within 6 h of collection by the same trained dedicated medical technologist as per the departmental standard operating procedure. The PRBCs were non-leukoreduced and suspended in plasma to maintain a haematocrit of 65%–70%. On the same day, 50 mL aliquots were prepared from the PRBC units using a sterile connecting device (Terumo Penpol, Thiruvananthapuram, India). The transfer bag aliquots (Terumo Penpol, Thiruvananthapuram, India) were stored under the same storage conditions at 2–6°C. The remaining PRBC units were transferred to the inventory for issuing to patients.

Aliquot sampling

Sampling from the aliquot was done after stripping the bag thrice using a tube stripper (Terumo Penpol, Thiruvananthapuram, India), to ensure a representative sample of the bag. Samples from these PRBC

aliquots were tested on days 1, 21 and 35 of storage. Hb and haematocrit were measured using a cell counter (LH 780, Beckman Coulter India Pvt. Ltd., Mumbai, India). PRBC samples were subsequently centrifuged at 2000*g* for 3 min and the supernatant was subjected to potassium and lactate dehydrogenase (LDH) levels using a Chemistry analyser (AU5800, Beckman Coulter India Pvt. Ltd., Mumbai, India) on the same day.

Plasma Hb was measured using the peroxidase method [15]. In this method, the peroxidase activity of the haem portion of the Hb molecule is utilized. It brings about the oxidation of benzidine by hydrogen peroxide to give a green colour, which changes to blue and green and finally reddish violet. The intensity of the colour developed is read photometrically at 515 nm.

Storage haemolysis was calculated using the formula [8]:

$$\text{Storage haemolysis}\,(\%) = \frac{(100 - \text{haematocrit}) \times \text{plasma Hb}\,(\text{g/dL})}{\text{Hb of PRBC}\,(\text{g/dL})}.$$

TABLE 1 Packed red blood cell (PRBC) parameters during storage.

Parameters	Day 1	Day 21	Day 35	*p*-value*
Potassium (mEq/L), Mean ± SD	7.95 ± 2.56	51.57 ± 10.49	72.37 ± 11.96	<0.01
LDH (U/L), Median (IQR)	590 (472–970)	3023 (2256–3848)	6132 (5127–8747)	<0.01
Plasma Hb (mg/L), Median (IQR)	103 (62–155)	563 (432–700)	1450 (1220–1696)	<0.01
Haemolysis (%), Mean ± SD	0.02 ± 0.02	0.08 ± 0.06	0.2 ± 0.09	<0.01

Abbreviations: Hb, haemoglobin; IQR, interquartile range; LDH, lactate dehydrogenase; SD, standard deviation.
*Using repeated measures ANOVA.

TABLE 2 Donor characteristics and potassium levels (mEq/L) of packed red blood cells (PRBCs) during storage.

Parameter	Day 1	Day 21	Day 35
Age (years)			
18–34 years ($n = 56$), Mean ± SD	8.25 ± 2.82	51.38 ± 11.24	73.04 ± 12.54
35–65 years ($n = 50$), Mean ± SD	7.61 ± 2.21	52.00 ± 9.74	71.41 ± 11.71
p-value*	0.20	0.76	0.49
Sex			
Male ($n = 98$), Mean ± SD	8.09 ± 2.59	52.38 ± 10.52	72.92 ± 12.24
Female ($n = 8$), Mean ± SD	6.20 ± 1.15	43.01 ± 5.51	64.32 ± 7.26
p-value*	**0.04**	**0.02**	0.05
Donation status			
FTD ($n = 52$), Mean ± SD	8.17 ± 2.56	51.03 ± 10.85	72.50 ± 11.45
FD ($n = 54$), Mean ± SD	7.73 ± 2.57	52.59 ± 10.24	72.05 ± 12.85
p-value*	0.38	0.54	0.85
Dietary pattern			
VD ($n = 36$), Mean ± SD	7.78 ± 2.19	49.69 ± 8.02	69.48 ± 9.11
NVD ($n = 70$), Mean ± SD	8.04 ± 2.74	52.46 ± 11.43	73.71 ± 13.25
p-value*	0.62	0.20	0.09
BMI (kg/m^2)			
HWD ($n = 48$), Mean ± SD	7.90 ± 2.66	50.38 ± 11.41	72.59 ± 12.38
OWD ($n = 58$), Mean ± SD	7.98 ± 2.50	52.74 ± 9.68	72.01 ± 12.02
p-value*	0.87	0.25	0.81
Uric acid (mg/dL)			
≤6 mg/dL ($n = 51$), Mean ± SD	7.74 ± 2.42	49.78 ± 7.75	69.58 ± 9.83
>6 mg/dL ($n = 55$), Mean ± SD	8.14 ± 2.69	53.43 ± 12.36	74.77 ± 13.54
p-value*	0.66	0.07	**0.03**

Note: Bold *p*-values indicate statistically significant results.
Abbreviations: BMI, body mass index; FD, frequent donor; FTD, first-time donor; HWD, healthy weight donor; NVD, non-vegetarian donor; OWD, overweight obese donor; SD, standard deviation; VD, vegetarian donors.
**p*-value calculated using Student's *t*-test.

Categorization for analysis

Blood donors were categorized into ≤35 (young) and >35 years (old) based on age; sex (male and female donors); first-time (blood donors who have not donated whole blood till date) and frequent (blood donors who have donated whole blood earlier at least once in the preceding year) based on donation status; vegetarian (never consumed non-vegetarian food in their life) and non-vegetarian based on dietary pattern, and healthy (BMI between 18.5 and 24.9 kg/m^2) and overweight (BMI ≥ 25.0 kg/m^2) based on BMI [16]. The UA cut-off level for analysis was 6 mg/dL based on laboratory reference range for ease of comparison amongst study subjects (males: 2.4–7 mg/dL, females: 2.4–5.7 mg/dL). The association of donor factors was then analysed with PRBCs potassium, LDH, plasma Hb and percentage haemolysis throughout storage. Percentage haemolysis is a parameter calculated using haematocrit, plasma Hb and Hb of PRBC, while a change in the levels of potassium, LDH and plasma Hb in PRBCs is a direct indicator of red cell lysis and hence, these parameters were selected for analysis.

Statistical analysis

As per the Drugs and Cosmetics Act, only 1% of the PRBCs and components prepared are subjected to quality control [13]. Considering an annual blood collection of 22,000 units, the sample size came out to be 92 subjects at a confidence level of 95% and a margin of error of 7.5%. For possible attrition, it was decided to include 10% extra subjects so the final sample size target was 100 subjects. A total of 109 blood donors were recruited, and data from 106 blood donors were analysed.

Data were compiled using MS Excel and analysis was performed using the Statistical package SPSS-20. For normally distributed quantitative variables, mean and standard deviation (SD) were used, while, for skewed data, median with an interquartile range was used. Blood donor characteristics and laboratory values of the blood donors or PRBCs were compared using parametric and non-parametric tests as appropriate: Student's *t*-test, and Mann–Whitney *U* test. Repeated measures analysis of variance (ANOVA) was applied for intergroup comparisons. Multiple regression analysis was performed to

TABLE 3 Blood donor characteristics and lactate dehydrogenase (LDH) levels (U/L) of packed red blood cells (PRBCs) during storage.

Parameter	Day 1	Day 21	Day 35
Age (years)			
18–34 years (n = 56), Median [IQR]	731 [471–1004]	3100 [1985–3985]	6142 [4839–9085]
35–65 years (n = 50), Median [IQR]	578 [468–972]	2960 [2348–3722]	6121 [5235–7731]
p-value*	0.24	0.92	0.94
Sex			
Male (n = 98), Median [IQR]	616 [481–984]	3045 [2302–3981]	6212 [5129–8901]
Female (n = 8), Median [IQR]	452 [414–536]	2291 [1935–2928]	5294 [4249–6106]
p-value*	0.02	0.05	0.06
Donation status			
FTD (n = 52), Median [IQR]	686 [473–1042]	2799 [1854–3627]	5760 [4680–7833]
FD (n = 54), Median [IQR]	578 [469–909]	3323 [2802–4407]	6550 [5559–9168]
p-value*	0.401	**0.006**	0.06
Dietary pattern			
VD (n = 36), Mean ± SD	747 ± 481	3769 ± 2091	7234 ± 3270
NVD (n = 70), Mean ± SD	830 ± 641	3184 ± 1575	6690 ± 2982
p-value**	0.5	0.11	0.39
BMI (kg/m^2)			
HWD (n = 48), Median [IQR]	590 [409–1043]	2784 [1855–3586]	5979 [4175–8048]
OWD (n = 58), Median [IQR]	590 [510–953]	3323 [2774.50–4443]	6192.50 [5469–9168]
p-value*	0.43	**0.003**	0.08
Uric acid (mg/dL)			
≤6 mg/dL (n = 51), Median [IQR]	570 [458–980]	2829 [2180–3630]	5705 [4570–7443]
>6 mg/dL (n = 55), Median [IQR]	731 [497–972]	3150 [2563–4428]	6700 [5652–9399]
p-value*	0.82	0.67	0.43

Note: Bold p-values indicate statistically significant results.
Abbreviations: BMI, body mass index; FD, frequent donor; FTD, first-time donor; HWD, healthy weight donor; IQR, interquartile range; NVD, non-vegetarian donor; OWD, overweight obese donor; SD, standard deviation; VD, vegetarian donors.
*p-value using Mann–Whitney test.
**p-value calculated using Student's t-test.

determine the association of donor characteristics with indicators of PRBC haemolysis on day 35 of storage. The *p*-value ≤0.05 was considered statistically significant.

RESULTS

Donor characteristics

Of 109 blood donors enrolled, 3 blood donors were excluded: 1 blood donor unit was HIV reactive and the other 2 donor units were under-collected. Data from 106 blood donors were included in the study.

In the present study, the majority were male ($n = 98$, 92.5%) and frequent ($n = 54$, 50.9%) blood donors. The mean age of the study population was 35.07 ± 9.8 years with a range of 19–60 years. Of 54 frequent blood donors, 94.4% ($n = 51$) had a donation frequency of three times in a year. Of 106 blood donors, 54.7% were overweight ($n = 58$) and 66% were non-vegetarians ($n = 70$). The majority ($n = 55$, 51.8%) of donors had serum UA levels >6 mg/dL with a mean value of 6.0 ± 1.25 mg/dL (range: 2.8–9.6 mg/dL).

The mean pre-donation Hb of donors was 14.75 ± 0.19 g/dL (males: 15.4 ± 0.51 g/dL; females: 12.5 ± 0.12 g/dL). The mean Hb of PRBCs was 22.4 ± 2.4 g/dL (males: 23.2 ± 3.2 g/dL; females: 19.5 ± 0.6 g/dL).

PRBC storage parameters

Mean potassium, median LDH, median plasma Hb and mean storage haemolysis of PRBCs were observed to increase over storage. Haemolysis in the PRBCs did not exceed the permitted limit of 0.8% throughout the storage. Repeated measures ANOVA for potassium, LDH, plasma Hb and haemolysis were statistically significant along with inter-group comparisons from days 1 to 21, 21 to 35 and 1 to 35 (*p*-value < 0.01) as shown in Table 1.

TABLE 4 Donor characteristics and plasma haemoglobin levels (mg/L) of packed red blood cells (PRBCs) during storage.

Parameter	Day 1	Day 21	Day 35
Age (years)			
18–34 years ($n = 56$), Median [IQR]	98 [62.33–153.45]	527.05 [368.12–725.19]	1380.50 [1191.82–1565.50]
35–65 years ($n = 50$), Median [IQR]	103.80 [60–154.20]	557.40 [450.62–645.25]	1499.50 [1220.92–1805.75]
p-value*	**0.02**	0.09	0.25
Sex			
Male ($n = 98$), Median [IQR]	99.5 [60.63–148.20]	543.05 [382.02–674.00]	1450.30 [1186.82–1711.75]
Female ($n = 8$), Median [IQR]	148.65 [51.48–180.72]	527.80 [289.75–784.50]	1442.25 [1363.75–3050.00]
p-value*	0.41	0.96	0.36
Donation status			
FTD ($n = 52$), Median [IQR]	88.20 [46.15–146.60]	586.30 [369.12–890.25]	1507 [1297.38–1892.90]
FD ($n = 54$), Median [IQR]	106.55 [72.32–154.20]	509.50 [388.18–605.75]	1358 [1145.15–1558.25]
p-value*	0.20	0.05	**0.02**
Dietary pattern			
VD ($n = 36$), Mean ± SD	127.26 ± 103.82	586.52 ± 386.94	1453.53 ± 418.10
NVD ($n = 70$), Mean ± SD	161.86 ± 222.41	706 ± 615.72	1668.43 ± 802.53
p-value**	0.38	0.29	0.14
BMI (kg/m^2)			
HWD ($n = 48$), Median [IQR]	103.40 [43.95–176]	591.20 [376.75–900]	1457.70 [1251.25–1851.75]
OWD ($n = 58$), Median [IQR]	100.70 [66.00–146.02]	499.50 [381.28–615.50]	1378.50 [1147.65–1613.50]
p-value*	0.79	**0.01**	0.13
Uric acid (mg/dL)			
≤6 mg/dL ($n = 51$), Median [IQR]	97 [63–146.60]	566.60 [358.60–780]	1450 [1255.60–1765]
>6 mg/dL ($n = 55$), Median [IQR]	105.30 [60–153.60]	534.50 [394–672]	1435 [1160–1699]
p-value*	0.82	0.67	0.43

Note: Bold *p*-values indicate statistically significant results.

Abbreviations: BMI, body mass index; FD, frequent donor; FTD, first-time donor; HWD, healthy weight donor; IQR, interquartile range; NVD, non-vegetarian donor; OWD, overweight obese donor; SD, standard deviation; VD, vegetarian donors.

*p-value using Mann–Whitney test.

**p-value calculated using Student's *t*-test.

Association of donor characteristics with potassium levels of PRBCs during storage

Significantly higher mean potassium values were observed in PRBCs from male donors than female donors on all days of storage (*p*-value: 0.04 day 1; 0.02 day 21; 0.05 day 35) and donors with higher UA values (>6 mg/dL) than donors with UA values ≤6 mg/dL on day 35 of storage (*p*-value: 0.03, Table 2).

Association of donor characteristics with LDH levels of PRBCs during storage

Median LDH values of PRBCs were significantly higher in male donors on day 1 (*p*-value: 0.02) and day 21 (*p*-value: 0.05) of storage and were also higher on day 35 though the difference was not statistically significant (*p*-value: 0.06). PRBCs from first-time donors had higher median LDH values on day 1 of storage while over the storage period, the values were greater in frequent donors with a significant difference on day 21 of storage (*p*-value: 0.006). PRBCs from overweight donors had higher median LDH values on days 21 and 35 of storage, and the difference was significant on day 21 (*p*-value: 0.003, Table 3).

Association of donor characteristics with plasma Hb levels of PRBCs during storage

Median plasma Hb values were higher in PRBCs from older donors throughout the storage period and the difference was statistically significant on day 1 (*p*-value: 0.02). Plasma Hb values were also significantly higher in first-time donors as compared with frequent donors on day 21 (*p*-value: 0.05) and day 35 (*p*-value: 0.02) of storage. Plasma Hb values were higher in PRBCs derived from healthy weight donors as compared with overweight donors throughout the storage period and the difference was statistically significant on day 21 of storage (*p*-value: 0.01). PRBCs from donors with UA levels ≤6 mg/dL had lower median plasma Hb on day 1 of storage. However, over the storage period, the plasma Hb values from blood donors with lower UA levels increased but were statistically non-significant (Table 4).

TABLE 5 Donor characteristics and storage haemolysis (%) of packed red blood cells (PRBCs) during storage.

Parameter	Day 1	Day 21	Day 35
Age (years)			
18–34 years ($n = 56$), Mean ± SD	0.019 ± 0.02	0.08 ± 0.05	0.19 ± 0.09
35–65 years ($n = 50$), Mean ± SD	0.020 ± 0.02	0.09 ± 0.07	0.21 ± 0.10
p-value*	0.99	0.32	0.4
Sex			
Male ($n = 98$), Mean ± SD	0.019 ± 0.02	0.08 ± 0.06	0.19 ± 0.08
Female ($n = 8$), Mean ± SD	0.025 ± 0.01	0.10 ± 0.08	0.30 ± 0.15
p-value*	0.49	0.31	**0.002**
Donation status			
FTD ($n = 52$), Mean ± SD	0.020 ± 0.02	0.09 ± 0.07	0.22 ± 0.11
FD ($n = 54$), Mean ± SD	0.018 ± 0.02	0.07 ± 0.05	0.18 ± 0.08
p-value*	0.72	0.08	0.43
Dietary pattern			
VD ($n = 36$), Mean ± SD	0.018 ± 0.02	0.076 ± 0.05	0.19 ± 0.06
NVD ($n = 70$), Mean ± SD	0.020 ± 0.02	0.086 ± 0.07	0.20 ± 0.11
p-value*	0.7	0.48	0.46
BMI (kg/m^2)			
HWD ($n = 48$) Mean ± SD	0.021 ± 0.02	0.099 ± 0.08	0.22 ± 0.12
OWD ($n = 58$) Mean ± SD	0.018 ± 0.02	0.069 ± 0.04	0.18 ± 0.07
p-value*	0.5	**0.02**	0.07
Uric acid (mg/dL)			
≤6 mg/dL ($n = 51$), Mean ± SD	0.020 ± 0.02	0.09 ± 0.08	0.22 ± 0.11
>6 mg/dL ($n = 55$), Mean ± SD	0.020 ± 0.02	0.07 ± 0.04	0.18 ± 0.07
p-value*	0.92	0.09	**0.03**

Note: Bold *p*-values indicate statistically significant results.
Abbreviations: BMI, body mass index; FD, frequent donor; FTD, first-time donor; HWD, healthy weight donor; NVD, non-vegetarian donor; OWD, overweight obese donor; SD, standard deviation; VD, vegetarian donors.
*p-value calculated using Student's *t*-test.

Association of donor characteristics with haemolysis of PRBCs during storage

Mean storage haemolysis was significantly higher in PRBCs from female donors on day 35 (p-value: 0.002). It was also higher in PRBCs from healthy weight donors as compared with overweight donors and the difference was statistically significant on day 21 of storage (p-value: 0.02). Over the storage period, mean haemolysis was observed to be higher in PRBCs from blood donors with lower UA levels (≤6 mg/dL) and the difference was statistically significant by the end of the storage period (p-value: 0.03, Table 5).

Multiple regression analysis

Multiple regression analysis was performed to determine the association between donor characteristics and parameters of PRBC haemolysis on day 35 of storage. A positive but statistically non-significant association was seen between donor UA levels and potassium levels of PRBCs (β coefficient: 1.77, 95% confidence interval [CI]: −0.10 to 3.64, p-value: 0.06). A statistically significant positive association was observed between donor UA levels and LDH levels of PRBCs (β coefficient: 715.52, 95% CI: 243.34–1187.71, p-value: 0.003).

DISCUSSION

PRBCs are one of the major cellular components of blood. The association between blood donor characteristics and PRBC quality has been a subject of interest in the recent past. However, the data are scarce in a developing country like ours. In the present study, we attempted to determine the association between donor characteristics and parameters of in vitro PRBC haemolysis during storage.

In our study, more than 60% of donors were below 35 years of age. This corroborates with the data on blood donor's age by the World Health Organization stating that the majority of the donors belong to younger age groups in low- and middle-income countries [17]. In the present study, mean potassium, median LDH, median plasma Hb and mean percentage haemolysis were comparable in both age groups over the storage period. The association between donor age and PRBC haemolysis during storage is debatable in the literature. Increased storage haemolysis has been observed with increasing age of males between 18 and 45 years with a decreasing trend in older donors as ageing reduces erythropoiesis, thereby leading to a decrease in RBC count, Hb level and haematocrit thus reducing haemolysis [6, 7, 18]. On the other hand, ageing may also increase haemolysis due to an increase in cholesterol/phospholipid ratio in the red cell membrane and oxidative stress altering the fluidity and deformability of red cells [18, 19].

The majority of donors were males (92.5%) in our study. Many female blood donors were deferred because of low Hb, low weight and poor venous access. In the present study, PRBCs prepared from male donors had significantly higher mean potassium and median LDH values while mean storage haemolysis was higher in PRBCs from female donors. In one of the studies, significantly higher storage haemolysis was observed in PRBCs prepared from male donors (0.41 ± 0.29%) as compared with female donors (0.35 ± 0.32%) at 39–42 days of storage. Besides this, it has been shown that RBCs from post-menopausal female blood donors and male donors showed higher mechanical fragility when compared with RBCs from pre-menopausal donors, suggesting that sex hormones could play a role in red cell haemolysis [20]. Studies with the inclusion of more female donors with adequate representation of both pre- and post-menopausal females could give a better insight into the association of donor sex with PRBC haemolysis.

PRBCs prepared from first-time donors had significantly higher median plasma Hb levels on day 35 of storage, while no association was seen with storage haemolysis. As per our National Regulatory Authority, males can donate whole blood after every 3 months (maximum donation frequency: 4 per year) while females are permitted to donate blood after every 4 months (maximum donation frequency: 3 per year). In our study, 94% of our frequent donors had a donation frequency of thrice a year. An increase in donation frequency was not seen to be associated with increased storage haemolysis in our study. Similarly, in another study, a decrease in RBC susceptibility to oxidative haemolysis was seen in frequent blood donors whereas no effect was observed on storage haemolysis [8].

No significant association of donor dietary habits with storage haemolysis in PRBCs was observed in our study. Literature reports that a vegetarian diet is associated with high levels of antioxidants [11]. However, to the best of our knowledge, no such study has been conducted to determine the association of vegetarian and non-vegetarian diets with PRBC haemolysis.

Median LDH values of PRBCs from overweight donors were higher than healthy weight donors. However, median plasma Hb and storage haemolysis were lower in PRBCs from overweight donors in our study and the difference was significant only on day 21 and not on day 35 of storage. On the contrary, Sparrow et al. found an overweight-obese BMI greater than 27 kg/m^2 in males to be significantly associated with higher haemolysis ($p < 0.0001$) with an odds ratio of 3 (95% CI: 1.3–6.7) which further increased to 4 (95% CI: 1.8–8.6) for obese males with BMI greater than 30 kg/m^2 [7]. We used a cut-off value of BMI ≥ 25 kg/m^2 to categorize donors into healthy weight and overweight donors as compared with the higher BMI cut-off used by Sparrow et al., which could be the reason for the differing results.

The blood donors were also segregated based on their serum UA values. It was observed that mean storage haemolysis was significantly lower in PRBCs from blood donors with high UA levels (>6 mg/dL) on day 35 of storage. In addition, median plasma Hb and mean LDH values were also lower in PRBCs from donors with high serum UA levels throughout the storage period. On multiple regression analysis also, a significant positive association was observed between donor UA levels and LDH levels of PRBCs. In corroboration with our results, authors in another study observed a statistically significant difference in mean percentage haemolysis in PRBCs between days 1 and 21 of storage from donors with low UA levels (<5 mg/dL) as compared with donors with high UA levels (>7 mg/dL). They concluded that high UA levels in donors have a protective effect on PRBCs during storage and UA can further be explored as a constituent of an additive solution [21].

Similarly, in another study, the authors evaluated the effect of the UA levels on haemolysis, plasma oxidant and antioxidant factors in leukoreduced PRBC units prepared from regular blood donors. The authors observed that PRBCs prepared from blood donors with UA levels >7.1 mg/dL showed significantly better intracellular calcium accumulation, total antioxidant capacity and low spheroechinocytes [13].

The present study had a few limitations. We analysed only in vitro haemolysis in PRBCs during storage. Other measures of haemolysis, such as oxidative and osmotic haemolysis, were not studied due to financial constraints. The data collected here are from PRBCs stored in 50 mL aliquots rather than standard storage bags. Hence, the data are not representative of standard clinical grade PRBCs (210–260 mL). In our study, due to the unequal distribution of male to female donors, the correlation between storage parameters and donor sex could not be confidently made. Moreover, the effect of donor characteristics on recipient outcomes was also not studied.

To conclude, donor characteristics do have an impact on in vitro haemolysis of PRBCs during storage. Prospective studies with a larger sample size and analysis of oxidative and osmotic haemolysis may give a better perception of the impact of inter-donor variability on the storability of PRBCs.

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B.D. collected and acquired the data; P.K. and K.M. conceptualized the study, collected, acquired and analysed the data, prepared the first draft of the manuscript; S.J. and A.T. analysed the data. R.K., T.S. and G.K. reviewed the manuscript; R.R. performed the statistical analysis.

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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Human immunodeficiency virus, hepatitis C virus and hepatitis B virus incidence in blood donors from 2000 to 2020 in France: Trends and lessons from haemovigilance surveillance

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Abstract

Background and Objectives: Data from 21 years (2000–2020) of haemovigilance were used to assess human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) incidence rates in repeat blood donors and the occurrence of transfusion-transmitted (TT) viral infections.

Materials and Methods: Blood donors who converted for HIV, HCV or HBV markers within serial three-year analysis periods were included. Epidemiological and virological data were retrieved from the national epidemiological donor database and were supplemented with information on blood components and the infection status of recipients of the previous negative donation (D.N-1) of donors who seroconverted.

Results: Incidence rates declined from 1.27 to 0.35/100,000 person-years for HIV, from 0.59 to 0.19 for HCV and from 1.66 to 0.18 for HBV. Risk factors and lookback for 232 HIV, 90 HCV and 74 HBV seroconversions were investigated. The main risk factor identified at post-donation interview was having sex with men (47.8% of males) for HIV and a sexual risk for HCV (30.6%) and HBV (37.1%). The viral loads and sequences were retrospectively tested in 191 HIV, 74 HCV and 62 HBV D.N-1 archived samples. Six (five HBV and one HIV-1) were positive all low viral loads. Two recipients were infected by red blood cells from two HBV seroconverting donors before the introduction of HBV-nucleic acid testing.

Conclusion: HIV, HCV and HBV incidence rates in blood donors declined over the two past decades in France. There is a very small risk of a blood component that tests negative entering the blood supply resulting in TT infections, especially after introduction of molecular assays in donor screening.

Keywords

blood donors; haemovigilance; HIV, HCV, HBV incidence

Highlights

- Human immunodeficiency virus (HIV), hepatitis C virus and hepatitis B virus (HBV) incidence rates in blood donors have declined over the two past decades in France.
- The systematic banking of a plasma sample of all donations led to the identification of retrospectively positive donations (five HBV-DNA and one HIV-1 RNA) with an extremely low viral load.
- There is a very small risk of a blood component that tests negative entering the blood supply resulting in transfusion-transmitted viral infections, especially after the introduction of molecular assays in donor screening.

INTRODUCTION

Measuring the incidence of infectious diseases transmitted by transfusion in the blood donor (BD) population is important for monitoring the safety of the blood supply. Several methods are currently used to estimate the incidence. Most of them are based upon newly infected repeat blood donors (RBDs), which is facilitated by the fact that the vast majority of blood donations in high-income countries are collected from such donors. This provides the opportunity to estimate the incidence in the blood supply and avoid tedious and expensive longitudinal cohort studies. Alternative approaches are based on yield rates from nucleic acid testing (NAT) and/or limiting antigen avidity assay when available, allowing incidence to be evaluated in first-time blood donors (FTBDs) as well [1].

The incidence rate in RBDs is also used along with the duration of the window period (WP) to calculate the residual risk (RR) of infections with human immunodeficiency virus (HIV), hepatitis B virus (HBV) or hepatitis C virus (HCV) [2, 3]. Indeed, it is now admitted that the major RR of transfusion-transmitted infections (TTIs) is due to donations given during the WP and that such risk is linked to the incidence of infections. Based on the incidence/WP model, the global RR of non-detection of viral infections in France during the 2019–2021 period has been estimated at 0.27 per million donations tested (0.058, 0.178 and 0.031 per million donations for HIV, HBV and HCV, respectively) [4, 5].

In addition, RBDs who recently acquired viral infections provide relevant information on the epidemiology and characteristics of recent infections, and lookback studies allow observed TTI to be compared with the estimated RR. A haemovigilance network, which collects information on adverse events and reactions in blood transfusions from healthcare organizations involved in the transfusion of blood components, is an essential tool to reach this goal.

We used data collected over 21 years of the French BD haemovigilance system, in place since 1994, to analyse trends in the incidence rates of HIV, HBV and HCV and the characteristics of BDs who have recently developed these viral infections. In addition, we analysed the results of lookback studies to assess the occurrence of TTI in recipients who were transfused with the last negative tested donation of RBDs who seroconverted and compared the data with the theoretical RR.

MATERIALS AND METHODS

BD surveillance and the haemovigilance process

In the past two decades, approximately 2.8 million donations from 1.5 million donors were collected per year in France. Donors represent around 4% of the general population between the ages of 18 and 70 and approximately 80% are donors who have made at least two donations in their lifetime. All donations are tested for several viral markers: anti-HIV-1/-2 antibodies (Abs), hepatitis B surface antigen (HBsAg), anti-hepatitis B core antibodies (anti-HBc Abs), anti-HCV Abs, anti-human T cell leukemia virus Abs (restricted to FTBDs in mainland France and in the Ile de la Réunion since 2019), and HIV-1, HCV and HBV-NAT.

Donors found positive for tested markers are recalled to be retested to confirm infection and are invited to complete a questionnaire collecting data including their risk factors for infection. All data are collated in a national epidemiological donor database, which contains demographic characteristics (age and sex), the most likely route of transmission, geographical origin (location of birth or ethnicity), as well as the virological characteristics (viral loads [VLs] and sequence data) of positive donors, together with the total number of donations and donors classified per status (FTBDs or RBDs).

According to our haemovigilance procedures, when an RBD is found positive for a viral marker, the archive sample of the most recent negative donation is requested for retrospective re-testing. The repository plasma sample from the previous negative donation (D.N-1, archived for 3 years) is subject to investigations at the National Reference Centre (NRC) for infectious risks in transfusion. This consists of analysis of the viral genome by NAT, VL and sequencing in order to confirm the safety of blood components from the D.N-1. If this sample is retrospectively found to be positive, a clinical lookback is carried out: the associated components are traced, recipients are identified and advice is given on follow-up and testing.

Blood donation testing

In the study period, all blood donations were screened for HIV Abs, HBsAg, HBc Abs and HCV Abs with PRISM assays (Abbott Diagnostics, Rungis, France) in mainland France and ARCHITECT assays (Abbott) in the overseas territories (French West Indies, Reunion

Island). HIV-1 and HCV-NAT screening was introduced in 2001 and performed in continental France in pool format by using either Chiron Procleix TMA HIV-1/HCV (Chiron, Emeryville, USA) in pools of 8 or Roche Cobas Ampliscreen HIV-1 and HCV (Roche Diagnostic, Meylan, France) in pools of 24, while it was performed in individual donation (ID) sample testing with Chiron assay in the overseas territories [6]. As of 2010, blood donations have been tested for viral genomes in ID testing with Procleix Ultrio assays (Grifols, Barcelona, Spain), including HBV-DNA testing.

A confirmatory and follow-up algorithm including HIV or HCV immunoblots and neutralization procedure for HBsAg was in place to classify repeatedly reactive samples by serology.

Investigations performed in archived samples at the NRC included plasma VL determination using the Cobas TaqMan assays (Roche Diagnostic) with a 95% limit of quantification (95% LOQ), at 34 copies/mL for HIV, 6 IU/mL for HBV and 15 IU/mL for HCV (data from package inserts). Molecular studies aimed at comparing viral strains when transfusion transmission was suspected based on NAT-positive archived donation and linked recipient NAT-positive samples consisted of sequencing at least two independent regions of the viral genome, as previously described [7–11].

Study population

BDs included in the study were those who donated between January 2000 and December 2020 and who converted from negative to confirmed positive for HIV, HCV or HBV (Abs and/or NAT for HIV and HCV, HBsAg and/or NAT positive for HBV) within a 3-year period. The 3-year study period was chosen because it corresponds to the period during which archived plasma samples are stored, so that information on the infectious status of previous negative donations is available.

Demographic, epidemiological and virological data were retrieved from the national epidemiological donor database and supplemented with information on blood components and the recipients of previous negative donations of seroconverters.

Incidence rate

RBDs included in this analysis were also used to assess HIV, HBV and HCV incidences in seven sequential 3-year periods from 2000–2002 to 2018–2020. Incidence was calculated as the number of donors who converted from negative to confirmed positive in one of the 3-year intervals, divided by the total person-years (PY) as previously described [3, 12].

Statistical analysis

To compare demographics between the different groups analysed, a chi-square analysis or Fisher's exact test, where appropriate, was used. Observations were considered statistically different if $p < 0.05$.

RESULTS

Seroconversions and incidence rates in RBDs

From January 2000 to December 2020, 58,160,984 blood donations were collected from 33,737,439 donors, of whom 26,322,760 (78%) were RBDs. The total number of seroconversions (SCs) was 241, 92 and 74 for HIV, HCV and HBV, representing 67%, 25% and 54% of the total number of HIV-, HCV- and HBV-positive RBDs, respectively (Table 1). Of these, 396 (232 HIV, 90 HCV and 74 HBV SC) were included in the study. No information was available for 11 HIV- and 2 HCV-infected donations.

Between 2000–2002 and 2018–2020 seven 3-year periods, incidence rates calculated as described above declined from 1.27 to 0.35 per 100,000 PY (divided by 3.6) for HIV, from 0.59 to 0.19 (divided by 3.1) for HCV and from 1.66 to 0.18 (divided by 9.2) for HBV (Figure 1).

Characteristics of seroconverted BDs

Among the 232 HIV SC, 184 were males and 48 were females (sex ratio 3.8), with a mean age of 37.8 and 35.2 years respectively (not significantly different). The mean inter-donation interval (between positive donation and D.N-1) was 316 days (median 227, range 28–1096). Information on risk factors was available for 228 donors (98.3%); 180 were men among which 47.8% reported having sex with men (MSM) with an increasing frequency between 2000–2002 (36.7%) and 2018–2020 (54.5%) (Figure 2); 34.4% reported a heterosexual sexual risk (including multiple sexual partnerships, a partner who is HIV positive or from a country with high HIV prevalence or sex work), 2.2% declared another risk factor (tattoo, healthcare professions, surgery, etc.) while for 15.6% the source of infection could not be identified. Among the 48 females, 35.4% had a sexual partner from an HIV endemic area, 18.8% had an HIV-positive sexual partner, 4.2% had another risk factor and 39.6% reported no risk factors (Table 2).

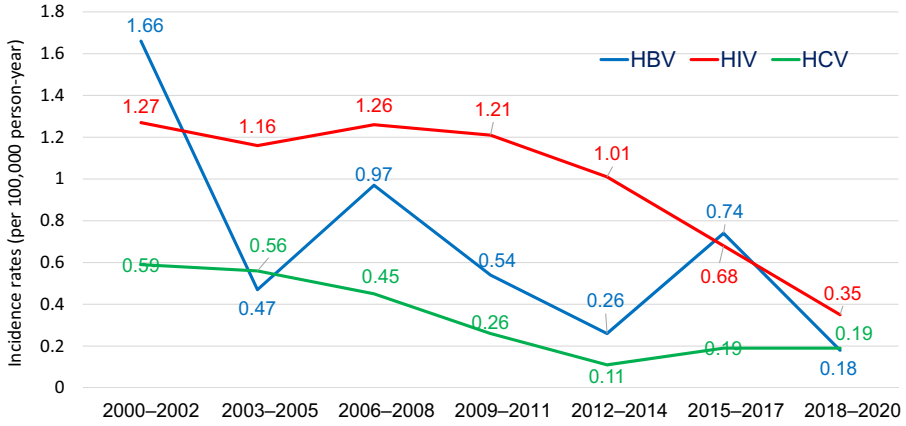
Among the 90 HCV SC, 34 were males and 56 were females (sex ratio 0.6), with a mean age of 45.6 and 41.5 years respectively (not significantly different). The mean inter-donation interval was 420 days (median 364, 66–1067). Seventy-five (83.3%) returned for the post-donation interview. Risk factors could not be identified for 29.3% of them, while 30.6% reported a sexual risk (out of whom 69% (16/23) had an HCV-positive partner), 18.7% had nosocomial exposure, 10.7% a healthcare professional risk and 4% reported drug use (Table 2).

Of the 74 HBV SC, 57 were males and 17 were females (sex ratio 3.3), with a mean age of 45.8 and 40.6 years respectively (not significantly different). The mean inter-donation interval was 243 days (median 159, 29–875). Information on risk factors was available for 62 donors (83.7%), of whom only 58.1% declared a risk factor. The main probable source of contamination was sexual for 37.1% (11.3% reported having an HBV-positive partner, 22.6% had an at-risk sexual contact and 3.2% were MSM) and 21% reported other probable

TABLE 1 Number of donations, donors, repeat blood donors (RBDs), RBDs positive for human immunodeficiency virus (HIV), hepatitis C virus (HCV) and hepatitis B virus (HBV), and HIV, HCV and HBV seroconvertions in previous 3 years from 2000 to 2020.

	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
N donations	2,475,102	2,409,484	2,459,663	2,465,382	2,498,298	2,512,795	2,575,273	2,713,582	2,818,689	3,001,321	3,004,863
N donors	1,530,000	1,522,000	1,537,000	1,523,000	1,535,900	1,506,082	1,527,209	1,635,578	1,643,493	1,701,018	1,662,994
N RBD	1,144,000	1,140,000	1,185,000	1,164,267	1,153,260	1,153,734	1,164,034	1,239,659	1,227,126	1,265,325	1,294,918
N HIV-positive RBDs	20	20	17	10	20	24	22	17	24	24	24
N HIV seroconversions	13	15	14	5	16	14	12	9	16	13	15
N HCV-positive RBDs	29	44	28	16	29	15	17	16	21	14	16
N HCV seroconversions	8	11	3	6	8	5	5	3	7	2	8
N HBV-positive RBDs	7	10	9	4	4	5	6	5	5	5	10
N HBV seroconversions	5	7	3	0	2	4	3	4	4	3	6
	2011	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**	**Total**
N donations	3,149,090	3,038,143	2,760,422	2,813,170	2,958,120	2,918,611	2,958,128	2,926,942	2,891,828	2,812,078	58,160,984
N donors	1,718,320	1,705,209	1,639,942	1,600,072	1,660,599	1,616,268	1,624,221	1,636,524	1,622,519	1,589,491	33,737,439
N RBDs	1,321,310	1,322,254	1,282,432	1,249,739	1,334,786	1,336,814	1,335,252	1,343,068	1,332,553	1,333,229	26,322,760
N HIV-positive RBDs	22	21	12	17	14	11	16	13	7	3	358
N HIV seroconversions	15	12	9	15	12	7	7	7	3	3	232
N HCV-positive RBDs	16	11	8	7	10	12	15	9	12	9	364
N HCV seroconversions	3	5	3	1	2	2	2	1	3	2	90
N HBV-positive RBDs	7	8	3	1	8	3	11	2	5	5	137
N HBV seroconversions	4	5	1	0	6	2	9	1	3	2	74

Note: Seroconversions: RBDs who converted from negative to confirmed positive within a 3-year period.
Abbreviation: FTBD, first-time blood donor.



HIV (*n*)	20	27	31	33	27	18	9
HCV (*n*)	13	13	11	7	3	5	5
HBV (*n*)	13	5	11	9	1	13	5

FIGURE 1 Trends in human immunodeficiency virus (HIV), hepatitis C virus (HCV) and hepatitis B virus (HBV) incidence in blood donors in France, 2000–2020. Incidence rates (expressed per 100,000 person-year [PY]) were calculated in seven consecutive 3-year periods (2000–2002 to 2018–2020) as the number of repeat blood donors (RBDs) who converted from negative to confirmed positive in the 3-year intervals for HIV, HCV or HBV markers, divided by the total PY (sum of days between the first and the last donation of positive and negative RBDs within the 3-year intervals/365). The number of incident cases is provided for each period.

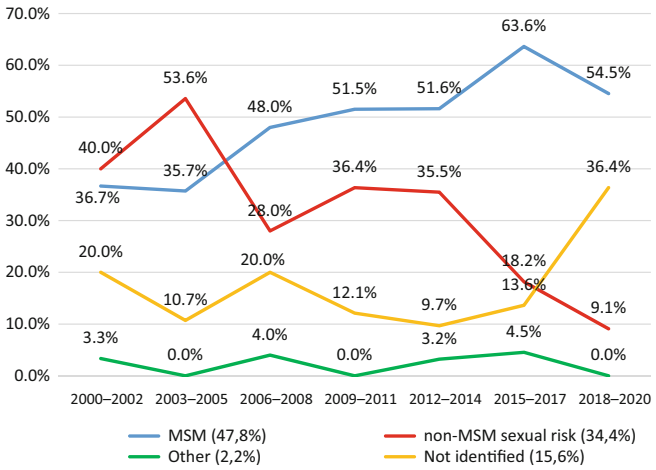


FIGURE 2 Trends in risk factors identified at post-donation interview in 180 male blood donors (BDs) who underwent a recent human immunodeficiency virus infection detected by BD screening over seven consecutive 3-year periods. MSM, men who have sex with men; non-MSM, sexual risk excluding MSM; other, healthcare professional risk.

sources of contamination, such as nosocomial or healthcare professional exposure (Table 2).

Lookback studies

Archived samples from D.N-1 were tested in 82.3% (191/232), 82.2% (74/90) and 83.4% (62/74) of HIV, HCV and HBV SC cases, respectively. Missing results were due to unavailable tubes or the loss of traceability but, in most cases, archived samples were not tested because during the post-donation interview, it had been shown that the donor had been infected between D.N-1 and the positive donation. Among the 327 D.N-1 samples tested overall, 6 were found to be retrospectively positive, all with a very low VL: 5/62 (8.1%) for HBV-DNA and 1/191 (0.5%) for HIV-1 RNA (Tables 3 and 4).

Out of the five patients who were transfused with blood products prepared from HBV-positive D.N-1, two were infected by the transfusion of red blood cell concentrates (Table 4). HBV isolates from donors and recipients showed 100% identities into genotypes A2 and B4, respectively, as previously described [7]. One recipient spontaneously recovered from infection while the second was successfully treated. No information regarding other blood products from these donations was available. Interestingly, the last D.N-1 that was retrospectively found to be HBV-DNA positive despite screening HBV-DNA negative was collected in 2019 from a donor with an HBc Ab negative occult B infection. Due to the donor's donation history, this case, described in detail elsewhere, was extensively investigated [4]. Briefly, of the nine previous donations given in the 3 years prior to the positive index donation, five were retrospectively found to be HBV-DNA-positive with an extremely low level, but none of the recipients included in the lookback were infected [4].

The lookback investigations from the HIV-positive D.N-1 revealed the absence of viral infection in the recipient transfused with the platelet concentrate which had been submitted to pathogen reduction, but transmission could not be excluded for the red blood cell concentrate recipient due to his/her death shortly after the transfusion [13].

TABLE 2 Demographic and epidemiological characteristics including risk factors of human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) seroconversions among repeat blood donors between 2000 and 2020.

		Males		Females		Inter-donation interval (days)	Risk factors	
Total		*N*	Age (years) Mean (range)	*N*	Age (years) Mean (range)	Mean–Median (range)		*N* (%)
HIV	232	184	37.8 (18–65)	48	35.2 (18–66)	316–227 (28–1096)	Males ($n = 180/184$)	86 (47.8%): MSM
								62 (34.4%): Sexual not MSM
								4 (2.2%): Other
								28 (15.6%): Not identified
							Females ($n = 48$)	17 (35.4%): Partner from endemic area
								9 (18.8%): Partner HIV positive
								2 (4.2%): Other
								20 (41.6%): Not identified
HCV	90	34	45.6 (20–70)	56	41.5 (18–65)	420–364 (66–1067)	All ($n = 75/90$)	16 (21.3%): Partner HCV positive
								14 (18.7%): Nosocomial
								8 (10.7%): Healthcare professional
								7 (9.3%): At-risk sexual partner (drug user, etc.)
								5 (6.7%): Other
								3 (4.0%): Drug use
								22 (29.3%): Not identified
HBV	74	57	45.8 (19–68)	17	40.6 (19–63)	243–159 (29–875)	All ($n = 62/74$)	14 (22.6%): Sexual
								13 (21%): Other
								7 (11.3%): Partner HBV positive
								2 (3.2%): MSM
								26 (41.9%): Not identified

Abbreviation: MSM, men who have sex with men.

TABLE 3 Summary of lookback investigations and outcomes of blood components and recipients from 396 included seroconversions.

	N donors	*N* D.N-1 retrospectively tested (n D.N-1 previously screened with NAT)	*N* D.N-1 positive (n D.N-1 previously screened with NAT)	*N* donations with fully completed lookback	*N* transfused donations	Recipients									
								Died		Not investigated		No information		Tested	Positive
						Total	%	*N*	%	*N*	%	*N*	%	*N*	
HIV	232	191 (154)	1 (1)	196	175	213	26.3	68	31.9	39	18.3	50	23.5	56	0
HCV	90	74 (51)	0	69	59	70	18.6	19	27.1	9	12.9	29	41.4	13	0
HBV	74	62 (31)	5 (1)	60	53	68	33.8	12	17.6	12	17.6	21	30.9	23	2
Total	396	327	6	325	287	351	26.2	99	28.2	60	17.1	100	28.5	92	2

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; NAT, nucleic acid testing.

TABLE 4 Lookback results of the six positive archived samples from N-1 donations.

	Donor						Recipient		
Virus	Year of positive donation	Gender/ age	Risk factor	Inter-donation interval (days)	Viral load of N-Viral load of N-1 positive donation	Stage of Stage of infection at D.N-1[a]	Transfused component	Transmission	References
HBV	2000	M/37	Heterosexual	72	Not available	Acute	RBC	No markers of past HBV infection, died before testing	
	2005	M/59	Not traced	95	<6 IU/mL	Acute	RBC	No recipient tested negative	
	2006	M/42	Not identified	74	<6 IU/mL	Acute	RBC	Yes	[7–11]
	2007	F/22	Not identified	102	<6 IU/mL	Acute	RBC	Yes	[7–11]
	2019	M/68	Not identified	182	<6 IU/mL	Occult B infection	RBCs + PLT + Plasma	No (but incomplete lookback)	[7–11]
HIV	2017	M/57	Not identified	135	<34 cps/mL	Acute	RBCs + PLT	No	[7–11]

Abbreviations: HBV, hepatitis B virus; HIV, human immunodeficiency virus; PLT, platelets; RBC, red blood cells.

[a]Acute stage was established by the detection of HBsAg and/or anti-HBc for HBV, and anti-HIV for HIV on the following sample(s), and occult B infection by the absence of evolution of the biological profile.

Information on blood components prepared from D.N-1 of all the 396 SC cases was missing for 71 of them. Of the remaining 327 donations, 287 resulted in one to three blood components transfused to 351 recipients (Table 3). Of these, 92 (26.2%) were tested despite our procedure, which stipulates that lookback should only be carried out on recipients of D.N-1 that tested positive on the archived sample: 56/213 (26.3%) for HIV, 13/70 (18.6%) for HCV and 33.8% (23/68) for HBV. Two of them were found to be HBV positive as described above.

DISCUSSION

This study aimed to characterize BDs who converted from negative to positive for HIV, HCV and HBV over seven successive 3-year periods, in order to describe and analyse the incidence of these infections in a 21-year study period in France. Furthermore, we capitalized on a haemovigilance system that has been in place in France for over 25 years to evaluate the occurrence of transfusion-mediated transmission.

The data reported here indicate a declining incidence of HIV, HCV and HBV infections in RBDs that were, respectively, 3.6 times, 3.1 times and 9.2 times lower in 2020 than in 2000. Apart from 2000–2002, the incidence of HIV has always been higher than HCV and HBV and was almost twice as high as HCV and HBV in 2018–2020, showing that new HIV infections occur more frequently than the other two viruses (Figure 1). This is illustrated by the higher percentage of SCs relative to the number of infected RBDs observed for HIV (67%), compared with HCV (24%) and HBV (54%). However, HIV incidence among BDs was 17 times lower than the estimated incidence in the general population (1.01/100,000 in 2012–2014 vs. 17/100,000 in 2014) and for HBV it was 1.7 times lower than in the general population (0.74 in 2015–2017 vs. 1.23/100,000 in 2016) [14, 15]. For HCV, a comparison between both populations was not possible because incidence in the general population is not available. The low incidence observed in the BD population is due at least in part to measures preventing infected donations from entering the blood supply, such as donor self-deferral or at-risk BD exclusion. For example, a recent study reported that countries with less stringent BD deferral criteria have higher HIV rates in their donor population [16].

An analysis of risk factors collected during the post-donation interview of positive donors provides insight into the most likely modes of acquisition of recent infections.

For HIV, the most common risk factor for males was MSM. Indeed, 56% of the 152 men with a recognized risk factor identified themselves as MSM. In France, MSM deferral for blood donation was reduced from permanent to 1 year since last sexual activity in July 2016 and to 4 months in April 2020 following reassuring risk analyses [17]. Notably, 8/12 HIV-positive male donors who gave blood between July 2016 and April 2020 (i.e., during the 12-month deferral period) and who reported MSM risk at the post-donation interview gave blood in the previous year although they would have been deferred with regard to the selection criteria in place at that time. This is consistent with non-compliance with BD selection criteria evidenced in a recent study performed in France showing that 0.73% [95% CI: 063–083] of male donors reported having sex with men in the 12 months prior to donation, the deferral period in place at that time [18]. In 2022, MSM-specific deferral criteria were removed for all blood donations. We could assume that a more individualized, behaviour-based deferral policy may increase donor adherence to the deferral criteria and ultimately increase blood safety.

For HCV, 43% (23/53 of donors with an identified risk factor) reported probably having been infected via a sexual route (16/23 had an HCV-positive partner). In the general population, newly acquired HCV infections have recently been observed in MSM, especially if they are co-infected with HIV [19, 20]. In our study, there were no self-reported MSM among the HCV SCs. In addition, the low number of donors reporting drug abuse ($N = 3$) is consistent with the decline in the incidence of HCV infection among drug users over the last two decades following the implementation of risk reduction policies in this population [21–23].

For HBV, being born in or having a relative from an endemic area has been reported as the most predictive factor for infection in chronically infected BDs, and recently acquired infections were most frequently associated with a sexual risk behaviour [4, 5, 24–26]. This is in line with the data since more than 68% of donors with acute infection (23/36 individuals with an identified risk factor) reported an at-risk sexual contact. However, the robustness of these findings is limited by the small numbers and proportions of interviewed HCV and HBV SC donors and uncertainty as to their representativeness.

Between 2000 and 2021, we identified five HBV and one HIV infections in retrospective testing of donations which were undetected as positive in routine screening. Of note, four of the five HBV donations were collected before the introduction of HBV-NAT and represent 13% of the 31 D.N-1 not screened for HBV-DNA but available for archive sample testing (Table 3). Two of them resulted in transmission to recipients. Assuming that these two transmissions could have been prevented by detection of HBV-DNA, this highlights the benefit of the introduction of NAT in reducing the RR of HBV infection.

Our haemovigilance procedures require patients transfused with components from the last negative donation of a seroconverted donor to be tested only if the archived sample is retrospectively positive. When the sample is no longer available, recipients are tested on a case-by-case basis according to the risk assessment. The review of 21 years of haemovigilance practice presented here shows that 92 of the 351 recipients involved in this analysis were tested (Table 3), whereas only 6 should have been tested given the 6 retrospectively positive D.N-1. The reasons for such off-protocol testing of recipients are probably multiple: (i) to save time for the rapid management of the patient in case of proven infection, (ii) to definitively rule out infection occurring with a donation with an undetectable VL. This strategy, while medically understandable, is questionable as it requires time and human and financial resources, could be a source of unnecessary anxiety for practitioners and patients and could create unfounded mistrust in the safety of blood components.

The main limitation of this study pertains to the focus on recent infections of less than 3 years for which samples were available in the frozen archive, which precluded the possibility of carrying out look-backs beyond this period and also possibly underestimated incidence rates. In addition, donors who have undergone SCs that were not identified by or reported to blood centres were excluded from the analysis. This has led to a possible underestimation of the number of transmissions. Nevertheless, the absence of HIV, HCV or HBV TTIs reported by the haemovigilance network since the introduction of NAT supports the reliability of our findings.

In conclusion, this study highlights the decline of viral incidence in BDs over the past two decades and shows that the epidemiological characteristics of BDs recently infected with HIV, HCV or HBV in France mirror those observed in the general population. These findings are reassuring with regard to the BD selection criteria, which are mostly driven by the knowledge of viral epidemiology in the general population. The extremely low number of viral transfusion transmissions reported here, even though possibly underestimated, demonstrated that there is a very low risk of a blood component that tests negative but contains infectious virus entering the blood supply, especially before the introduction of molecular diagnostics assays in blood screening [4, 5, 27–30]. Lookback investigations offer many other opportunities than simply proving or ruling out TTI, such as characterizing the factors that determine the transmission of infectious agents through blood and the determination of minimal infectious doses [13, 31, 32]. In addition, a well-organized haemovigilance system can be mobilized rapidly in the event of a new infectious disease emerging with potential transfusion implications, as recently demonstrated with COVID-19 [33]. Our extensive experience also highlights the importance of preserving donor samples, not only for TTI research but also for epidemiological studies [34, 35], although the short retention period of archived samples limits our ability to investigate newly identified or emerging infectious diseases that may have long clinical latency periods.

While national haemovigilance systems are well established in some countries, there is a lack of effective blood safety surveillance in many settings, such as in resource-limited areas. Continuous surveillance through a national haemovigilance network is fully justified and should be encouraged where it is not yet implemented.

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S.L. coordinated and contributed to the study design, collected and analysed the data and wrote this article; C.S., S.J. and L.C. acquired the data and contributed to the data analysis; J.Y.P. contributed to the study design; P.G., E.P., P.M., P.R., F.L. and P.T. contributed to the study and reviewed and edited the manuscript.

CONFLICT OF INTEREST STATEMENT

S.L., S.J., P.G., J.Y.P., L.C., E.P., P.R., P.M. and P.T. are employed by Etablissement Français du Sang, the French blood transfusion service in charge of collecting, manufacturing, testing and issuing blood components in France.

DATA AVAILABILITY STATEMENT

Anonymised data will be provided upon a justified request to the corresponding author.

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ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Incidence of acute haemolytic transfusion reaction among ABO-incompatible recipients transfused with A_3 blood: A case series

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Abstract

Background and Objectives: ABO antigens are among the most immunogenic, but the haemolytic risks of ABO incompatibilities involving a donor with a weak ABO phenotype are little documented.

Materials and Methods: This retrospective case series assessed the incidence of acute haemolytic transfusion reaction (AHTR) among ABO-incompatible recipients of A_3 blood in Québec (Canada). Transfusion safety officers reported laboratory AHTR indicators measured ≤24 h pre- and post-transfusion. Because the AHTR case definition of Québec's Hemovigilance System (QHS) leaves significant room for clinical judgement, a two-step approach was used to assess potential cases: Step 1 consisted in a highly sensitive—but unspecific—initial screen that identified all candidate cases per QHS case definition, and Step 2 consisted in a detailed review of candidate cases by two haematologists.

Results: Nine donors initially typed as Group B ($N = 1$) or O ($N = 8$) were subsequently found to display an A_3B or A_3O phenotype. Eighty-one recipients received ABO-incompatible blood, including 53 (65.4%) with interpretable data. Of these, 29 (54.7%) were classified as candidate cases after Step 1. Following Step 2, no conclusive evidence of AHTR was found: Abnormal pre- versus post-transfusion changes appeared modest, within normal range, insufficient to ascertain AHTR, or were consistent with a pre-existing condition unrelated to AHTR. Two candidate cases had a QHS-reported transfusion reaction; both were unrelated to AHTR.

Conclusion: In this case series, no conclusive evidence of serious AHTR was found among ABO-incompatible recipients who were inadvertently transfused with A_3 blood.

Keywords

acute haemolytic transfusion reaction, blood group incompatibility, haemovigilance, red blood cell transfusion, weak ABO subgroups

Highlights

- The haemolytic risks of ABO incompatibilities involving a donor with a weak ABO phenotype are little documented.

- In this case series, no conclusive evidence of serious AHTR was found among ABO-incompatible recipients who were inadvertently transfused with A_3O or A_3B red blood cells.
- These results suggest that no serious AHTR ensued from these transfusions.

INTRODUCTION

Acute haemolytic transfusion reaction (AHTR) is a rare but potentially life-threatening transfusion-related complication of immune or non-immune origin [1, 2]. Immune AHTR can result from the transfusion of red blood cells (RBCs) presenting cell-surface antigens that are incompatible with the recipient's antibodies. AHTRs due to ABO incompatibilities tend to show the most severe presentation [3], but their incidence has significantly declined in Québec (Canada) since the early 2000s due to the implementation of several safeguards (notably to prevent 'wrong blood in tube' errors and other patient identification errors) [4].

A number of rare, weak ABO subgroups (e.g., A_3, A_x, A_{el}, B_3, B_x, B_{el}) are associated with low levels of the A or B antigens on the surface of RBCs [5], which can make the interpretation of serologic test results difficult. A 1959 case report described a Group O female patient who accidentally received one packed RBC unit of A_X blood [6]. The patient subsequently experienced several signs and symptoms of AHTR, which suggests at least some weak A subgroups can cause AHTR [6].

However, we are unaware of more recent documented cases in which weak A or weak B blood was transfused to an ABO-incompatible recipient. Furthermore, the weak A phenotype identified in the aforementioned report might have been identified as a standard A phenotype with modern, highly sensitive reagents. Therefore, the haemolytic risks of ABO incompatibilities involving a donor with a weak ABO phenotype are little documented.

Our institution recently observed nine donors who were initially identified as Group O or B and were found to have an A_3O or A_3B phenotype at a subsequent donation. The RBC units collected before the detection of this typing discrepancy were transfused to 81 recipients, including Group O or B recipients or recipients who underwent stem cell transplant (SCT). Given the unclear haemolytic risks associated with such ABO incompatibilities, this case series investigated the potential incidence of AHTR among these recipients.

MATERIALS AND METHODS

Study design

This was a retrospective case series that identified ABO-incompatible recipients. Corresponding donors were initially typed O or B, but no anti-A1 on reverse grouping was detected at a subsequent donation. These donors were found to have an A_3O or A_3B phenotype through sequencing of the *ABO* locus (described in more detail below). All discrepancies were detected between 23 April 2020 and 12 March 2021 by Héma-Québec, the sole blood operator in Québec (Canada). Of note, no change in testing procedure, reagent or instrumentation occurred during this period. Before April 2020, donors with weak ABO subgroups were deferred from donating blood, but hospitals were not notified of weak ABO typing discrepancies. At the time, this practice was not evidence-based, as no recent studies had evaluated the outcomes associated with ABO incompatibilities involving a donor with a weak ABO subgroup (hence the need for the current case series).

As per Héma-Québec's quality control procedures, a corrective and preventive action was undertaken, which led to the conduct of the current study. Hospital transfusion safety officers (TSOs) were asked to complete an Excel form to report recipients' AHTR indicators. No institutional review board was required as this was a non-interventional, retrospective study that aimed to validate the quality of existing operations.

ABO typing

The initial typing results were obtained by using an automated microplate system (PK7200 from 2004 to 2010 and PK7300 from 2011 to 2021; Beckman Coulter, Fullerton, CA), with antisera, A1 and B cells purchased from Beckman Coulter. Samples with typing discrepancies subsequently underwent further manual serologic testing (i.e., immediate centrifugation, room temperature [RT] incubation for 30 min and warming at RT + 4°C for 60 min) with reagents purchased from various manufacturers.

Sequencing of the ABO locus

Genomic DNA was extracted from blood (in EDTA-containing collection tubes) by using the QIAamp mini blood kit (Qiagen Inc., Canada) following the manufacturer's instructions. Exons 2–7 of the *ABO* gene were PCR-amplified using primers described by Goebel et al. [7] and analysed by Sanger sequencing (ABI 3730xl, Applied Biosystems). Results were compared to the NG_006669 *ABO* reference sequence.

Recipient outcomes

For each recipient, TSOs were asked to report the following AHTR indicators of interest within 24 h pre- and post-transfusion (where applicable): haemoglobin (Hb) concentration; lactate dehydrogenase (LDH) concentration; haptoglobin concentration; results of polyclonal, immunoglobulin G-specific and C3-specific direct antiglobulin test (DAT; positive or negative); plasma appearance (icteric or normal); total and indirect bilirubin concentration; and haemoglobinaemia (present or absent). They were also asked

TABLE 1 Québec Hemovigilance System case definition of acute haemolytic transfusion reaction.

AHTR may be suspected based on the following (unspecific) symptoms
Fever, chills, hot flashes
Hypotension
Icteria
Haemoglobinuria (reddish or dark urine)
Diffuse bleeding
Disseminated intravascular coagulation
Low back pain, or abdominal and thoracic pain
Nausea, vomiting, diarrhoea
Oligoanuria
AND ≥1 of the following laboratory findings
Haemoglobinaemia
Elevated levels of unconjugated bilirubin
Haemoglobinuria
Elevated LDH and aspartate transaminase levels
Reduced haptoglobin levels
Reduced Hb levels OR unsatisfactory increase in Hb levels

Abbreviations: AHTR, acute haemolytic transfusion reaction; Hb, haemoglobin; LDH, lactate dehydrogenase.

whether a transfusion reaction was reported to Québec's Hemovigilance System (QHS).

These indicators were selected based on the AHTR case definition of QHS (Table 1) [8]. This definition includes the laboratory test results that are essential for diagnosis and certain symptoms (e.g., fever, hypotension)—which can support the diagnosis but are not essential. Therefore, data on symptoms were not collected because of (1) their limited specificity for AHTR, (2) their dispensable nature for diagnosis and (3) the anticipated heterogeneity in the reporting of clinical data (as some products were transfused 20 years ago). This approach additionally alleviated the burden of data collection for TSOs amid the COVID-19 pandemic and a severe staff shortage in Québec.

Data interpretation

Given the anticipated heterogeneity in the reporting of AHTR indicators, we restricted our analysis to recipients with at least some interpretable data, defined as recipients with available pre- and post-transfusion data for ≥1 indicator. As for several other haemovigilance systems, the QHS case definition of AHTR lacks clarity for certain criteria, which leaves room for clinical judgement when determining whether certain signs or symptoms are consistent with AHTR. We used a two-step approach to address this potential caveat: (1) An initial screen was performed to identify all possible cases (termed 'candidate cases'), defined as recipients with ≥1 potentially abnormal or inconclusive AHTR indicator per the QHS case definition; and (2) two haematologists specializing in transfusion medicine subsequently reviewed candidate cases to determine the likelihood of AHTR.

Step 1: Initial screen

The initial screen was designed to account for the lack of specificity of certain criteria in the QHS case definition. For example, the QHS case definition mentions that AHTR can be characterized by the following: (1) an increase in LDH levels, (2) a decrease in haptoglobin levels and (3) an increase in indirect bilirubin levels [8]. However, no threshold is specified for these changes. For these indicators, recipients exhibiting any change consistent with AHTR were thus conservatively classified as candidate cases.

The QHS case definition additionally mentions that AHTR can be characterized by a decrease or unsatisfactory increase in Hb levels [8], without specifying any threshold. For this indicator, we thus conservatively classified recipients exhibiting any decrease in Hb levels or an increase ≤10 g/L (i.e., the expected increase for a non-haemorrhagic adult transfused with one RBC unit) [9] as candidate cases.

For certain indicators, only pre- or post-transfusion data were available. Continuous or categorical indicators were not considered in data interpretation if only pre-transfusion data were available. Continuous indicators (e.g., biomarker levels) were considered as inconclusive if only post-transfusion data were available, because the unavailability of pre-transfusion results makes it impossible to assess causality with transfusion. Categorical indicators (e.g., positive vs. negative DAT) were considered as normal if only a post-transfusion result inconsistent with AHTR was available, and as inconclusive if only a post-transfusion result consistent with AHTR was available (as the abnormal result could be due to another pre-existing condition).

Categorical indicators that were abnormal pre- and post-transfusion were not considered to be potentially indicative of AHTR, as these were likely due to another condition.

Step 2: Review of candidate cases

While highly sensitive, the initial screen lacked specificity, hence the need for the clinical judgement of two haematologists (C.L. and N.R.) in Step 2. It was planned that a third haematologist would be consulted to resolve any disagreement between the two haematologists (but this situation did not occur). Reviewers exercised their clinical judgement to assess the likelihood of AHTR considering the full set of available indicators—a process similar to that used by the QHS.

RESULTS

Donors

One donor was initially typed as Group B and eight donors as Group O (Table 2). At a subsequent donation, the automated platform

failed to adequately detect anti-A1 in the blood of these donors, thereby generating typing discrepancies that were initially confirmed by manual serologic testing (Table S1).

These discrepancies were then resolved through sequencing of the *ABO* locus. All donors were carriers of the *ABO*A3.01* allele (Table 2). Eight donors had this allele in conjunction with various *O* alleles containing the c.261delG deletion (yielding an A_3O phenotype) and one in conjunction with the typical *ABO*B.01* allele (yielding an A_3B phenotype).

These donors gave a total of 93 packed RBC units from 2002 to 2020, including 82 that were transfused to Group O or B recipients and one transfused to a recipient who underwent SCT, for a total of 83 transfused RBC units transfused across 46 hospitals (Table 2 and Figure 1). The 10 other RBC units were either transfused to compatible Group A or AB recipients or were discarded by the hospital blood bank for various reasons. For 57 of these 83 transfusions, data were available on further compatibility testing performed at the hospital blood bank, and no unexpected incompatibilities were detected by immediate spin (for 25 transfusions), anti-human globulin crossmatch (for 23 transfusions) or electronic crossmatch (for 9 transfusions). Moreover, 39 donations were subject to confirmatory typing at the hospital blood bank, and no typing discrepancy was noted.

Recipient outcomes

These 83 RBC units were transfused to 81 unique recipients, including 2 recipients who received two RBC units (Figure 1; Table S2 for outcomes of all recipients). Of these, 53 recipients (65.4%) had interpretable data, and their AHTR indicators were analysed in Step 1 (see Table S3 for outcomes of recipients with interpretable data).

Step 1: Initial screen

Following the initial screen, 24 (45.3%) recipients exhibited no evidence of AHTR (Figure 1). The remaining 29 recipients had ≥1 potentially abnormal or inconclusive AHTR indicator and were considered as candidate cases in Step 2 (Table 3).

Step 2: Review of candidate cases

Overall, there was no conclusive evidence of serious AHTR in these 29 recipients. Among those with an unsatisfactory increase

TABLE 2 *ABO* genotype and ABO phenotype of included donors.

	ABO genotype		ABO phenotype		RBC units transfused to	
Donor #	Allele 1	Allele 2	Previous donation(s)	Last donation (before deferral due to weak ABO subgroup)	Group O or B recipients	Recipients who underwent SCT
1	*ABO*A3.01*	*ABO*O.01.01*	O	A_3O	11	0
2	*ABO*A3.01*	*ABO*O.01.67*	O	A_3O	3	0
3	*ABO*A3.01*	*ABO*O.01.26*	O	A_3O	2	0
4	*ABO*A3.01*	*ABO*O.01.01*	O	A_3O	2	0
5	*ABO*A3.01*	*ABO*O.01.75*	O	A_3O	4	0
6	*ABO*A3.01*	*ABO*O.01.01*	O	A_3O	18	0
7	*ABO*A3.01*	*ABO*O.01.68*	O	A_3O	5	1
8	*ABO*A3.01*	*ABO*O.01.01*	O	A_3O	35	0
9	*ABO*A3.01*	*ABO*B.01*	B	A_3B	2	0

Abbreviations: RBC, red blood cells; SCT, stem cell transplant.

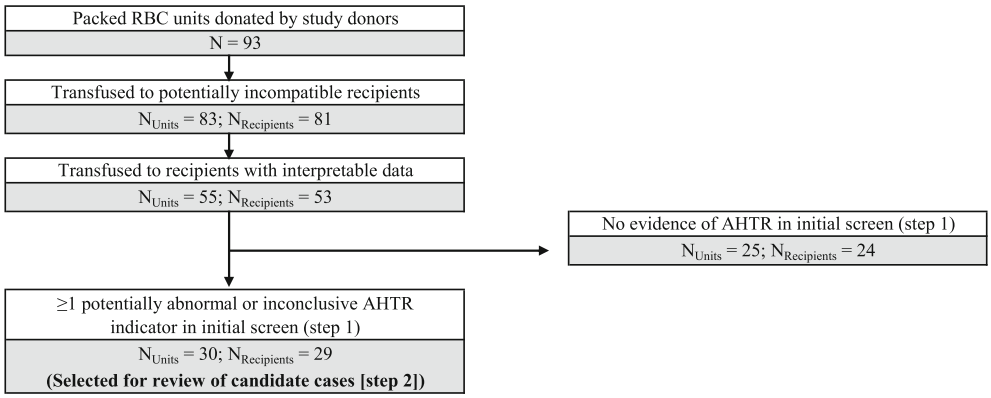

FIGURE 1 Flowchart illustrating the analytical process. AHTR, acute haemolytic transfusion reaction; RBC, red blood cells.

TABLE 3 Acute haemolytic transfusion reaction indicators were observed among candidate cases, ordered by change in haemoglobin levels.

	Recipient ID	Change in Hb levels (g/L)[a]	Reported transfusion reaction	LDH (U/L)[b]	Haptoglobin levels (g/L)	Direct antiglobulin test[c]			Plasma appearance	Total bilirubin[b] (μmol/L)	Indirect bilirubin[b] (μmol/L)
						Polyclonal	Immunoglobulin G-specific	Immunoglobulin C3-specific			
Decrease or unsatisfactory increase ($N = 15$)	1	−4	No	–	–	–	–	–	–	–	–
	2	−4	No	–	–	–	–	–	–	–	–
	3	−3	Yes	170 to 174[d]	NA to 1.91[d]	– to NA	–	–	NA to Normal	12 to 11[d]	–
	4	−1	No	–	–	–	–	–	–	62.3 to NA	24.9 to NA
	5	1	No	92 to NA	–	–	–	–	Normal to NA	6.0 to NA	3.7 to NA
	6	3	No	–	–	–	–	–	–	–	–
	7	3	No	–	–	–	–	–	Normal to Normal	108 to 120	NA to 28
	8	5	No	–	–	–	–	–	–	–	–
	9	6	No	112 to NA	–	–	–	–	–	18 to NA	–
	10	7	No	NA to 139	NA to 3.21	NA to –	–	–	–	NA to 28	–
	11	7	NA	–	–	– to NA	–	–	Normal to NA	4 to NA	3 to NA
	12	8	No	–	–	–	–	–	–	–	–
	13	8	No	–	–	–	–	–	–	12 to 18	–
	14	9	No	313 to NA	–	–	–	–	–	–	–
	15	9	No	219 to NA	–	–	–	–	–	9 to NA	–
Satisfactory increase in Hb levels ($N = 14$)	16	10	No	–	–	–	–	–	–	92.9 to 101.7	–
	18	10	No	209 to 250	–	– to –	–	–	Normal to NA	5.6 to 11.0	–
	23	13	No	730 to 463	–	– to NA	–	–	–	NA to 6.1	–
	25	18	No	–	NA to 1.11	–	–	–	NA to weakly icteric	NA to 25	NA to 11
	30	20	No	–	–	–	–	–	Normal to Normal	NA to 9.1	NA to 5.6
	33	22	No	NA to 157	–	–	–	–	Normal to NA	7 to NA	–
	37[e]	25	No	843 to 1150	–	+ to +	+ to +	+ to +	Icteric to Icteric	43 to 65	35 to 59
	39	26	Yes	–	–	– to –	–	–	Normal to Normal	–	–
	40	26	No	581 to 706	–	–	–	–	Normal to NA	8 to 17	–
	41	26	No	NA to 458	–	–	–	–	–	NA to 21	–
	42	27	NA	NA to 575	–	–	–	–	Normal to NA	NA to 11.9	–

TABLE 3 (Continued)

Recipient ID	Change in Hb levels (g/L)[a]	Reported transfusion reaction	LDH (U/L)[b]	Haptoglobin levels (g/L)	Direct antiglobulin test[c]			Plasma appearance	Total bilirubin[b] (μmol/L)	Indirect bilirubin[b] (μmol/L)
					Polyclonal	Immunoglobulin G-specific	Immunoglobulin C3-specific			
44	32	No	–	–	–	–	–	–	NA to 19	–
48	38	No	–	–	–	–	–	Normal to Normal	8 to 9	–
50	42	No	–	–	–	–	–	–	22.7 to 43.8	–
Interpretation according to initial screening criteria (Step 1)										
Normal indicator										
Potentially abnormal indicator										
Inconclusive owing to unavailability of pre-transfusion measurement										
No measurement available, or only pre-transfusion measurement available for given indicator										

Note: Haemoglobinaemia is not shown, because none of the candidate cases had interpretable data for this indicator.
Abbreviations: Hb, haemoglobin; ID, identifier; LDH, lactate dehydrogenase; NA, not available.
[a]Per Step 1 criteria, any decrease in Hb levels, or an increase ≤10 g/L, was considered as a potentially abnormal indicator.
[b]Per Step 1 criteria, any increase was considered as a potentially abnormal indicator for AHTR.
[c]Only recipient #37 had a positive DAT, and the degree of reactivity did not change pre- and post-transfusion.
[d]For this recipient, the exact numerical values were within the range considered normal by the hospital.
[e]Two RBC units were transfused to the same recipient.

or decrease in Hb levels ($N = 15$), 11 had no other abnormal or inconclusive indicator (Table 3), which is insufficient to ascertain AHTR. Recipient #7 additionally exhibited an increase in total bilirubin levels, but pre-transfusion levels appeared elevated. Recipient #10 had three inconclusive indicators, but available post-transfusion results appeared within normal range. Recipient #13 exhibited a marginal increase in total or indirect bilirubin, with post-transfusion levels seemingly within normal range. Recipient #3 was the only patient in this group who experienced a QHS-reported transfusion reaction (Table 3). More detailed outcomes were obtained through direct interaction with the treating physician who completed a chart review. This was a Group O, elderly male patient admitted for lower gastrointestinal bleeding, oxygen desaturation and a suspected pneumonia for which intravenous antibiotics had been initiated. Besides a (non-significant) decrease in Hb levels, all other available indicators were normal, including LDH levels, haptoglobin levels, plasma appearance and total bilirubin levels (Table 3). Based on this information, the treating physician, the two reviewers and the TSO agreed that the reported transfusion reaction was a febrile non-haemolytic transfusion reaction.

Among candidate cases with a satisfactory increase in Hb levels ($N = 14$), one (recipient #39) experienced a QHS-reported transfusion reaction without any other potentially abnormal indicator (Table 3); the cause was a minor allergic reaction. Six recipients (#16, #18, #37, #40, #48 and #50) exhibited increased LDH levels, or increased total or indirect bilirubin levels. However, the patterns of these increases were generally not consistent with AHTR: Pre-transfusion levels were sometimes elevated (LDH: for recipients #37, #40 and #18; bilirubin: for recipients #16 and #37), and some increases were modest or within normal range (bilirubin: for recipients #18, #40 and #48). Only recipient #50 exhibited a meaningful increase in total bilirubin levels, but the concomitant (and substantial) increase in Hb levels was not consistent with AHTR. Several recipients (#23, #25, #30, #33, #41, #42 and #44) had one or more inconclusive indicators, but all exhibited strong increases in Hb levels that were not consistent with AHTR. Only recipient #37 had a positive DAT, and the degree of reactivity did not change pre- and post-transfusion. Further, the eluate of this recipient did not contain anti-A, and the recipient had autoimmune haemolytic anaemia.

Of note, recipients #57, #79 and #80 died within 24 h post-transfusion (Table S2). No other data were available for these units, except for recipient #79 who had seemingly normal post-transfusion Hb levels (115 g/L).

DISCUSSION

In this case series, none of the 53 recipients with interpretable data exhibited conclusive evidence of serious AHTR following transfusion with A_3O or A_3B blood. Only two recipients experienced a transfusion reaction that was reported to the haemovigilance system (i.e., QHS), and both reactions had a known or plausible cause other than AHTR. No transfusion-related death was reported to QHS. Following the initial screen (i.e., Step 1), 29 recipients exhibited ≥1 potentially abnormal or inconclusive indicator. However, potentially abnormal pre- versus post-transfusion changes appeared modest, remained within normal range, were insufficient to ascertain AHTR or were consistent with a pre-existing condition unrelated to AHTR. To the best of our knowledge, this is the largest case series of ABO incompatibilities involving weak ABO donors.

Nonetheless, our study does not rule out the possibility that ABO incompatibilities involving an A_3 donor elicits an asymptomatic immune response (e.g., weakly positive DAT alone, without haemolysis), which may not have been recognized by the treating physician [1]. Mild AHTR-related symptoms might also have been (mistakenly) attributed to other pre-existing conditions, and hence not be reported in medical charts. However, if such symptoms manifested, there were no reported serious adverse reactions to the recipients.

Taken alone, the absence of transfusion reactions reported to the QHS is reassuring given the robustness of this haemovigilance system established in 2000. Over 20 years, the annual number of reports to the QHS has been relatively stable (i.e., ~2500–2900 reports/year) [10]. In 2019, there were 989.7 events per 100,000 units (i.e., 2968 reports for 299,879 units transfused) reported to the QHS, compared with 184.1 per 100,000 units (i.e., 4248 reports for 2,306,983 units) reported to the United Kingdom-based Serious Hazards of Transfusion (SHOT) [10, 11]. This comparison suggests that awareness of transfusion reactions is relatively high in Québec, so that a clinically significant AHTR is likely to be noticed and reported to the QHS. The rate of QHS-reported ABO incompatibilities was 7.2 per 100,000 RBC units transfused in 2000, fell to 0.5 in 2009, and has been stable since then (i.e., 0.5–1.4 per 100,000 units from 2009 to 2019) [10]. TSOs use standardized case definitions and submit forms to report suspected transfusion reactions to the QHS.

AHTR case definitions vary across jurisdictions, but the QHS case definition [8] is largely similar to that of the US-based Centers for Disease Control and Prevention (CDC) [12] and the UK-based SHOT [13]. Nearly all laboratory measures included in the QHS definition are also included in the CDC or SHOT definition. The only exception is spherocytosis, which is included in the CDC and SHOT definitions but not in that of the QHS. Other country-specific elements include decreased fibrinogen (CDC only), plasma discolouration consistent with haemolysis (CDC only) and a positive crossmatch (SHOT only). Overall, these differences are relatively minor and suggest our results may be extrapolated to case definitions used in many other jurisdictions.

All *ABO*-sequenced donors were carriers of the *ABO*A3.01* allele, consistent with other studies in which A_3 was the most frequent weak A subgroup [5, 14–16]. Since 2018, 40 individuals analysed at our Immunohematology Reference Laboratory had a discordant ABO phenotype, and 23 (57.5%) were carriers of the *ABO*A3.01* allele (Figure S1). Of note, A_3 (along with A_x) ranks among the weak A subgroups with the highest levels of A antigen expression [17, 18]. Therefore, the apparent absence of serious AHTR observed in our study likely applies to other weak A subgroups with lower A antigen levels.

This study is subject to some limitations. First, owing to the retrospective nature of our study and the fact that some transfusions occurred 20 years ago across 30 hospitals, a large number of laboratory measures were unavailable or incomplete, which hindered data interpretation. Missing data may be due to lack of testing—in which case AHTR was likely not suspected—or data trimming after the mandatory 5-year retention period [19]. Second, data on AHTR-related symptoms were not collected. However, collecting data on AHTR-related symptoms was deemed too burdensome for TSOs amid the COVID-19 pandemic and a severe staff shortage in Québec. Further, these data would have been of limited use since (1) AHTR-related symptoms are unspecific and dispensable for diagnosis per the QHS and SHOT case definitions [8, 13], and (2) clinically overt signs of AHTR should have prompted further laboratory testing. Third, data on recipients' medical conditions were unavailable. The recipient population included in this study is, therefore, likely heterogeneous and may include trauma patients and patients with a chronic condition who regularly need blood transfusions (e.g., for sickle cell disease). Many of these health conditions may cause abnormal laboratory test results seemingly consistent with AHTR, in which case our approach would overestimate the incidence of AHTR. In this regard, the apparent absence of conclusive signs of serious AHTR is reassuring. Fourth, the cause of death of the three recipients could not be ascertained based on the data available to us. Lastly, our ability to ascertain AHTR was limited by the amount of missing data, which we addressed by categorizing some indicators as 'inconclusive'. Albeit imperfect, this handling of missing data was complemented by clinicians' reports to the QHS, which should be generated each time a transfusion reaction is suspected. The QHS is a robust haemovigilance system with a relatively high and stable rate of reporting.

In this case series, no conclusive evidence of serious AHTR was found among ABO-incompatible recipients who were inadvertently transfused with A_3O or A_3B blood. Our results suggest no serious AHTR ensued from these transfusions, although mild or subclinical AHTR cannot be ruled out. Based on the present analysis, Héma-Québec obtained approval from the national regulator (i.e., Health Canada) not to notify hospitals when a donor with a weak, discordant ABO subgroup is identified. These donors are redirected to plasma for fractionation and are no longer eligible to donate whole blood.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflict of interest related to this manuscript.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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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

Plasma resuscitation improves and restores intestinal microcirculatory physiology following haemorrhagic shock

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Abstract

Background and Objectives: Intestinal ischaemia–reperfusion injury following resuscitated haemorrhagic shock (HS) leads to endothelial and microcirculatory dysfunction and intestinal barrier breakdown. Although vascular smooth muscle machinery remains intact, microvascular vasoconstriction occurs secondary to endothelial cell dysfunction, resulting in further ischaemia and organ injury. Resuscitation with fresh frozen plasma (FFP) improves blood flow, stabilizes the endothelial glycocalyx and alleviates organ injury. We postulate these improvements correlate with decreased tissue CO_2 concentrations, improved microvascular oxygenation and attenuation of intestinal microvascular endothelial dysfunction.

Materials and Methods: Male Sprague–Dawley rats were randomly assigned to groups ($n = 8$/group): (1) sham, (2) HS (40% mean arterial blood pressure [MAP], 60 min) + crystalloid resuscitation (CR) (shed blood saline) and (3) HS + FFP (shed blood + FFP). MAP, heart rate (HR), ileal perfusion, pO_2 and pCO_2 were measured at intervals until 4 h post-resuscitation (post-RES). At 4 h post-RES, the ileum was rinsed in situ with Krebs solution. Topical acetylcholine and then nitroprusside were applied for 10 min each. Serum was obtained, and after euthanasia, tissues were harvested and snap-frozen in liquid N_2 and stored at −80°C.

Results: FFP resuscitation resulted in sustained ileal perfusion as well as rapid sustained return to baseline microvascular pO_2 and pCO_2 values when compared to CR ($p < 0.05$). Endothelial function was preserved relative to sham in the FFP group but not in the CR group ($p < 0.05$).

Conclusion: FFP-based resuscitation improves intestinal perfusion immediately following resuscitation, which correlates with improved tissue oxygenation and decreased tissue CO_2 levels. CR resulted in significant damage to endothelial vasodilation response to acetylcholine, while FFP preserved this function.

Keywords

fresh frozen plasma, haemorrhagic shock, microcirculation, physiology, resuscitation, transfusion, trauma

This study was presented at Eastern Association for the Surgery of Trauma, 35th EAST Annual Scientific Assembly, Austin, Texas, January 11–15, 2022.

Highlights

- Haemorrhagic shock (HS) results in derangements of the intestinal microcirculation which are significantly improved with plasma resuscitation.
- The improved microcirculatory flow resulting from plasma transfusion correlates with improved tissue oxygenation and decreased tissue CO_2.
- Plasma transfusion and resuscitation restore normal physiology of the intestinal microcirculatory environment following HS, possibly allowing for less intestinal barrier breakdown and systemic physiological derangements.

INTRODUCTION

Microvascular alterations are an integral part of the pathophysiology of haemorrhagic shock (HS). Just as the microcirculatory flow continues to be diminished following restoration of central haemodynamics, decreased oxygenation of the microcirculation can occur despite normal global oxygenation [1]. Under normal physiological conditions, the intestinal mucosa cells experience significant blood flow variations and tissue oxygenation. Intestinal epithelial cells frequently exist at a low pO_2, termed 'physiological hypoxia'. Countercurrent oxygen exchange from arterioles to venules allows for maintenance of the high-energy requirement of the intestinal barrier during fluctuations in oxygen supply [2–4]. However, this compensatory mechanism cannot overcome prolonged alterations in microvascular flow, such as in HS, resulting in microvascular ischaemia and subsequent barrier breakdown.

Endothelial dysfunction and interstitial oedema further exacerbate this microvascular ischaemia [5]. Previous studies have demonstrated the positive effects of plasma resuscitation on the endothelial glycocalyx, intestinal microcirculation, enterocyte damage and the intestinal barrier [6–8]. We hypothesized that fresh frozen plasma (FFP) resuscitation would improve intestinal microcirculatory flow, increase intestinal pO_2, decrease intestinal pCO_2 and improve endothelial function following HS.

MATERIALS AND METHODS

All studies were performed at the Robley Rex Veterans Affairs Medical Center in Louisville, Kentucky, an American Association for the Accreditation of Laboratory Animal Care (AAALAC) approved facility. The protocol was approved by the Research and Development Committee, Research Animal Safety Subcommittee and Research Safety Subcommittee. Male Sprague–Dawley rats were housed in the Veterinary Medicine Unit, allowed food and water ad libitum and were acclimated on a 12-h light/dark cycle for at least 7 days prior to use. Daily body weights (BWs) were obtained, and protocols were performed when BW ranged between 250 and 300 g. Induction with ketamine/D-xylazine anaesthesia was performed via intraperitoneal (ip) injection (0.1 mL/100 g BW, 91 mg/kg and 9.1 mg/kg ip) and maintenance was via subcutaneous (sc) ketamine injections (0.1 mL/100 g BW) as needed to maintain the surgical plane of anaesthesia. Body temperatures were regulated using a servo-controlled heating pad and a rectal thermistor to remain at 37.0 ± 1.0°C.

ARRIVE 2.0 guidelines

The following statements pertain to the ARRIVE guidelines 2.0. There were 32 Sprague–Dawley outbred rats used for the three experimental groups ($n = 8$/group), which were sham haemorrhage (no HS, no RES), HS plus resuscitation with shed blood and saline and HS plus shed blood plus FFP. The animals were randomly assigned to groups. An additional 12 rats were used to obtain FFP. The sample size was determined by power analysis for analysis of variance (ANOVA) using the laser Doppler flow (LDF) in the terminal ileum as the primary variable. All animals in the experimental groups are included in the study. Randomization was performed by coin toss: first toss heads = sham, second toss heads = HS + crystalloid resuscitation (CR) and second toss tails = HS + FFP. Animals were added to groups until the $n = 8$ needed for the group were completed. Blinding was not possible since the investigator performing the study had to know HS versus sham and CR versus FFP. All rats were anesthetized to a surgical plane of anaesthesia prior to and during the protocol. The ARRIVE recommended set was referenced during the drafting of this paper.

FFP donor surgery

The medial neck site for surgery was shaved, and 4% chlorhexidine scrub was used for sterilization. After loss of blink and withdrawal reflexes, a 1-cm incision was performed for surgical access to the left carotid artery, which was cannulated with PE50 (polyethylene [PE]) tubing (Intramedic PE50 tubing, Clay Adams Division of Becton Dickinson & Co., Parsippany, NJ, USA) that had been soaked in 1 mL NS with 10 IU heparin/mL. Blood was withdrawn under deep surgical anaesthesia. In a green top tube, the blood was spun at 2500 rpm for 15 min. The plasma was slowly pipetted into a sterile orange top tube and snap-frozen in liquid nitrogen. The FFP was stored in an ultra-freezer (−80°) for no more than 10 days prior to use.

Animal experimental groups surgical preparation

After the loss of blink and withdrawal reflexes, the rats underwent tracheostomy (Intramedic PE240 tubing, Clay Adams Division of Becton Dickinson & Co., Parsippany, NJ, USA), and cannulation of the right carotid artery (PE50), jugular vein (PE90), left femoral artery

(PE50) and left femoral vein (PE50). Rats spontaneously breathed room air throughout. PE lines were coated with heparin (10 IU/mL) prior to placement, and lines were filled with normal saline (NS). Mean arterial blood pressure (MAP) and heart rate (HR) were continuously monitored (carotid artery) throughout the experimental protocol (BPA-400, DigiMed Signal Analyzers, Louisville, KY, USA). Lines were also used for haemorrhage, resuscitation, blood sampling and administration of intravenous (iv) fluids.

Experimental groups

Rats were randomly assigned to groups with $n = 8$ rats per group. Three groups were performed in this experiment: sham, CR and FFP. Sham rats had similar surgical preparation and manipulation but had no HS or RES. CR rats were haemorrhaged to 40% of baseline (BL) MAP for 60 min and resuscitated with shed blood plus two equal volumes of warmed NS. FFP rats received shed blood plus one equal volume of FFP (see below).

HS model

HS was initiated by the withdrawal of blood (1 mL/min) into a 10 mL syringe that had been prewarmed and coated with heparin (10 IU) before use. The haemorrhage syringe was placed on the heating pad (37.0 ± 1.0°C) and frequently turned to prevent clot formation. Withdrawal of blood was continued until MAP decreased to 40% of BL MAP. Further withdrawal or reinfusion of blood was performed to maintain a 40% BL MAP for the duration of 60 min HS period (Figure 1).

CR protocol

The volume of blood withdrawn was recorded and the blood was returned over 5 min. For example, if a final total of 7.5 mL of blood was withdrawn during HS, the blood was returned using a syringe pump at the rate of 1.5 mL/min. After the 5-min blood return, two equal volumes of NS were administered over 25 min. If 7.5 mL of blood was haemorrhaged, 15 mL of warmed NS was infused over 25 min at a rate of 0.6 mL/min. The total period for RES was 30 min.

FFP resuscitation protocol

On the day of the experiment, the FFP was thawed in warm water to 37°C before use. One equal volume of FFP to match the volume of withdrawn blood was administered over 25 min via the femoral vein cannula after the withdrawn blood reinfusion was complete. At the completion of the injection, MAP and HR were compared with the initial BL levels. MAP and HR were considered stable (i.e., less than 10% variability from BL) for all HS/FFP rats.

Measurement of ileal perfusion

Ileal perfusion was measured by LDF using a Perimed 4001 system (Perimed AB, Järfälla, Sweden) using an integrated LDF probe (seven sites). The integrating flow probe was held in place on the ileum near the site where pO_2 and pCO_2 were measured and was kept in place until the measurements were stable. The perfusion was recorded for 5 min at each time point and averaged.

Measurement of ileum pO_2 and pCO_2 levels

Through a 3-cm midline laparotomy, the terminal ileum was identified by moving back from the ileocecal junction approximately 6 cm. A 4-cm section of terminal ileum was sutured (5-O silk) to a small piece of soft polyvinyl chloride in situ. pO_2 and pCO_2 levels were measured by microelectrodes held onto the surface of the bowel. Micromanipulators attached to a wooden base were used to manoeuvre the

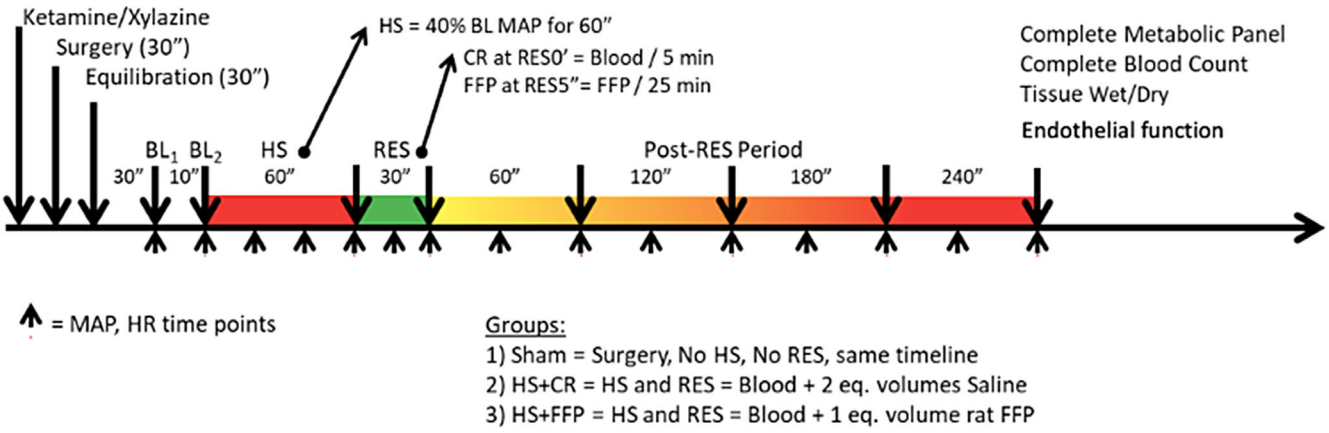

FIGURE 1 Experimental timeline and protocol. BL, baseline; CR, crystalloid resuscitation; FFP, fresh frozen plasma; HR, heart rate; HS, haemorrhagic shock; MAP, mean arterial pressure; RES, resuscitation.

microelectrodes into place. Increased pCO_2 is thought to be a reliable representation of oxygen availability and metabolic activity within the intestine. pO_2 was measured by DO-166MAT-1 microelectrode using DO-166MT-1 amplifier and DO-166MT-1 attenuator box (Lazar Research Laboratories, Inc., Los Angeles, CA, USA) and Jenco Model 6230N mV metre, and data were recorded on an ASUS laptop computer (Arrow DO and Arrow ION software, Jenco Instruments, Inc., San Diego, CA, USA). pCO_2 was measured with GS-136CO-1 micro-CO_2 electrode, amplifier and metre. pO_2 and pCO_2 electrodes were standardized at 0% and 100% CO_2 medical gas cylinders. Readings were made at each time point over 5 min and averaged. Calibration was checked against the gas standards at the end of the protocol.

Evaluation of endothelial function following HS/RES

The ileum was washed in situ with Krebs solution (no glucose, 37.0°, pH = 7.40), followed by topical acetylcholine (10^{-5} M) and nitroprusside (NP, 10^{-4} M) for 10 min each. LDF was measured following each of these applications.

Tissue oedema

Tissue samples from lung and intestine were harvested, washed, blotted dry, weighed for a BL wet weight and then dried in an oven for 3 weeks. Weekly weights were obtained until stable, and tissue dry

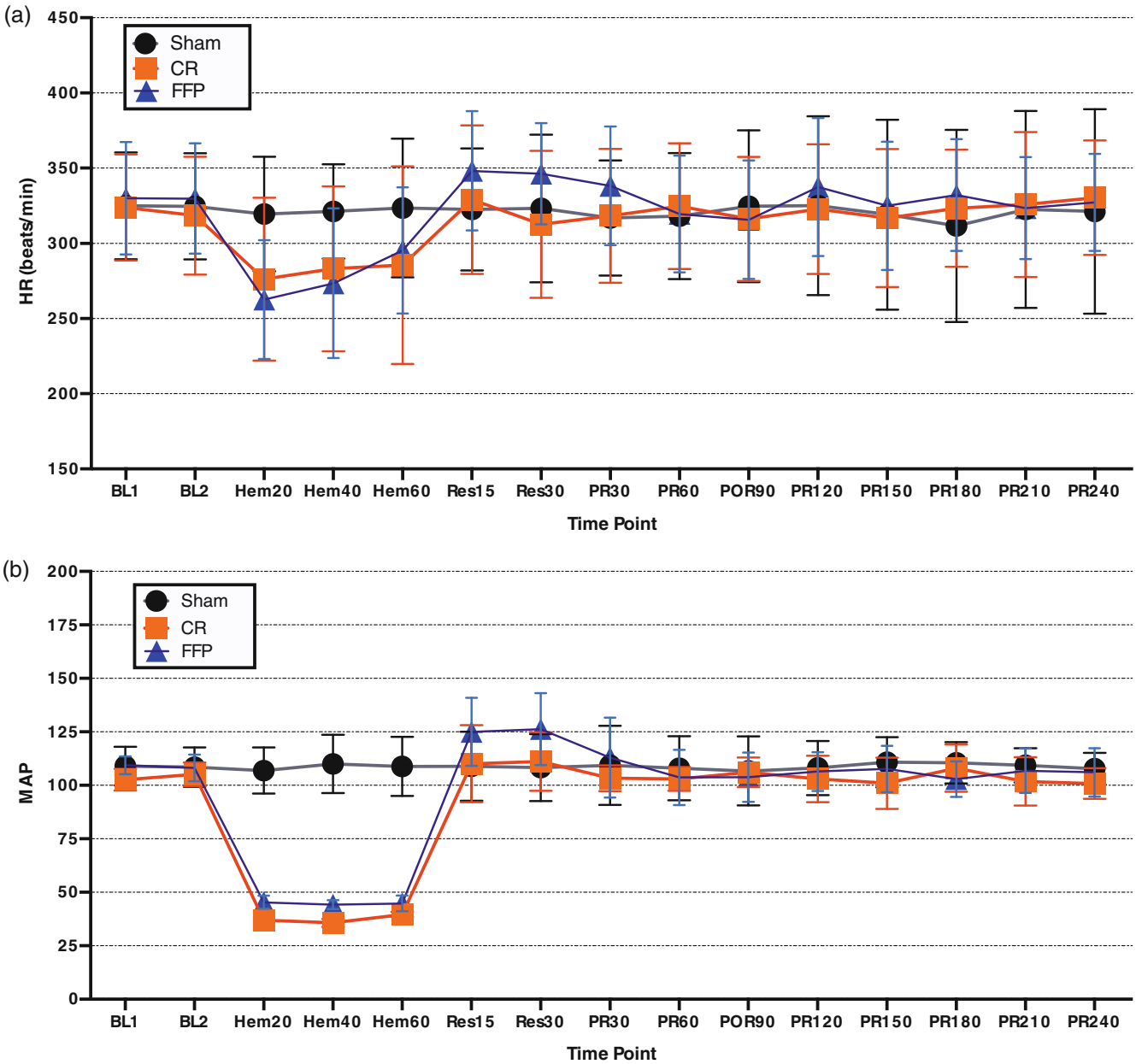

FIGURE 2 Central haemodynamics during baseline (BL), haemorrhagic shock (HS), resuscitation (RES) and post-RES (PR). (a) Heart rate (HR) in beats per minute. (b) Mean arterial pressure (MAP) in mmHg. Values are mean ± SD. CR, crystalloid resuscitation; FFP, fresh frozen plasma.

weights were compared to the wet weights for an evaluation of tissue oedema. The data are shown as a ratio of wet weight/dry weight.

Syndecan-1 enzyme-linked immunosorbent assay (ELISA)

Ileum and serum samples were obtained at the completion of the protocol, rinsed in cold NS, blotted dry on gauze, snap-frozen in liquid N_2 and stored at −80°C until the day of the ELISA protocol. A rat specific syndecan-1 ELISA kit was used (LSBio, Seattle, WA, USA). On the day of the protocol, serum and ileum tissue samples were prepared according to the manufacturer's directions.

Statistics

Data are expressed as mean ± standard deviation (SD), and differences between groups were assessed by one-way or two-way ANOVA. Differences across time points in groups were determined by ANOVA for repeated measures (REMANOVA). The null hypothesis was rejected a priori at $p < 0.05$. When differences between groups were found, Tukey–Kramer's honestly significant difference test, Bonferroni's test or Dunnett's test was used for post hoc analysis.

RESULTS

Central haemodynamics and LDF

Central haemodynamic values are shown in Figure 2. There were no differences between groups at BL in MAP or HR. Figure 2a shows the HR in beats per minute (bpm) and Figure 2b shows MAP (mmHg). Both MAP and HR values decreased during the HS period and returned to BL levels throughout the RES and post-RES periods. LDF values as a measure of microvascular perfusion in sham and treatment groups are shown in Figure 3. The decrease to 40% of BL MAP during the HS period resulted in a drop of less than 40% of BL LDF levels (i.e., from greater than 55 at BL to about 15–20 during HS in the CR and FFP groups). During the first 15 min of resuscitation, LDF increased more significantly in the CR group than in the FFP group (64.14 vs. 45.71, $p = 0.018$). At the end of the 30-min resuscitation period, LDF was improved considerably in the FFP group compared to CR or sham. Sham and CR demonstrated no statistically significant difference at 15 min into resuscitation (R15) to 150 min of post-resuscitation (post-RES150). Throughout the post-RES period, LDF in the CR group continued to decline, despite continued normalized central haemodynamics (Figure 2).

Measurement of ileum pO_2 and pCO_2 levels

Using microelectrodes, pO_2 and pCO_2 levels were measured and compared at protocol time points. Statistical significance was calculated with respect to change from BL values. Figure 4a,b show the average pO_2 and average percent change from BL for the animals in each group respectively (Figure 4). During the haemorrhage period, pO_2 demonstrated a precipitous decrease in both shocked groups. After 15 min of resuscitation, both FFP and CR had improved significantly from the end of the HS period but had not yet reached sham values. After the 30-min resuscitation period, both groups returned to sham values. At 60-min post-RES, the CR group began to show signs of decompensation, significantly different than both sham and FFP groups ($p < 0.001$ and $p = 0.02$, respectively). Oxygenation continued

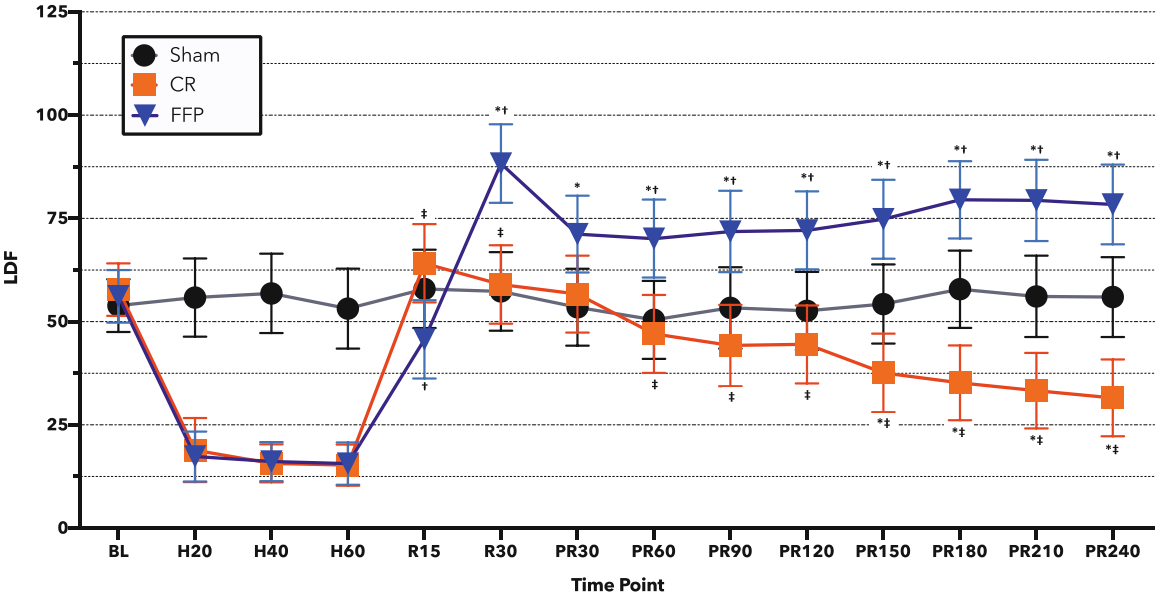

FIGURE 3 Results of laser Doppler flow (LDF) measurements at baseline (BL), HS (H), RES (R) and post-RES (PR). Values are mean ± 95% CI; *$p < 0.05$ versus sham, †$p < 0.05$ versus crystalloid resuscitation (CR), ‡$p < 0.05$ versus fresh frozen plasma (FFP).

to decrease sharply in the CR group, whereas in the FFP group, pO_2 remained stable, if not improved over sham at various points throughout the post-RES period.

pCO_2 levels and change from BL are shown in Figure 5a,b. Baseline values in the sham, FFP and CR groups were not statistically different. Microvascular pCO_2 again progressively increased in both shocked groups throughout the HS period. After 60 min of HS, both CR and FFP demonstrated significant pCO_2 increases from their BL values relative to sham. There was a slight difference between the FFP and CR groups (2.83, $p = 0.03$). However, this was not thought to impact the overall trend in results. After 15 min of resuscitation, both CR and FFP values had started to decrease towards BL but remained significantly increased over sham values. CR initially demonstrated a more rapid improvement than the FFP group and showed the lowest pCO_2 value at the end of the resuscitative period. At this time, pCO_2 values steadily increased and were significantly higher than sham beyond 1-h post-RES. FFP values continued to decrease and were substantially lower than sham at the end of the post-resuscitative period (5.40, $p < 0.0001$).

Evaluation of endothelial function following HS/RES

Endothelial function was evaluated by measuring LDF after the application of acetylcholine (Ach) and then subsequently, sodium

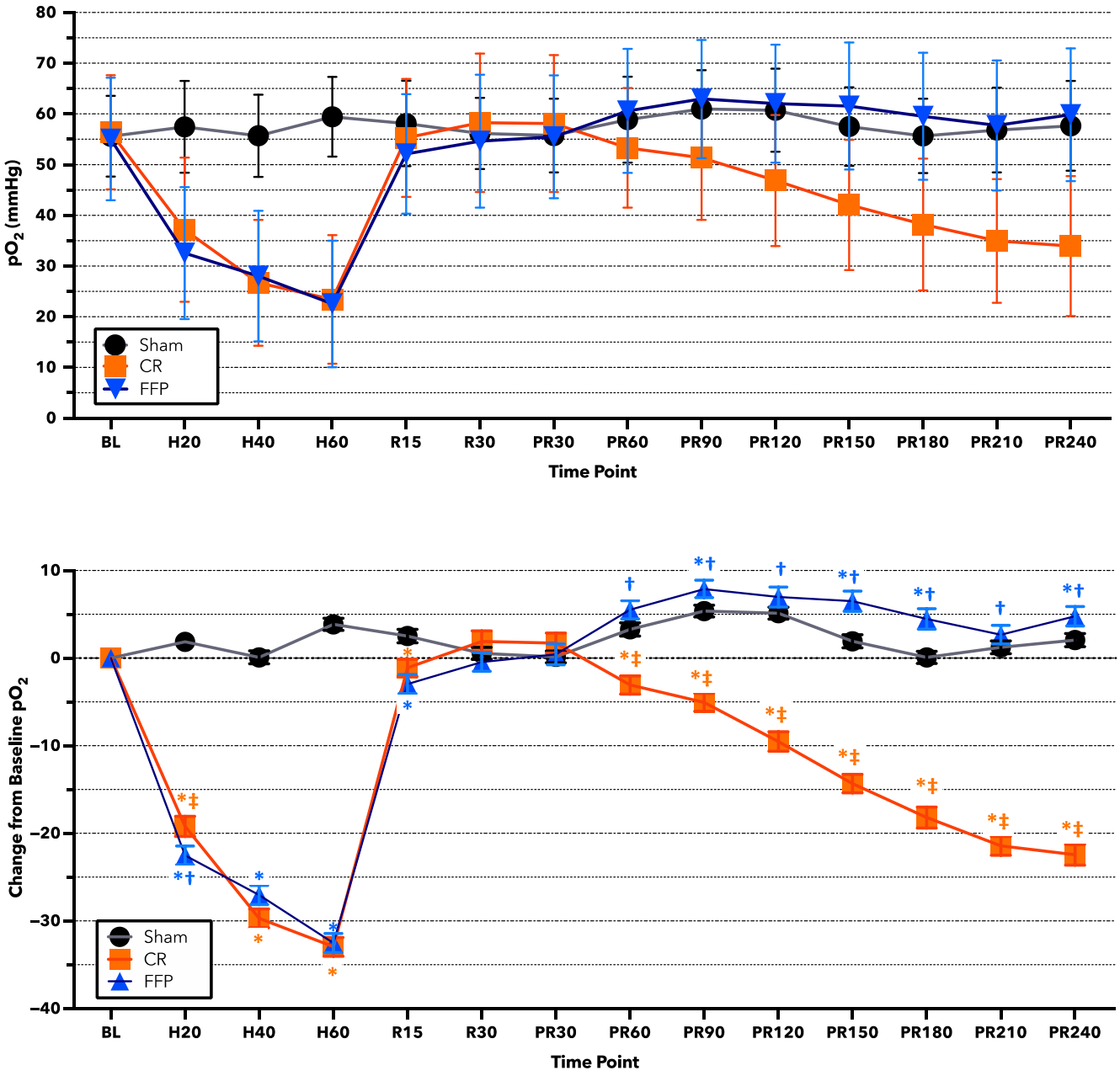

FIGURE 4 pO_2 (a) and change from baseline (BL) pO_2 (b) at each time point, HS (H), RES (R) and post-RES (PR). Values are mean ± SD for (a) and mean ± 95% CI for (b); *$p < 0.05$ versus sham, †$p < 0.05$ versus crystalloid resuscitation (CR), ‡$p < 0.05$ versus fresh frozen plasma (FFP).

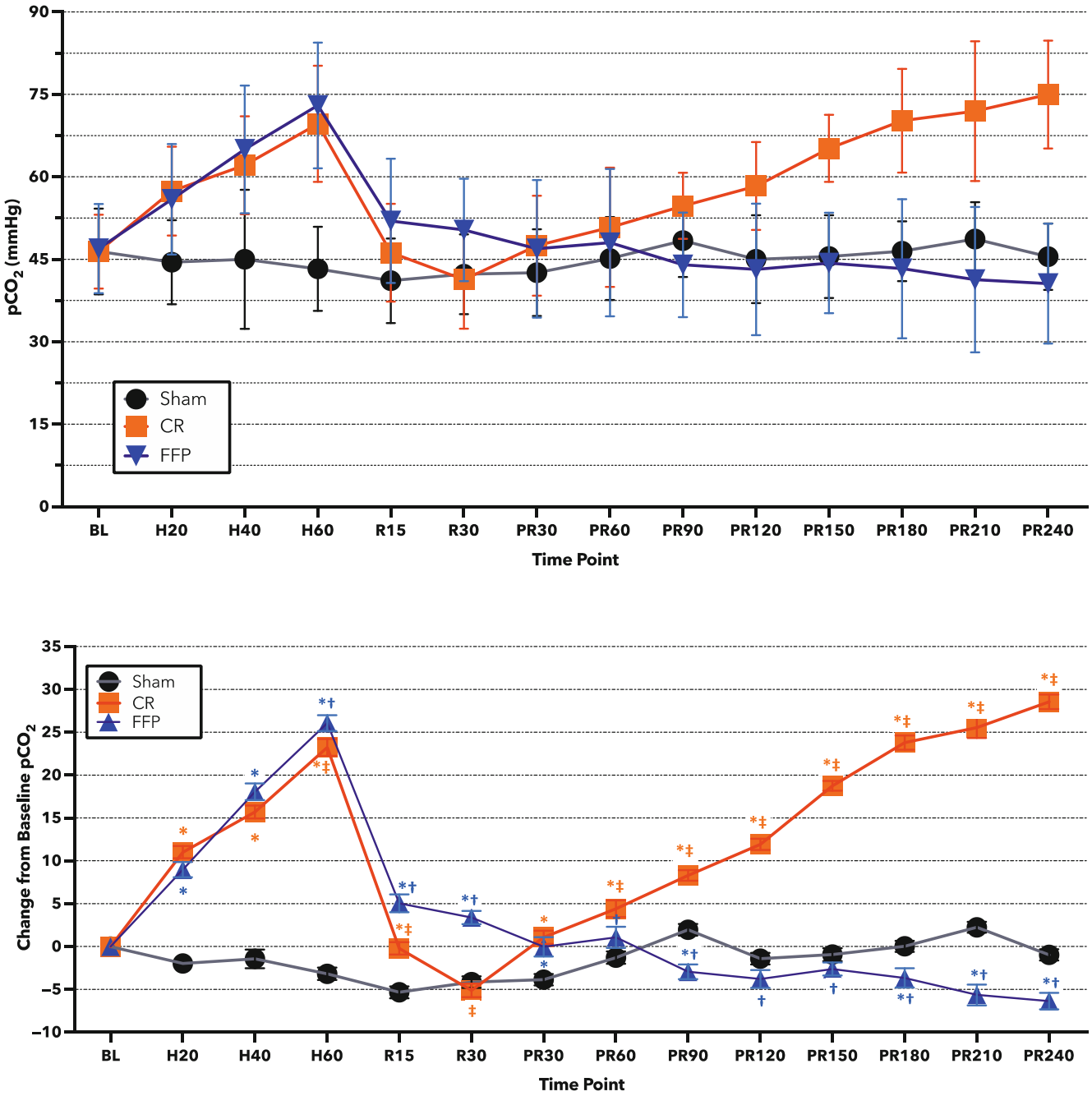

FIGURE 5 pCO_2 (a) and change from baseline (BL) pCO_2 (b) at each time point, HS (H), RES (R) and post-RES (PR). Values are mean ± SD for 5a and mean ± 95% CI for 5b; *$p < 0.05$ versus sham, †$p < 0.05$ versus crystalloid resuscitation (CR), ‡$p < 0.05$ versus fresh frozen plasma (FFP).

NP (Figure 6). Acetylcholine elicits vasodilation via endothelial-dependent pathways, whereas sodium NP, a nitric oxide donor, demonstrates endothelial-independent vasodilation. At BL, as previously mentioned in the LDF results, all groups showed a similar flow. Acetylcholine application resulted in significant increases in inflow in both sham and FFP groups. CR flow did not significantly increase over BL and was considerably lower than both sham and FFP ($p < 0.05$). There were no statistically significant differences in flow following the application of NP. Tissue oedema was measured by comparing wet to dry weights of both lung and ileal tissue (Figure 7). Both tissues are thought to be adversely affected by interstitial oedema following HS. CR resulted in significantly higher ratios than sham and FFP groups in both tissues measured ($p < 0.05$). Finally, syndecan-1 levels were measured in serum and ileum tissue (Figure 8). Plasma levels of syndecan-1 in both the CR and FFP groups were significantly increased compared to sham with CR having the largest concentration. Tissue levels of syndecan-1 were lowest in the CR group while FFP concentrations approached the levels noted in the sham animals.

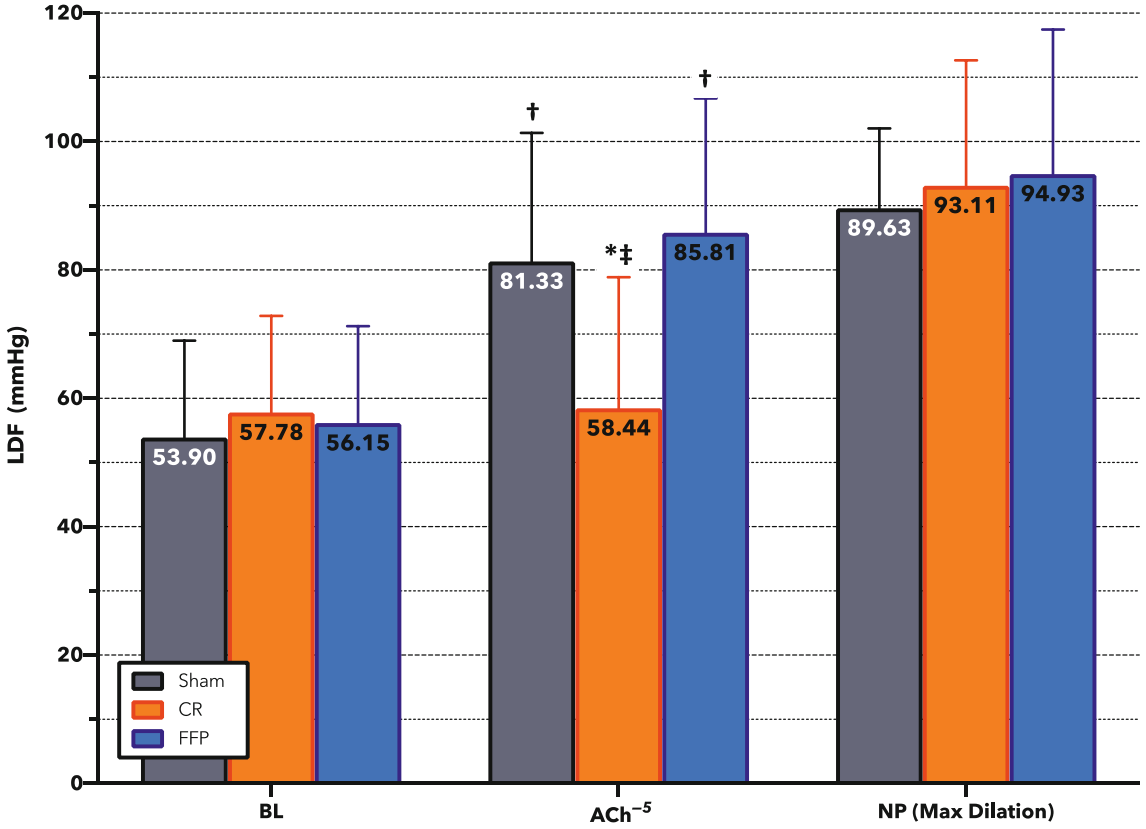

FIGURE 6 Results of laser Doppler flow (LDF) measurements at baseline (BL) and after application of acetylcholine (Ach) and nitroprusside (NP). Values are mean ± SD; *$p < 0.05$ versus sham, †$p < 0.05$ versus crystalloid resuscitation (CR), ‡$p < 0.05$ versus fresh frozen plasma (FFP).

DISCUSSION

This experiment examined various aspects of the intestinal microvasculature to further investigate the effect of HS/RES on the physiology of intestinal microcirculation. As hypothesized, FFP-alleviated signs of microvascular dysfunction following HS as evidenced by improved perfusion, increased pO_2, decreased pCO_2 and improved endothelial function. CR resulted in further decompensation. Although there is a relationship between oxygenation and blood flow, examining blood flow alone does not demonstrate a complete picture of tissue ischaemia. For this reason, we chose to measure LDF, physiological variables and endothelial function following the post-RES period.

Measuring LDF in microvessels allows for the measurement of the microvascular effects of vasomotion, spontaneous oscillations in vascular tone and diameter and RBC velocity within the intestinal microcirculation [9]. These changes in the microvasculature occur secondary to local humoral input and predominate during HS/RES. FFP LDF was increased above CR throughout the entire post-resuscitative period. LDF provides a measurement of flow motion. Increased flow motion, as demonstrated in the FFP group, has been demonstrated in other therapeutic interventions that resulted in positive outcomes [9, 10] [11]. We previously demonstrated improved flow 3-h post-RES; this study confirms that this increased perfusion continues as the CR group's flow continues to decline.

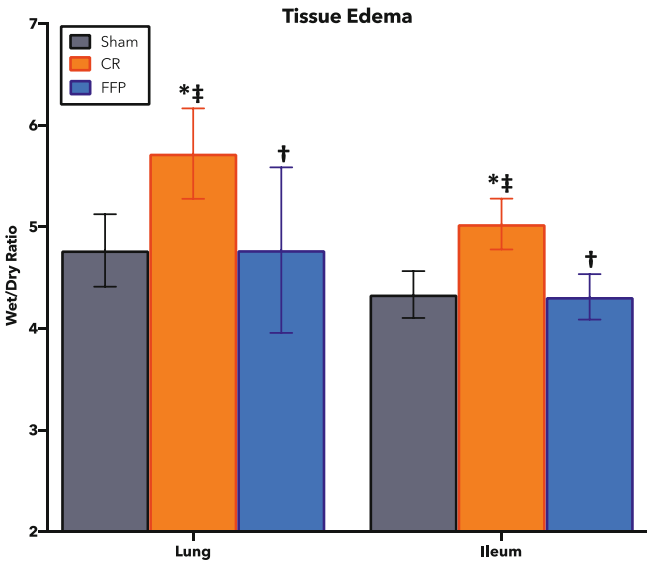

FIGURE 7 Results of the wet-to-dry ratio of ileum and lung tissue to quantify tissue oedema. Values are mean ± SD; *$p < 0.05$ versus sham, †$p < 0.05$ versus crystalloid resuscitation (CR), ‡$p < 0.05$ versus fresh frozen plasma (FFP).

Alterations in microcirculatory oxygenation are not as well documented as changes in microcirculatory flow due to HS/RES. Oxygenation within the microcirculation can be measured using

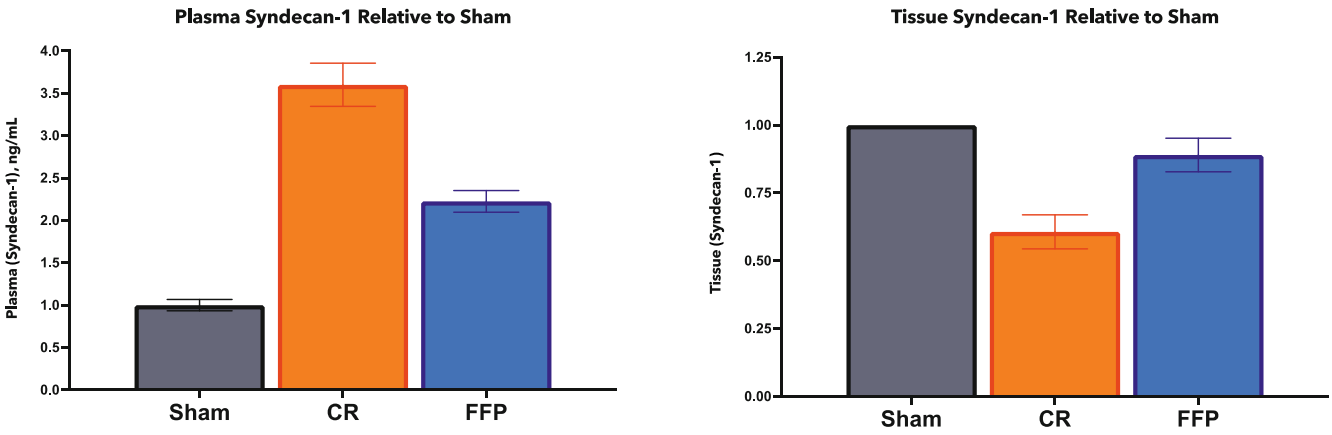

FIGURE 8 Tissue and plasma concentrations of Syndecan-1. Values are mean ± SD; *$p < 0.05$ versus sham, †$p < 0.05$ versus crystalloid resuscitation (CR), ‡$p < 0.05$ versus fresh frozen plasma (FFP). Values are mean ± SD; *$p < 0.05$ versus sham, †$p < 0.05$ versus CR, ‡$p < 0.05$ versus FFP.

microelectrodes for perivascular and tissue oxygen partial pressure or phosphorescence quenching microscopy, a non-invasive technique. This experiment made use of the microelectrode method allowing for the measurement of pCO_2 concurrently. Measuring pO_2 and pCO_2 provides for a more thorough examination of tissue ischaemia and metabolism following HS. To our knowledge, this is the first study comparing these variables between CR and FFP resuscitation. As expected, the decreased flow was accompanied by a simultaneously decreased pO_2 and increased pCO_2. Oxygenation and LDF returned to BL by the end of the 30 min resuscitative period in both groups, with supraphysiological LDF in the FFP group. Although a return to BL pCO_2 values lagged in the FFP group, overall changes in LDF and pO_2 in the two treatment groups demonstrated a relative inverse change in the pCO_2 values.

HS resulted in a significant decrease in microvascular pO_2 in both shocked groups, comparable to other studies examining HS's effects on the microvasculature [9]. The extent and development of intestinal injury following HS are directly related to hypoxia within the villus [12–14]. We have previously demonstrated that FFP resuscitation decreases intestinal damage and intestinal barrier dysfunction following HS [7, 8]. The sustained increase in pO_2 following resuscitation with FFP shown in this study helps to explain these results. Tissue hypoxia not only leads to decreased availability of adenosine triphosphate but also adversely affects cellular immune responses [15], further exacerbating tissue damage and restoration following HS.

HS resulted in significant increases in intestinal microcirculatory pCO_2, up to 150% of BL values. Baseline and HS levels of pCO_2 were similar to those previously reported in the literature [9]. Intestinal pCO_2 measurements are thought to be reliable representations of oxygen availability and regional tissue perfusion [16]. FFP's sustained improvement in microcirculatory pCO_2 exemplifies improved oxygenation, tissue perfusion and overall decrease in tissue damage seen in this group.

HS leads to vascular hyporeactivity, which is thought to be partially responsible for the decompensation and tissue damage following HS [17, 18]. One group examined vascular responsiveness to norepinephrine following HS. It has been well demonstrated that plasma resuscitation protects the endothelium through the stabilization of the endothelial glycocalyx. Vasodilation was measured in this experiment following either sodium NP or acetylcholine. NP, a NO-donor, causes vasodilation through endothelium-independent pathways, whereas ACh relies on the endothelial release of NO to elicit vasodilation. This allowed us to distinguish the changes in endothelial function following HS and resuscitation. Both treatment groups demonstrated full vasodilatory capabilities when treated with NP. Therefore, it is likely that HS does not damage the vasodilatory machinery itself, but the endothelium's ability to respond to vasoactive mediators. Just as FFP protects the endothelial glycocalyx, FFP appears to protect the vasodilatory function of the endothelium. In contrast, CR led to decreased responsiveness of the endothelium to ACh. The combination of syndecan-1 and vasodilation preservation demonstrates FFP's protective effect on both endothelial structure and function.

In this study, we also examined the effects of resuscitative methods on intestinal and pulmonary oedema following HS. Oedema of the villus, in conjunction with decreased vasodilation, further reduces the pressure gradient between arterioles and venules in the intestinal villi and impedes microcirculatory flow [14]. FFP resuscitation was again superior to CR, demonstrating no statistically significant difference from sham values.

This study should be interpreted considering several limitations. In order to minimize variation, only male rats were used due to the significant differences in haemodynamic response to HS between male and female rats. The return of shed whole blood prior to CR or FFP resuscitation must be noted but is required to allow reproducible survival following the HS insult. Likewise, animals in the CR group received twice the volume (2× shed blood volume) when compared to the FFP group (equal volume shed blood) during the resuscitation phase. This discrepancy is needed to ensure animals in the CR arm survive the experimental protocol. Although animals in the FFP group received blood products donated from at least two animals, there is

significant variability between different aliquots of single donor plasma that must be considered.

In conclusion, overall, FFP resulted in improved microcirculatory physiology following HS. Improved LDF correlated with both improved oxygenation and decreased levels of carbon dioxide within the ileal microcirculation. Furthermore, FFP was found to preserve vasodilatory response to acetylcholine and reduce intestinal and pulmonary oedema. These variables help explain the superiority of FFP over CR with regard to intestinal damage and barrier function. Although inherent physiological differences exist between humans and our experimental model, these findings provide further evidence of the beneficial effects of FFP utilization during acute resuscitation in HS.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest, financial or otherwise, to declare.

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

Vox Sanguinis ISBT International Society of Blood Transfusion

Genetic diversity of Gerbich alleles in Brazilians reveals an unexpected prevalence of the GE:−2,−3,4 phenotype

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Abstract

Background and Objectives: Gerbich (GE) blood group system carries high-frequency antigens and the absence of them leads to rare phenotypes: GE:−2,3,4, GE:−2,−3,4 and GE:−2,−3,−4. Their serological differentiation is limited and misclassification of Gerbich phenotypes may occur, but this can be avoided by molecular characterization. This study aimed to characterize the molecular background responsible for rare Gerbich phenotypes in Brazilian population.

Materials and Methods: We selected eight samples from patients with anti-Ge, six from their relatives and nine samples with normal expression of Gerbich antigens. Serological tests were performed in gel and red blood cells (RBCs) were tested with anti-Ge2 and anti-Ge3. Monocyte monolayer assay (MMA) was performed. Molecular investigation was performed with allele-specific polymerase chain reaction and DNA sequencing.

Results: Patient plasma samples reacted with all commercial RBCs. Patient RBCs showed negative results with anti-Ge2 and anti-Ge3. Using MMA two of eight antibodies were clinically significant. Exon 3 was not amplified in any of the patient samples and in two samples from relatives, suggesting the presence of *GE*01.-03/GE*01.-03*. By sequencing, we identified the genetic variability that interferes with the definition of deletion breakpoints, thus two options of genetic structure were suggested to be responsible for the GE:−2,−3,4 phenotype.

Conclusion: This study showed for the first time the genetic diversity of *GYPC* alleles for carriers of Gerbich-negative phenotypes in a Brazilian population and showed an unexpected prevalence of the GE:−2,−3,4 phenotype. It also demonstrated the importance of using molecular tools to correctly classify Gerbich phenotypes for selection of variants in antigen-matched transfusions.

Keywords

Brazilians, Gerbich alleles, Gerbich genotype, Gerbich phenotype, *GYPC*

Highlights

- We report here the first study of the *GYPC* gene in Brazilian individuals with rare Gerbich (GE)-negative phenotypes.
- The study shows the unexpected predominance of the GE:−2,−3,4 phenotype.
- Molecular tools for the classification of Gerbich-negative phenotypes are important for antigen-matched transfusions.

INTRODUCTION

The Gerbich (GE) blood system (ISBT 020) consists of 13 antigens carried on single-pass type I membrane glycoproteins, called glycophorin C (GPC) and glycophorin D (GPD) [1]. Rare Gerbich phenotypes such as Yus (GE:−2,3,4), Gerbich (GE:−2,−3,4) and Leach (GE:−2,−3,−4) are related to the development of antibodies against high-prevalence antigens [2]. As there are no commercial antisera for typing Gerbich antigens, antibody identification depends on the availability of rare red cells and sera with antibodies from individuals previously alloimmunized [3]. However, sometimes these 'in-house' reagents are not well characterized and misclassification of Gerbich phenotypes may occur. Thus, characterization at the molecular level can be a tool to overcome this limitation.

The single *GYPC* gene is responsible for the expression of both glycophorins (GPC and GPD), as a result of translation initiation at two different sites (Figure 1). GPD, a truncated version of GPC, is identical to residues 22–128 of GPC lacking the N-terminal amino acid residues [4, 5]. Protein assays demonstrated that the Ge2 antigen is located on N-terminus of GPD, but not on GPC, while Ge3 is

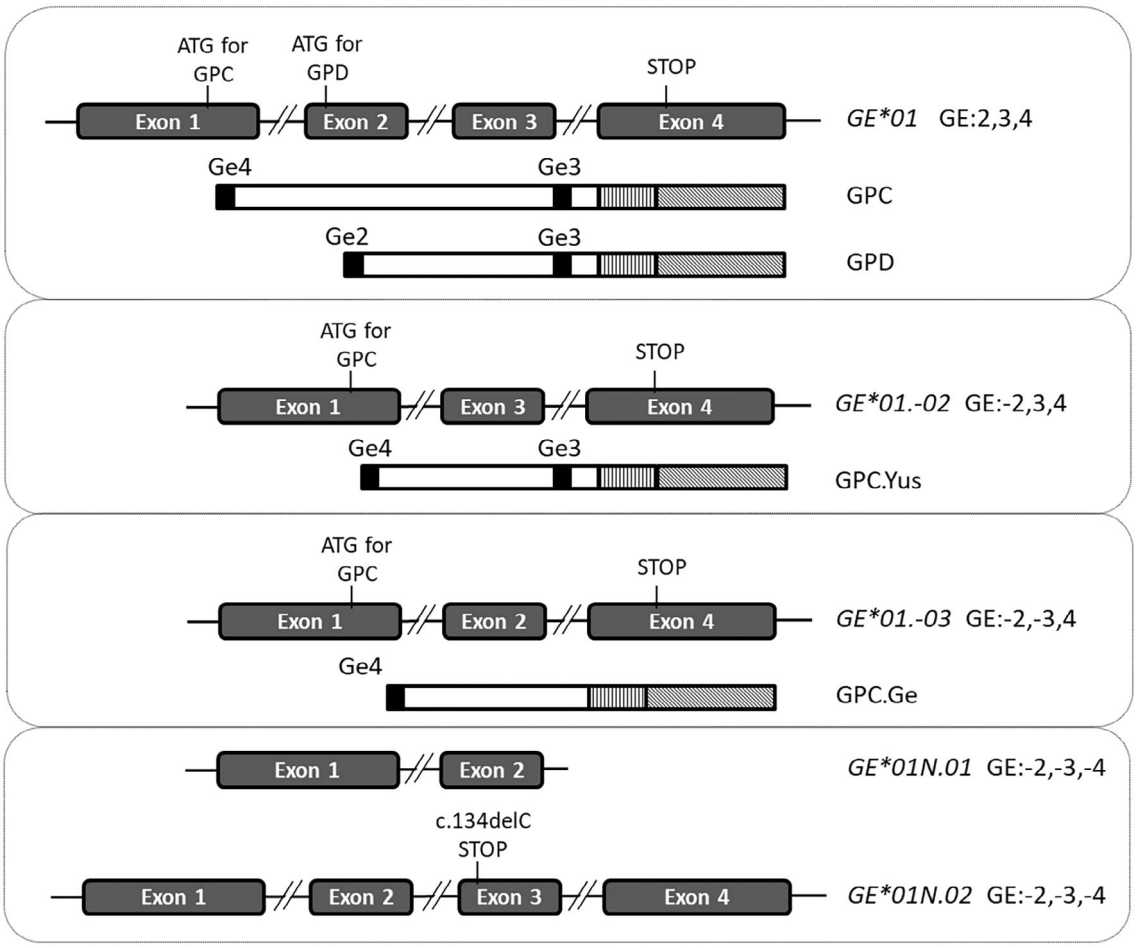

FIGURE 1 The genomic organization of *GYPC* and corresponding structure of glycophorins in wild-type and rare Gerbich (GE) phenotypes. Grey boxes represent exons. In glycophorins, black boxes represent antigens, white boxes represent the extracellular portion of the protein, vertical lines represent the transmembrane region and diagonal lines represent the intracellular portion. GE, Gerbich; GPC, glycophorin C; GPD, glycophorin D.

present in both proteins. Although amino acid sequence present in GPD is also present in GPC, probably anti-Ge2 recognizes an amino acid sequence only when it is in the conformation of the N-terminus of GPD and not when it is an internal sequence within GPC. Also, the Ge2 determinant could involve the free amino group of GPD and the adjacent amino acid sequence. GPC also carries Ge4 antigen that is situated near the N-terminus and therefore is not on GPD.

Figure 1 shows the genetic organization of *GYPC* and the structure of the glycophorins. *GYPC* comprises four exons, wherein exons 1–3 encode the extracellular domain of GPC and exon 4 encodes the membrane-spanning and intracellular domains [4]. Exons 2 and 3 of *GYPC* and their flanking regions of approximately 3600 base pairs are highly homologous, sharing 95% identity among repeat regions 1 and 2 [3]. They likely arose from a duplication event, although exon 3 contains a 27-nucleotide insertion not present in exon 2 [4]. It is suggested that this high homology contributes to unequal crossing-over resulting in deletion events responsible for deletion of high-frequency antigens (Ge2, Ge3 and Ge4) [6, 7]. The Yus (GE:−2,3,4) phenotype is the result of exon 2 deletion (*GE*01.-02*), which leads to the loss of Ge2 antigen, whereas the Gerbich (GE:−2,−3,4) phenotype is a consequence of exon 3 deletion (*GE*01.-03*) [3, 8, 9] in which Ge2 and Ge3 antigens are absent. The Leach phenotype (GE:−2,−3,−4) is characterized by a complete absence of GPC and GPD proteins and has been associated with two molecular events: an extended deletion from intron 2 to intron 4, with complete absence of exons 3 and 4 (*GE*01N.01*) and a cytosine deletion in 134 nucleotides resulting in a frameshift and premature generation of a stop codon (*GE*01N.02*) [6, 10].

Yus and Gerbich phenotypes are rare, being found in 1:44,000 Caucasian individuals [4]. Otherwise, these phenotypes are more common in endemic regions for malaria, such as Papua-New Guinea, probably due to a natural selection of the negative health effects of malaria, because the malarial merozoite form of *Plasmodium falciparum* uses GPC and GPD as a receptor to invade human red blood cells (RBCs) [11, 12].

Clinical significance of Gerbich antibodies is questionable, most of which are not involved in serious haemolytic transfusion reactions [4, 13], although anti-Ge3 has been related to haemolytic disease of the foetus and newborn [14]. In vitro studies have shown that, during pregnancy and postpartum, Gerbich antibodies not only cause classical macrophage-dependent erythroid cell destruction, but also inhibit early erythroid progenitor cell proliferation [13].

Serologic classification of Gerbich phenotypes and the correct identification of anti-Ge2 and anti-Ge3 are complex. Anti-Ge2 is the most common Gerbich alloantibody [4]. It is found in GE:−2,3,4 individuals, but it is also the most frequently detected antibody in the GE:−2,−3,4 and GE:−2,−3,−4 phenotypes. Despite that, some individuals with GE:−2,−3,4 phenotype make anti-Ge3 without anti-Ge2 [3, 15, 16]. Thus, the identification of the antibody formed by a patient does not necessarily reflect his phenotype, requiring well-characterized sera with anti-Gerbich antibodies or the use of molecular methods.

Determining the Gerbich genotype and the specific breakpoint of the deletion is challenging due to the high homology between repeat regions and large deletions involving variant alleles. In order to characterize Gerbich system in our population, this is the first study in Brazil performing genetic screening for carriers of Gerbich-negative alleles. As the Yus phenotype has been described in the literature as the most frequent worldwide and the restricted access to well-characterized serum with anti-Ge3 and anti-Ge4, most of the patients enrolled in this study were classified as Yus (Ge:−2,3,4) and the antibodies produced as anti-Ge2. However, with *GYPC* analysis, we demonstrated here that GE:−2,−3,4 is more prevalent in our population and a specific molecular background was described, for which we proposed two options of deletion breakpoints.

METHODS

Samples

We analysed a total of 23 samples. Eight of them were from alloimmunized patients and six were relatives of these patients. Nine samples with normal expression of Gerbich antigens were also included as controls to better define the molecular changes present in our population. All patients were treated in hospitals located in the State of São Paulo. Samples from six patients were sent to our reference laboratory for investigation. All of them were initially typed as GE:−2,3,4 using a patient's serum containing an antibody identified as anti-Ge2. Ex295 and Ex273 samples were serologically analysed by other reference laboratories. In the Ex295 sample, an anti-Gerbich not classified was identified in the patient's serum and in the Ex273 sample the GE:−2,−3,4 phenotype was identified. Molecular analysis was performed in all samples included in the study. Patients and their relatives are listed in Table 1. The study was approved by our local Ethics Committee and conducted in accordance with our Institution Review Board (IRB).

Serological tests

Antibody screening and identification were performed by gel test in low ionic strenght solution and papain using RBC panels containing the most common erythrocyte antigens (BioRad Laboratories, Hercules, CA or Grifols, Barcelona, Spain), according to the manufacturer's instructions.

Patient samples (with the exception of P157 and Ex295) and a relative (D1089) were also tested for Ge2 and Ge3 expression using gel cards with sera derived from alloimmunized patients and from the serum cells and rare fluids (SCARF) repository (anti-Ge2 A3, anti-Ge2 A8 and anti-Ge3.2 A4). Patients' sera were tested with red cells available from our cryopreserved collection.

Monocyte monolayer assay

To predict the clinical significance of anti-Ge, monocyte monolayer assay (MMA) was performed as previously described [17] in six

TABLE 1 Samples included in the study with description, serological results and genotype.

ID	Description	Antigen determination			Crossing match with RBCs	Genotype	Phenotype
		Anti-Ge2 A3	Anti-Ge2 A8	Anti-Ge3 2A4			
P132	Patient with anti-Ge	N	N	N	P46: N; P54: w; P109: N	*GE*01-03.01/GE*01-03*	GE:−2,−3,4
P230	Patient with anti-Ge	N	N	N	D1089: N	*GE*01-03/GE*01-03*	GE:−2,−3,4
P157	Patient with anti-Ge	NT	NT	NT	P54: N	*GE*01-03/GE*01-03*	GE:−2,−3,4
P46	Patient with anti-Ge	N	N	N	D47: 2+	*GE*01-03/GE*01-03*	GE:−2,−3,4
P54	Patient with anti-Ge	N	N	N	Ge(−2,3,4)[a]: N	*GE*01-03/GE*01-03*	GE:−2,−3,4
P109	Patient with anti-Ge	N	N	N	Ge(−2,3,4)[a]: N	*GE*01-03/GE*01-03*	GE:−2,−3,4
Ex273	Patient with anti-Ge	N	N	N	Ge(−2,3,4)[a]: N	*GE*01-03/GE*01-03*	GE:−2,−3,4
Ex295	Patient with anti-Ge	NT	NT	NT	P132: N; Ex296: 2+ Ge(−2,3,4)[b]: N	*GE*01-03/GE*01-03*	GE:−2,−3,4
D1089	P230 sister	N	N	N		*GE*01-03/GE*01-03*	GE:−2,−3,4
D891	P157 sister	NT	NT	NT		*GE*01-03/GE*01-03*	GE:−2,−3,4
ex296	Ex295 grandson	NT	NT	NT		*GE*01/GE*01-03*	GE:2,3,4
ex300	Ex295 daughter	NT	NT	NT		*GE*01/GE*01-03*	GE:2,3,4
ex301	Ex295 daughter	NT	NT	NT		*GE*01/GE*01-03*	GE:2,3,4
D47	P46 brother	NT	NT	NT		*GE*01/GE*01-03*	GE:2,3,4

Abbreviations: N, negative; NT, not tested; w, weak.
[a]RBC from own inventory.
[b]RBC from serum cells and rare fluids (SCARF) program.

samples (P46, P54, P109, P157, D891 and D1089). Using an optical microscopy, 600 monocytes were counted to determine the percentage of reactive monocyte (RBC adhered and phagocytized). MMA results <4% were considered negative while results ≥4% were considered positive.

Molecular tests

NG_007479.1 was used as reference gene and to define the location of the single nucleotide variants (SNVs) we considered the nucleotide position in the introns. All samples were submitted to allele-specific polymerase chain reactions (AS-PCRs) for exons 2 and 3 using the set of primers GE13-i1+2203F/GYP2R and GYPC3F/GE25-i3-503R, respectively, and a set of specific primers to human growth hormone as internal control. Sanger sequencing was performed to determine the breakpoint of deletions. For this, different sets of primers were used for PCR and sequencing, as listed in Table 2 and in Figure 2. We were unable to sequence the entire gene and the sequenced regions were from IVS1-2140 to IVS2-1250; from IVS2-938 to IVS2+15; from IVS2+280 to IVS3-1735 and from IVS3-490 to IVS3-758.

PCR was performed with 50–100 ng of DNA, 0.2 μM of each primer, 0.4 mM of each dNTP, 2.0 mM of $MgCl_2$, 1.0 U Taq DNA polymerase and 1× reaction buffer. The reagents were obtained from Cellco (Cellco Biotech, São Carlos, Brazil). PCR amplification was performed in

TABLE 2 Primer sequences used in this study.

Primer name	Sequence	Reference
GE13-i1+2203F	GGACGCAGCTGCCAAATCT	Gourri et al. [3]
GYPC-i1-978F	GCCAAGTCCTGACCTGATAC	Gourri et al. [3]
GYPC-i1-561F	TGTGGTCTCTTTGCGAATG	Gourri et al. [3]
GYPC2F	CTCTGCCTCCACCACAATG	This article
GYPC-i2-336F	AGTTTTGGTGAGGGAGGTTG	Gourri et al. [3]
GYPC3F	TGGAGAATCTTCCTCTCTGACC	This article
GYPC2R	GCTGCTTAAAGGGCTCTGTG	This article
GYPC-i2-1283R	GGATCACAGAAATATCAGT	Gourri et al. [3]
GYPC3R	TGCTTGAAGGGCTCTGTGAT	This article
GYPC-i3-773R	TTGGGGAAGGTTGTGTGG	Gourri et al. [3]
GE25-i3-503R	TGCCTTCCTAAAATGATTCTCC	Gourri et al. [3]

Veriti™ 96-well Thermal Cycler (Applied Biosystems, Foster City, CA) using the following conditions: denaturation at 95°C for 15 min, followed by 28–35 cycles of 95°C for 60 s, 57°C for 40 s and 72°C for 150 s, and a final extension step of 72°C for 10 min. After purification, sequencing reaction was performed with BigDye™ Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corporation, Austin, TX) and sequencing was performed on a 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA). CLC software was used for sequencing analysis, in which all the sequences were aligned with both homologous repeated regions.

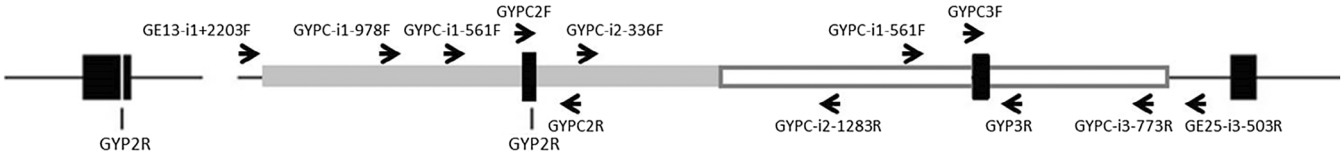

FIGURE 2 Schematic sequence of *GYPC* and position of primers in the gene. The grey box represents repeat region 1 and white box represents repeat region 2. Above the sequence are forward primers and below are reverse primers.

The SNVs were searched in GenBank and their frequency was collected from the NCBI ALFA project [18].

RESULTS

Serological and MMA results

The eight plasma samples from patients and two from their relatives (D891 and D1089) had an antibody reacting from weak (+) to 2+ with all commercially available reagent red cells by indirect antiglobulin testing at 37°C. Negative reactions were observed in red cells treated with papain. Crossmatching performed with Ge:−2 RBCs (previously serologically characterized) from our collection was negative, suggesting the presence of anti-Ge2 in all samples. Only two antibodies were demonstrated to be clinically significant according to the MMA results (P46 with 26% of activated monocytes and P157 with 21%).

Patient RBC samples were also tested with anti-Ge2 A3, anti-Ge2 A8, anti-Ge3 2A4, and all of them gave negative results with the three antisera, which reacted 2+ with control samples, validating the results. Table 1 summarizes the serological findings.

Molecular analysis

Using the AS-PCR, *GYPC* exon 2 was amplified in all DNA samples of patients and relatives, excluding the presence of the Yus type. Exon 3 was not amplified in any of the patients' samples and in two samples from relatives (samples D1089 and D891), suggesting the *GE*01.-03/GE*01.-03* genotype. Molecular genotypes found in patients and relatives are described in Table 1.

By sequencing, using different sets of primers, heterozygosity was identified in the samples from relatives (with the exception of D1089 and D891), which presented a wild-type allele (*GE*01*) in heterozygosity with the *GE*01.-03* allele. Although the P132 sample failed to amplify exon 3 by AS-PCR, it also showed a heterozygous composition, with both *GE*01.-03* alleles, but with different breakpoints. We detected the *GE*01.-03.01* allele in a heterozygous status with the allele reported in the present study (see below). It also occurred in the other samples with GE:−2,−3,4 phenotype.

Characterization of deletion breakpoints

To investigate deletion breakpoints, extensive molecular analysis was performed on control samples ($n = 9$) and on samples with *GE*01.-03/GE*01.-03* genotype with sufficient genetic material, totalizing eight samples, which consist of seven patients and one relative (D891). Samples with *GE*01.-03/GE*01.-03* genotype that had extensive molecular analysis were called *case samples*.

The same molecular background leading to exon 3 deletion was observed in all case samples. The DNA sequences were very similar to the *GE*01.-03.02* allele, but due to the genetic variability found in the control samples, another different breakpoint responsible for exon 3 deletion can be suggested. Two possible start sites of deletion are represented in Figure 3 as first (*GE*01.-03.02*) and second (proposed herein) options. As the control samples showed genetic variability in the SNVs IVS2+745G>A and IVS3+863insAG, it was not possible to define whether the deletion started from IVS2+913 (first option, as already described by Gourri et al. [3] and named as *GE*01.-03.02* genotype) or starting from IVS2+494 (second option, not yet described).

Besides that, the determination of deletion endpoint is also uncertain due to the presence of IVS3+907C>G at a frequency of 61% in control samples and 100% in case samples. These data suggest that sequencing of this region in case samples is part from intron 3 and not from intron 2, as established in *GE*01.-03.02* genotype.

GYPC molecular variability

Analysing the *GYPC* sequencing and comparing with the reference gene and SNV prevalence reported by the NCBI ALFA project [18], as highlighted in Table 3, the nucleotide changes that are predominant in case samples and differ between control samples and/or previously reported frequencies. In addition to the aforementioned SNVs that interfered with the definition of the deletion breakpoint, other molecular changes were predominantly observed in the case samples. Focusing on SNVs located in intron 1, we observed SNVs commonly found in our population and present in 100% of case samples. We emphasized SNVs c.IVS1-661G>A and c.IVS1-68G>A, in which wild-type G alleles are reported in 99% of individuals according to NCBI ALFA. Although both SNVs (A alleles) were present in homozygosity in all case samples, wild-type G alleles were present in 70% and 30% of control samples in SNVs c.IVS1-661G>A and c.IVS1-68G>A, respectively.

DISCUSSION

We herein report the results of the investigation of samples from Brazilian patients with antibodies against Gerbich antigens and their relatives. All patients and two relatives were serologically

Repeat Region 1

Gene localization	IVS2+489	IVS2+494	IVS2+745	IVS2+912-913	IVS2+958	IVS2+988	IVS2+992
Reference sequence	A	C	G	AG	C	C	A
Control D851	A	C	A	AG	C	C	A
Control D1096	A	C	R	AG	C	C	A
Control D1081	A	C	A	AG	C	C	A
Control D1080	A	C	R	AG	C	C	A
Control D1061	A	C	A	AG	C	C	A
Control D1075	A	C	A	AG	C	C	A
Control D1083	A	C	G	AG	C	C	A
Control D1085	A	C	G	AG	C	C	A
Control D257	A	C	R	AG	C	C	A
*GE*01.-03.02*			A	AG			
1st option	A	C	**A**	**AG**			
2nd option	A	C					

Repeat Region 2

Gene localization	IVS3+440	IVS3+445	IVS3+696	IVS3+862	IVS3+907	IVS3+937	IVS3+941
Reference sequence	G	G	A	--	C	T	A
Control D851	G	G	A	AG	G	T	A
Control D1096	G	G	A	AG/--	S	T	A
Control D1081	G	G	A	AG	G	T	A
Control D1080	G	G	A	AG/--	S	T	A
Control D1061	G	G	A	AG	G	T	A
Control D1075	G	G	A	AG	G	T	A
Control D1083	G	G	A	.--	C	T	A
Control D1085	G	G	A	.--	C	T	A
Control D257							
*GE*01.-03.02*					G	T	
1st option					G	T	A
2nd option			**A**	**AG**	G	T	A

FIGURE 3 GYPC reference sequence (NG_007479.1) of repeat region 1 is shown in the top frame and the equivalent sequence of repeat region 2 is shown in the bottom frame. Sequences found in control samples were described separately due to the variability of the sequence. Reference wild-type was included, as well as the sequence of *GE*01.-03.02* with deleted sequence in grey. At the bottom of the frame, sequence of case samples is proposed by two options of deletion breakpoints (in grey). All case samples presented the same nucleotides at these positions. Outlined with dotted lines are conserved regions.

characterized with the GE:−2,−3,4 phenotype, later confirmed by molecular tests. Our results showed that the GE:−2,−3,4 phenotype is prevalent in our population and a specific molecular background was found common to all. This is the first Brazilian study that characterized the molecular basis and structure of the *GYPC* deletion in Brazilian patients.

Gerbich-negative phenotypes are rare worldwide, with the exception of malaria-endemic areas, including the Middle East and Papua New Guinea, where the high frequency of rare Gerbich phenotypes is probably consequence of the presence of *P. falciparum* [4]. Among the rare Gerbich phenotypes, GE:−2,3,4 has been found in Europeans, in the Middle East and in people from African origin [19]. On the other hand, GE:−2,−3,4 phenotype has been described as rarer, being observed with extreme rarity in Europeans and Africans and sporadically in Iraqis, Native Americans, Japanese and Polynesians [19]. Despite this, our results showed a predominance of GE:−2,−3,4 phenotype in Brazilian patients. It is important to emphasize that our study was carried out with alloimmunized patients and we cannot state that this phenotype is more frequent than GE:−2,3,4 in our population, because the molecular investigation was not performed in the general population and alloimmunization could be a bias.

TABLE 3 Polymorphisms with discrepant frequency among NCBI ALFA [18], control samples and case samples.

		NCBI ALFA [18]		Control samples ($n = 9$)		Case samples ($n = 8$)	
c.IVS1-780G>C	rs2165227	G = 0.59	C = 0.41	G = 0.3	C = 0.7	G = 0	C = 1.0
c.IVS1-739G>A	rs501866	G = 0.42	A = 0.58	G = 0.3	A = 0.7	G = 0	A = 1.0
c.IVS1-734G>T	rs935021	G = 0.44	T = 0.56	G = 0.3	T = 0.7	G = 0	T = 1.0
c.IVS1-702C>T	rs935022	C = 0.43	T = 0.57	C = 0.3	T = 0.7	C = 0	T = 1.0
c.IVS1-661G>A	rs2077378	G = 0.99	A = 0.009	G = 0.7	A = 0.3	G = 0	A = 1.0
c.IVS1-638G>A	rs2019405	G = 0.89	A = 0.1	G = 0.44	A = 0.56	G = 0	A = 1.0
c.IVS1-68G>A	rs192963041	G = 0.999	A = 0.001	G = 0.3	A = 0.7	G = 0	A = 1.0
c.IVS2+528C>T	rs74499727	C = 0.74	T = 0.26	C = 0.61	T = 0.39	C = 1.0	T = 0
c.IVS2+745G>A	**rs382844**	**G = 0.43**	**A = 0.57**	**G = 0.39**	**A = 0.61**	**G = 0**[b]	**A = 1.0**[b]
c.IVS2+1560C>G	rs13432961	C = 0.79[a]	G = 0.2	C = 0.5	G = 0.5	–	–
c.IVS3+862insAG	**rs1553470184**	**NR**	**NR**	**Wild = 0.37**	**insAG = 0.62**	**Wild = 0**[b]	**insAG = 1.0**[b]
c.IVS3+907C>G	**rs10174393**	**C = 0.64**[a]	**G = 0.36**	**C = 0.37**	**G = 0.62**	**C = 0**	**G = 1.0**
c.IVS3-748T>C	rs10184704	T = 0.75	C = 0.25	T = 0.58	C = 0.42	T = 0	C = 1.0

Note: Bold values are the SNVs that interfered in deletion breakpoint definition.
Abbreviations: –, sequence deleted; NR, not reported.
[a]Latin American C = 1.0.
[b]If not consider deleted region (see Figure 3).

This study clearly showed the high risk of misclassification of the Gerbich phenotype when molecular characterization is not available. Serological characterization of GE:−2,−3,4 phenotype is challenging, as individuals with this phenotype frequently develop anti-Ge2, but do not always produce anti-Ge3. Moreover, serum with anti-Ge3 is scarce. As a consequence, patients can be erroneously classified as GE:−2,3,4, while the correct phenotype is GE:−2,−3,4, as occurred with patients at our institution. Accurate characterization is important for antigen-matched transfusion, considering that patients with the GE:−2,3,4 phenotype can be transfused with RBC units with the GE:−2,−3,4 phenotype but not vice versa. Furthermore, this classification is very important for the correct registration of these phenotypes in the rare donor programme. The Brazilian National Rare Blood Registry [20] lists 22 GE:−2,3,4 donors and no GE:−2,−3,4 donors, which is contradictory with the results shown in the present study. The aforementioned difficulty to define Gerbich phenotype comprising the limitation of serological methods and the lack of access to Gerbich genotyping may contribute for these conflicting results.

GYPC has a complex structure due to large regions with high level of homology. These repeat regions show 95% of homology, a fact that hinders the design of molecular protocols for Gerbich genotyping [4]. Well-designed primers, optimized PCRs and careful sequence analysis are needed to avoid misinterpretations of Gerbich genotypes. Gourri et al. [3] described a set of primers very useful for classification of Gerbich alleles and for *GYPC* investigation. For this study, we selected some primers that allowed us to characterize the deletions, even when they were heterozygous.

Our results show the genetic diversity of *GYPC* in Brazilians, highlighting some SNVs that are much more prevalent in samples with *GE*01-03* allele than previously described in the literature, even when compared with data from Latin America [18]. The genetic diversity found close to the deletion breakpoints impairs the exact definition of the *GYPC* structure in Brazilians with the rare Gerbich phenotype. Our analysis showed a molecular background similar to the *GE*01-03.02* allele; however, different starting and ending breakpoints are entirely possible, which may allow the recognition of a new allele responsible for the GE:−2,−3,4 phenotype.

Figure 3 aligns the *GYPC* homologous repeat region 1 and 2 and contains the nucleotides that differ in both repeat regions to explain the two proposed breakpoints. As described in the literature, for the *GE*01.-03.02* allele, which we consider as the first option, adenine and adenine followed by a guanine (in bold in Figure 3) found in the case samples belonging to IVS2+745 and IVS2+912–913, respectively, being part of the repeat region 1. However, the reference base of IVS2+745 is a guanine and its respective base in the repeat region 2 (IVS3+696) is an adenine, and we found an adenine in all case samples. Furthermore, the presence of IVS2+745G>A SNV in control samples (61% have IVS2+745A, Table 3) allows us to suggest that it is from the repeat region 1. Reinforcing this hypothesis, the next difference between regions 1 and 2 is IVS2+912AG, which are deleted in the repeat region 2 (IVS3+863–864) and all case samples have AG at IVS2+912–913 nucleotides.

On the other hand, in the second option, we consider that the nucleotides IVS2+745 and IVS2+912–913 are part of the deleted sequence because the adenine located in IVS2+745 in the first option (that has guanine as a reference base) could perfectly belong to IVS3+696, which has adenine as a reference base. Furthermore, in the reference *GYPC* sequence, the next point that differs the repeat regions 1 and 2 is the presence of an adenine and a guanine in IVS2+912–913 and their absence in IVS3+863–864 (the respective nucleotides in repeat region 2). In contrast to the reference sequence, IVS3+863–864AG is present at a frequency of 62% in the

control samples, thus, we can suggest that the detected AG is from the repeat region 2.

The endpoint of deletion may also be questionable. The fragment deletion described for *GE*01.-03.02* ends at IVS3+937, because the nucleotides differ between the two repeat regions at this point, being a cytosine in IVS2+988 (repeat region 1) and a thymine in IVS3+937 (repeat region 2), as shown in Figure 3. However, in the control samples, 30 bases before IVS3+937, we observed a molecular change (IVS3+907C>G) at a frequency of 61%. This SNV was observed only in the repeat region 2, while the respective base in the repeat region 1 is a conserved cytosine (IVS2+985C). All case samples were homozygous for IVS3+907G, that is, they had the SNV found only in repeat region 2, which strongly suggests that this nucleotide belongs to repeat region 2 and that the deletion ends at nucleotide IVS3+907.

In summary, this study revealed for the first time the genetic diversity of *GYPC* in Brazilian individuals with rare Gerbich-negative phenotypes and showed an unexpected prevalence of the GE:−2,−3,4 phenotype. It also demonstrated the importance of using molecular tools in the classification of Gerbich-negative phenotypes for correct selection of variants in antigen-matched transfusions.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest relevant to the manuscript.

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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Use of computational biology to compare the theoretical tertiary structures of the most common forms of RhCE and RhD

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Abstract

Background and Objectives: Computational biology analyses the theoretical tertiary structure of proteins and identifies the 'topological' differences between RhD and RhCE. Our aim was to identify the theoretical structural differences between the four isoforms of RhCE and RhD using computational biological tools.

Materials and Methods: Physicochemical profile was determined by hydrophobicity and electrostatic potential analysis. Secondary and tertiary structures were generated using computational biology tools. The structures were evaluated and validated using Ramachandran algorithm, which calculates the single score, *p*-value and root mean square deviation (RMSD). Structures were overlaid on local refinement of 'RhAG-RhCE-ANK' (PBDID 7uzq) and RhAG to compare their spatial distribution within the membrane.

Results: All proteins differed in surface area and electrostatic distance due to variations in hydrophobicity and electrostatic potential. The RMSD between RhD and RhCE was 0.46 ± 0.04 Å, and the comparison within RhCE was 0.57 ± 0.08 Å. The percentage of amino acids in the hydrophobic thickness was 50.24% for RhD while for RhCE it ranged between 73.08% and 76.68%. The RHAG hydrophobic thickness was 34.2 Å, and RhCE's hydrophobic thickness was 33.83 Å. We suggest that the C/c antigens differ exofacially at loops L1 and L2. For the E/e antigens, the difference lies in L6. By contrast, L4 is the same for all proteins except Rhce.

Conclusion: The physicochemical properties of Rh proteins made them different, although their genes are homologous. Using computational biology, we model structures with sufficient precision, similar to those obtained experimentally. An amino acid variation alters the folding of the tertiary structure and the interactions with other proteins, modifying the electrostatic environment, the spatial conformations and therefore the antigenic recognition.

Keywords

computational biology, hydrophobicity, RhCE, RhD, tertiary structures

Highlights

- RhD and RhCE/ce each have a different physicochemical profile, based on hydrophobicity analysis, electrostatic potential analysis, secondary structure analysis and comparison of tertiary structures.

- Differences among the five proteins could explain the specific antigen recognition.
- We believe that explaining the differences in these proteins by selectively replacing the model proposed by Wagner and Flegel is not correct because it does not allow us to demonstrate the structural differences between the Rh variants.

INTRODUCTION

The attachment of the cytoskeleton to the plasma membrane determines the flexibility or stiffness of the erythrocyte. Ankyrin-1 is a key element connecting the membrane through Band 3 to the cytoskeleton via Spectrin; this complex contributes to the maintenance of the biconcave shape of erythrocytes [1]. Vallese et al. described that the binding domain between the membrane and ankyrin-1 has three different constitutive elements: transmembrane (RhCE and AQP1), extramembrane (cytosolic band-3 domains) and peripheral adaptors (protein 4.2) [2].

The Rh heterotrimer is the anchor between the cytoskeleton and the membrane through the interaction between the N and C termini of RhCE and the first five ankyrin-1 repeats [3]. Interestingly, in the study by Vallese et al. [2] RhD was not detected within the Rh heterotrimer structure, although they used RhD-positive blood. They only observed multiprotein complexes with RhCE, possibly due to the diversity of the RhAG–Rh complex. Another explanation would be that RhD is not associated with the Band 3 ankyrin complex and is alternatively associated with the 4.1R binding complex as in mice [4].

In the 1990s, analysis of the hydrophobic regions of the RhD amino acid (AA) sequence yielded three models of secondary structure. They describe between 11 and 13 transmembrane domains, but the position of the amino terminus varies [5–9].

One of the most influential studies on the three-dimensional description of the RhD protein is that of Cherif-Zahar et al [6]. The studies that use it to explain the D variants substitute the mutant AA residues in the secondary structure of the protein but do not consider the effects on the tertiary structure and the interaction with the membrane, that is, the hydrophobic thickness [10, 11].

Based on these studies, the notion that only AA changes in the exofacial loops (L) affect tertiary structure, and therefore only these alter antibody recognition, has gained acceptance in transfusion medicine. However, the outcome of any AA substitution should affect the physicochemical properties and functionality of the protein [12].

The role of protein structural prediction has gained importance

Computational biology combines physicochemical, biochemical and molecular biology knowledge with quantum mechanics, allowing us to understand variations in the molecular microenvironment and how small chemical changes in pH, temperature or molarity can affect protein interactions. However, predicting protein structures remains an extremely difficult task. The two main problems are calculating the free energy and finding its global minimum.

Depending on whether similar structures are found in the protein data base (PDB) library, protein structure prediction can be divided into template-based modelling and free modelling. Two factors determine the success of structural genomics: determination of experimental structure of optimally selected proteins and efficient computer modelling algorithms [13].

The methods chosen to predict protein structure can be divided into four main classes: first principle without database information; first principle with database information; fold recognition and threading; and comparative modelling and sequence alignment strategies [14].

Conroy et al. proposed one of the first RhD tertiary structures [15]. Recently, some studies have attempted to explain the variations between associations within the RhAG–RhD–RhCE complex [10, 16] and the identification of weak RhD variants by AA substitution [11].

The aim of the present study is to identify the theoretical structural differences between the four forms of RhCE and RhD using computational biological tools.

MATERIALS AND METHODS

We used AA sequences Q02094-1 (RhAG), Q02161-1 (RhD) and NP_065231.4 (RHCE*01) as reference. We removed the initial methionine [6] and substituted the residues indicated in The International Society of Blood Transfusion (ISBT) catalogue in the analysed forms.

Primary structure analysis

Hydrophobicity analysis of the aminoacidic sequence was performed with ProtScale from Expasy using the Kyte and Doolittle scale with a window of nine AAs.

Secondary structure analysis

Secondary structural elements were determined using the prediction algorithm of PBDsum structural analyses [17].

Tertiary structure analysis

The comparison consisted of four steps. (i) theoretical construction (modelling, evaluation and validation); (ii) calculation of the electrostatic potential (EP); (iii) structural alignment and (iv) analysis of protein position within the lipid bilayer.

Theoretical construction

The tertiary structural design was done using the server I-Tasser. This protocol combines sequence-based domain parsing, single-domain structure folding, inter-domain structure assembly and structure-based function annotation in a fully automated pipeline [18].

The six structures were evaluated and validated by uploading the PDB files to two servers with different algorithms.

1. The PBDsum uses the Ramachandran algorithm, which allows visualizing energetically valid regions for the dihedral angles of the backbone ψ versus φ of AA residues in the protein structure [17].
2. ModFOLD8 provides a single score and a *p*-value relating to the predicted quality of a single three-dimensional (3D) model of a protein structure [19].

Calculation of EP

The models were uploaded into the webPIPSA server tool [20], which link between the modifications in EP calculations and the topological properties of the membrane (visual inspection of the EP distribution). The result is a tree-like diagram (epogram) that puts the EP of the query sequence into relation with other proteins for which potential data are available. The analysis identifies both the surface area modifications in each model and the electrostatic distance between structures (Å).

Structural alignment

We align the structures within the 7UZQ model obtained by Vallese using electron microscopy with a resolution of 2.17 Å to verify that the built models were similar to any model obtained experimentally.

To assess the significance of the alignment of sequences and to know the probability that a pair of biologically significant AA residues occupy the same place within the alignment, using the IPBA-webserver, we calculated the global root mean square deviation (RMSD) of atomic positions expressed in Å [21].

The theoretical tertiary structures were compared using an alignment approach and plotted using Geneious 2015 and Pymol.

Analysis of protein position within the lipid bilayer

We determined hydrophobic thickness, transmembrane passages and exofacial loops using OREMPRO [22]. The RhAG structure was used to compare the membrane structure and analyse the coupling sites with the Rh proteins.

We plotted the transmembrane passages with Protter using the modifications of the OREMPRO and Geneious algorithms to visualize the distribution of AAs along the membrane.

To compare the space occupied by proteins in the membrane, we plotted the structures in a 'surface' style. The models are irregular structures; therefore, we construct an imaginary trapezoid seen from above the complex and calculate its area by considering as vertices the alpha-carbons of the four exofacial AAs located superficially in L1, L2, L4 and L6 (Ser_{36}(L1), Pro_{101}(L2), Pro_{230}(L4) and Trp_{352}(L6)).

RESULTS

Primary structure

The raw data of similar AA frequencies between the five forms reveal an obvious identity. On average, all proteins have 263.4 non-polar or hydrophobic AA, 98.6 polar uncharged, 16 negatively charged and 38 positively charged. The only difference appears to be that RhD has 25% more acidic AA and 25% fewer basic AA than the RhCE isoforms, with 17 AA exchanged for others with a different charge.

However, we must consider that the sequences differ by approximately 8% (34–35 AA). Thus, within the sequence, there are on average nine changes for every 100 AA, the probability of finding a substitution in RhD for RhCE is 1 of 11 (34.5/416), and furthermore, the probability of changing the nature of the AA is on average 0.494. And yet these differences will remain balanced.

Hydrophobicity analysis

The differences in the five hydrophobicity plots can be associated with the 35 substitutions in RhD and RhCE (Figure 1a).

Secondary structure

Features such as type, total number of helices and number of AAs per turn were similar. Although all had two β-sheets at the same positions (Thr_{31}-His_{32} and Val_{105}-Val_{106}), the different type of β-turns that each protein has was crucial for the structural variation (Table 1).

If we locate the five significant changes that distinguish the forms of RhCE analysed in this work, we found that the substitutions p.Cys15Trp and p.Ser67Asn do not change the structure of any of the proteins, the side chains are distributed on the outside of the helix. The p.Ile59Leu change cuts the helix in two, but the structure is conserved in all four forms. The substitutions p.Ser102Pro and p.Pro225Ala do not change the structure, probably because they occur within a β-turn (Figure 1b).

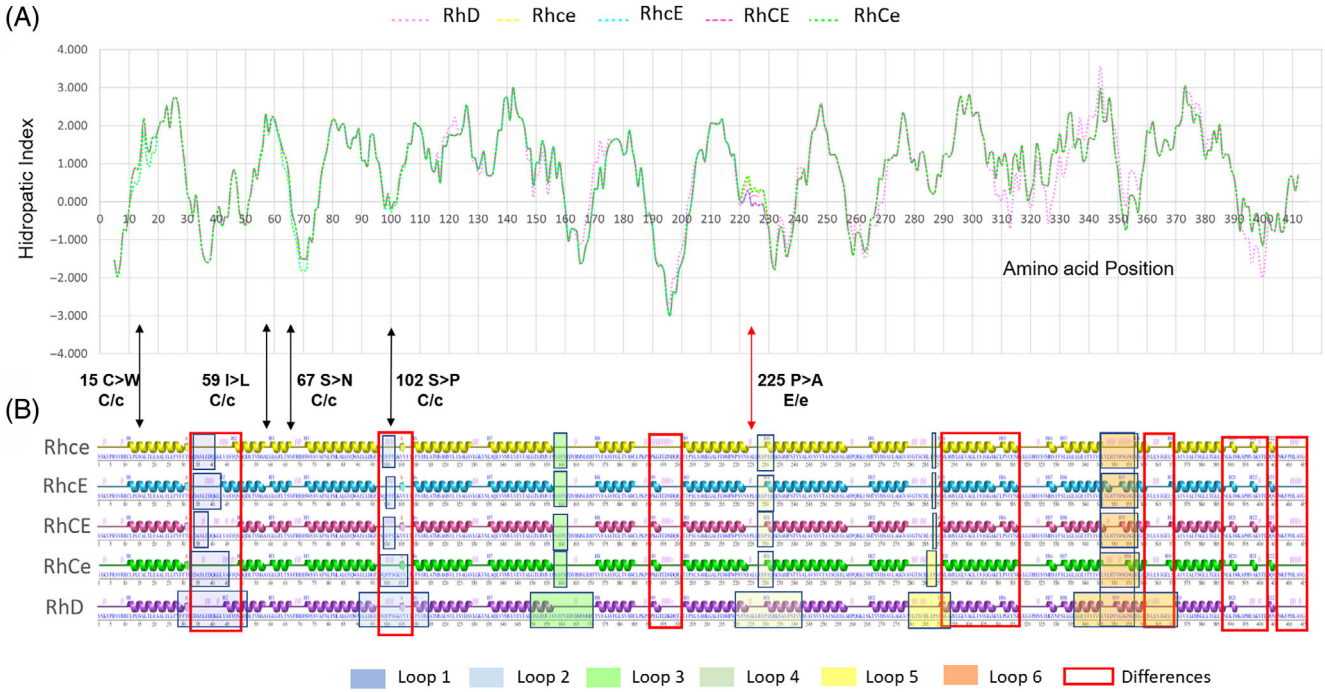

FIGURE 1 (a) Hydrophobicity profile of RhD and RhCE proteins. Labelling of each protein is above the graph. (b) Secondary structures of Rh proteins. The shaded areas represent the exofacial loops and the red rectangles represent the structural differences. The arrows indicate the five changes leading to the RhCE forms.

TABLE 1 Description of the secondary structure of the five forms analysed.

	β-turn						Helix			
	Type						Type			
	I	II	IV	VIII	Total β-turn	Mean of distance Cα1 to Cα4 (Å)	H	G	Total of helix	Mean of AA per turn
RhD	14	2	16	0	32	5.63	17	5	22	3.72
RhCe	12	1	21	1	35	5.61	16	6	22	3.88
RhCE	15	1	16	0	32	5.56	17	6	23	3.71
RhcE	12	1	17	1	31	5.63	17	6	23	3.71
Rhce	13	2	25	1	41	5.50	17	5	22	3.73

Note: First section: types, quantity and the average length of β-turns. Second section: type and quantity of helix, and average amino acid (AA) per turn.

Seven significant structural differences are observed in the five proteins at residues 33–50, 190–201, 291–317, 345–356, 360–370, 387–402 and 407–416. None of the changes that distinguish the forms of RhCE are found in these regions, but at least 10 residues that distinguish RhD from RhCE are found there (Figure 1b).

Tertiary structure

Theoretical construction of the tertiary structure

Modelling

The tertiary structures of the RhD, RhCE and RhAG proteins were modelled by homology, using the 3hd6A (RhCG) template with an identity greater than 31.36%.

Evaluation and validation

The PBDsum server returns the statistical analysis of the Ramachandran plot. It compares about 118 structures with a resolution of at least 2.0 Å and an *R*-factor of at most 20.0. A good quality model would be expected to have more than 90% in the most favoured regions. We calculated RhD 92.0%, RhCe 91.3%, RhCE 90.7%, RhcE 92.9%, Rhce 91.6% and RhAG 92.3%.

The confidence and *p*-values obtained by ModFOLD8 were lower than $p < 0.001$ for the six proteins.

Calculation of the EP

Analysis of EP shows that there are differences in charge distribution that alter the surface area in each model, as well as the electrostatic

distance that varies the solubility of the five Rh forms. Figure 2 shows the epogram with the four electrostatic distances. Only RhCE and RhCe have the same distance.

From the tertiary structure, the EP values were calculated. The obtained models were plotted in 'surface' mode because it allows us to contrast the positive (red) and negative (blue) dipoles in a spatial environment (Figure 3).

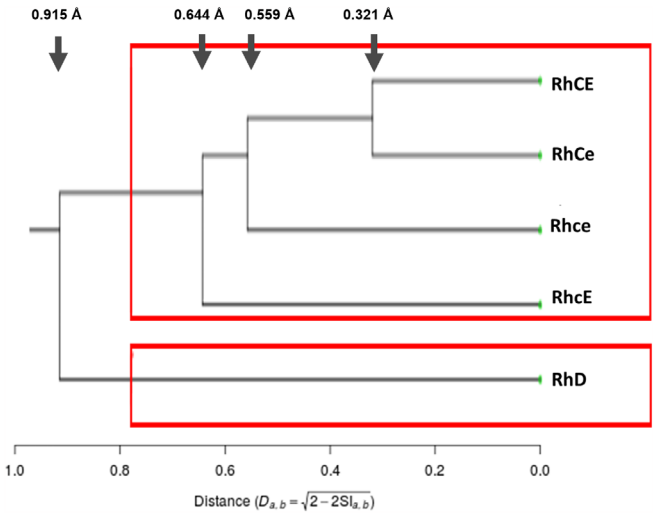

FIGURE 2 Dendrogram that relates the electrostatic potential of the RhD sequence to other proteins. The distances range from 0.321 to 0.915 Å.

Structural alignment

We compared the three-dimensional shape and conformation of the Rh proteins with the 7UZQ template. The alignment was used to identify the equivalences between the structures based on the information of the three-dimensional conformations of the template, since the method can be applied only to sequences of experimentally obtained structures. The result is a superposition of the atomic coordinate sets. The RhCE proteins aligned on the RhCE and RhD with the RhAG.

The calculation of global RMSD shows the divergences between RhD and RhCE. The average RMSD of the inter-Rh comparison (RhD vs. RhCE) is 0.46 ± 0.04 Å, and the intra-RhCE comparison gives an average RMSD of 0.57 ± 0.08 Å (Table 2).

Analysis of protein position within the lipid bilayer

The percentage of transmembrane AA of RhD is 50.24% and RhCE ranges from 73.08% to 76.68%. The exofacial AAs are also different; RhD has 33.89% whereas RhCE has 7.45%–11.3%. However, the amount of cytoplasmic AAs is the same for all five proteins (Table 3). In all cases, the terminal amino and carboxyl groups were cytoplasmic, but they differed in the number of transmembrane steps; only RhCe had 11, while the others had 12.

The percentage of AA in the transmembrane region determines its thickness. The RhAG thickness is 34.2 Å; on average, the four RhCE forms have 33.83 Å. However, in RhD, the membrane is

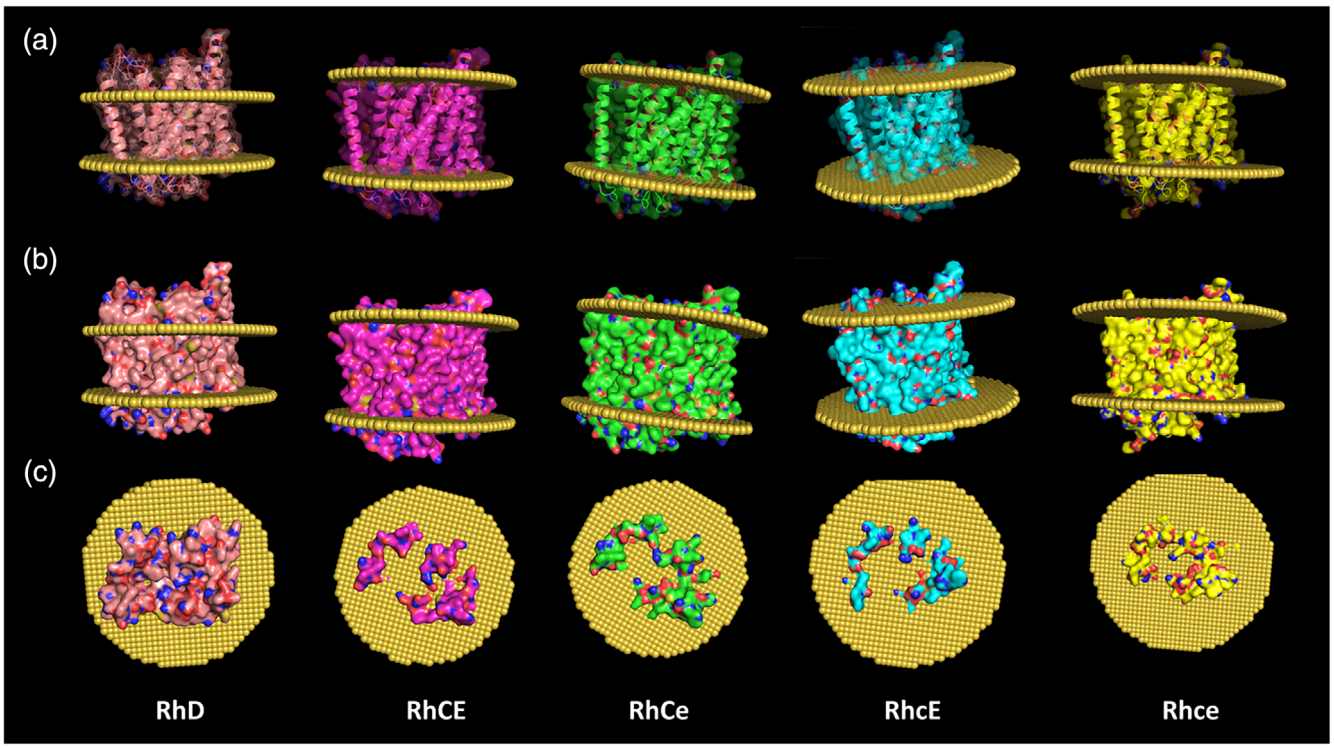

FIGURE 3 Tertiary structures of Rh proteins. Lateral view, (a) cartoon mode, (b) surface mode (positive [red] and negative [blue] dipoles). (c) Apical view in relation to the erythrocyte membrane. The identification of each protein is found at the bottom.

32.38% thinner (23 Å). The difference in the transmembrane region between RhD and RhAG is 11 Å. In Figure 4, we show the overlap of RhD over RhAG and RhCE over RhAG; this is to show that the thickness of the transmembrane region and AA coupling is more feasible between RhCE/RhAG than RhD/RhAG.

TABLE 2 Comparison of the root mean square deviation (RMSD) of the atomic positions in Å, determined using IPBA.

RMSD (Å)					
	RhD	**RhcE**	**Rhce**	**RhCe**	**RhCE**
RhD		0.47	0.5	0.46	0.41
RhcE	0.47		0.62	0.63	0.48
Rhce	0.5	0.62		0.64	0.62
RhCe	0.46	0.63	0.64		0.53
RhCE	0.41	0.48	0.62	0.53	

As suggested by Vallese et al [2], the AA of Rh proteins interacting with RHAG1, RHAG2 and ankyrin-1 is conserved in the five proteins in the transmembrane regions. However, in RhD, residues Phe_{27} and Tyr_{28} are located in L1 (Figure 4).

From the PBDsum detailed secondary structure assignment, we extracted the structure and number of each residue and calculated the percentage of identity of the five proteins. The percentage between RhcE/RhD was 91.58%, although the AA sequence most similar to RhD was RhCe (Table 3).

Figure 3 shows the orientation of the tertiary structures. The exofacial RhD loops are 3.89 times longer than the RhCE forms (Figure 3a,b). Apically, the exofacial topology is different for the five proteins (Figure 3c).

The perimeter of the trapezium is 89.51 Å in RhD and 97.98 ± 0.78 Å in RhCE. The L1–L4 distance is similar among the five proteins, and L4–L6 is the largest difference (Table 4).

TABLE 3 The first three columns describe the percentage of amino acids in the regions: Transmembrane, exofacial and cytoplasmic. The hydrophobic thickness refers to the calculated thickness of the membrane based on the percentage of amino acids in this region.

					Comparison between the four forms of RhCE and RhD according to their structure (% similarity)		
Protein	**%Trans**	**%Exof**	**%Cyto**	**Hydrophobic thickness (Å)**	**1a structure**	**2a structure**	**3a structure**
RhD	50.24	33.89	15.87	23.0			
RhcE	75.24	8.17	16.59	35.1	91.346	91.587	91.707
Rhce	76.68	7.93	15.38	33.3	91.587	87.981	89.183
RhCe	73.08	11.30	15.63	34.5	92.067	90.144	89.784
RhCE	76.68	7.45	15.87	35.0	91.827	90.505	91.106

Note: % Trans, percentage of transmembrane amino acids. % Exof, percentage of exofacial amino acids; % Cyto, percentage of cytoplasmic amino acids.

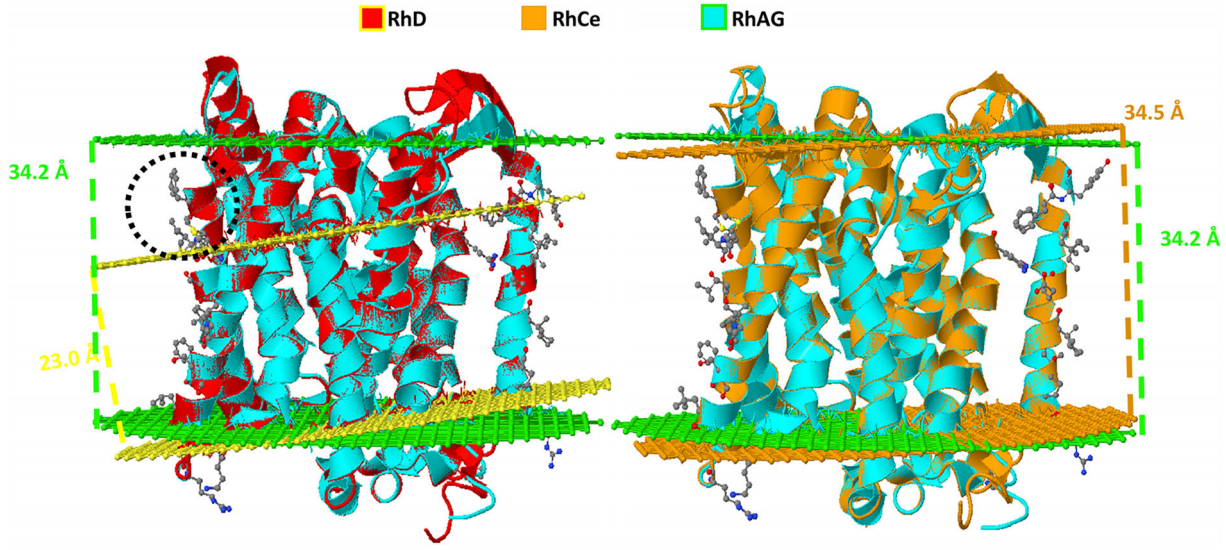

FIGURE 4 Alignment of RhD and RhCE on RhAG. Note the difference in thickness of the transmembrane regions between RhD and RhAG. The amino acids involved in the association with RhAG1, RhAG2 and ankyrin-1 are centred in ball and stick. The black circle indicates residues Phe_{27}, Tyr_{28} in loop 1 of RHD, which make the coupling with RhAG less stable. Membranes are shown in RhAG green, RhD yellow and RhCE orange. Protein identification is at the top of the figure.

The four RhCE antigens differ in the combination of microdomains and secondary structures found in four regions. The difference between the C and c antigens are in L1 (Ser_{36}-Leu_{37}) and L2 (Pro_{101}), and between the E and e antigens, it is in L6 (Val_{351}-Met_{357}). L4 (Arg_{228}-Pro_{230}) is different only in the Rhce form (Figure 5).

Figure 6 presents a schematic summary of the four structural levels of the five proteins studied, highlighting the exofacial topological differences, transmembrane distribution and similarity of cytoplasmic regions.

TABLE 4 Distance between the Cα of the four exofacial amino acid and perimeter of the trapezoid formed from the five shapes analysed.

	Distance (Å)				
	L2–L6 Pro-Trp 101–352	L1–L2 Ser-Pro 36–101	L4–L1 Pro[a]-Ser 230–36	L6–L4 Trp-Pro[a] 352–230	Perimeter
RhD	33.6	16.42	18.67	20.82	89.51
RhcE	36.71	17.9	18.09	24.54	97.24
RhCE	37.3	18.44	17.77	25.38	98.89
Rhce	36.25	18.91	19.69	23.52	98.37
RhCe	37.44	18.22	17.84	23.92	97.42
Mean	36.26	17.978	18.412	23.636	97.98

Abbreviation: L, loop.
[a]In RhD, Gly replaces Pro in 230.

DISCUSSION

The scheme of Wagner and Flegel [23] was the cornerstone for revealing the differences between RhD and RhCE. Their figure shows the secondary structure of a single protein and highlights the 35 AA positions that distinguish the forms of RhCE and RhD. This is logical, assuming that the genes they encode are highly homologous. Since then, studies have suggested that transmembrane changes do not affect exofacial loops or that the changes should involve more than one AA. However, altering a single AA affect not only the structure but also the solubility profile of the protein, to the extent that recognition by antibodies is altered. The RHCE*ceRT [24] and RHCE*ceSL.02 [25] alleles are examples of how the change of one

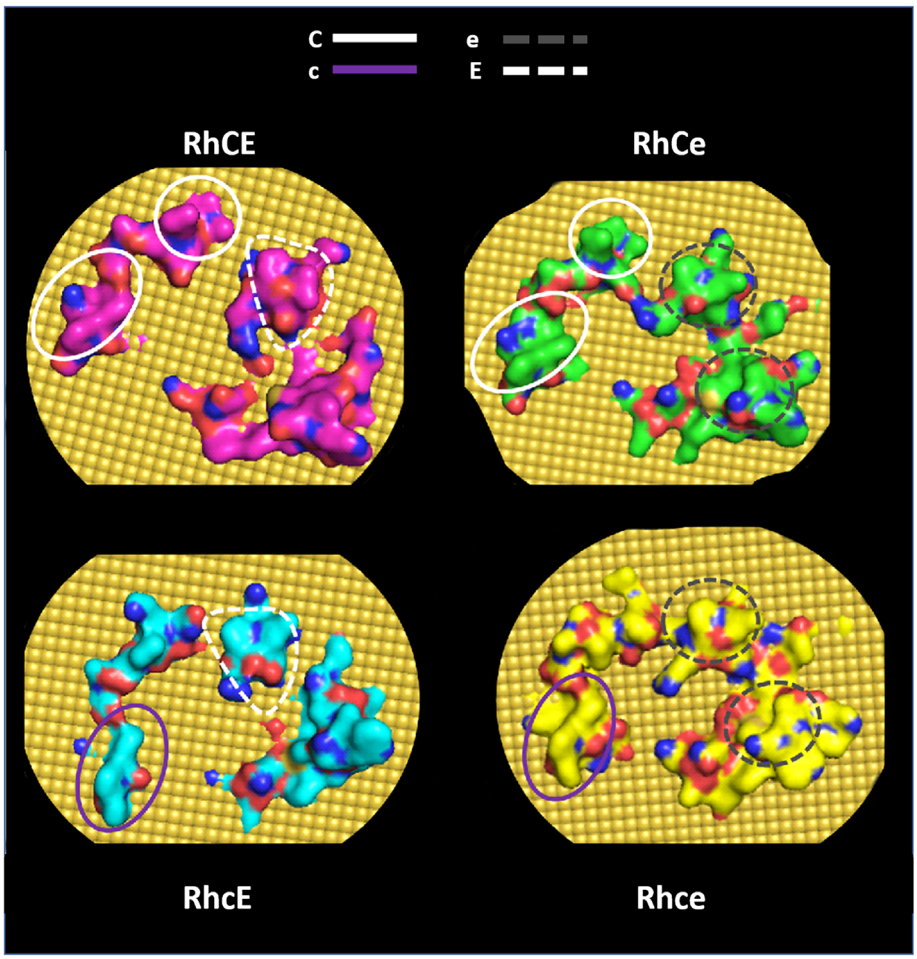

FIGURE 5 Apical view of possible antigenic sites. The differences between E/e are in L6, while those of C/c are in L1 and L2. Rhce is the only one with differences in L4. Solid lines identify C (white) and c (purple) antigens. Dashed lines represent E (white) and e (grey).

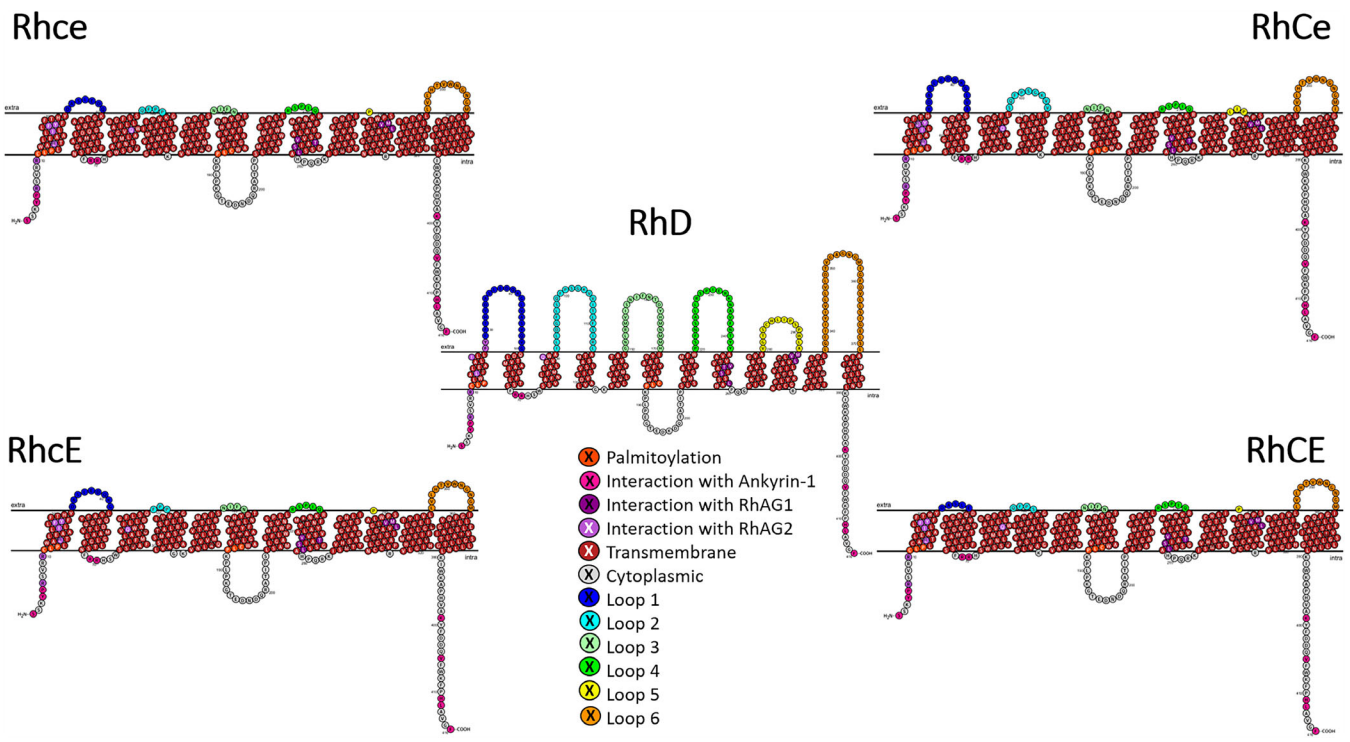

FIGURE 6 Secondary structures of the RhD and RhCE. Note the difference in the length of the exofacial loops of RhD compared with RhCE proteins. Amino acids interacting with ankyrin-1, RhAG1 and RhAG2 in the transmembrane and cytoplasmic regions are labelled. The colour identification code is within the image.

AA (p.Arg154Thr and p.Ser122Leu, respectively) exposes RhD epitopes usually lacking.

We derived the effects of AA substitutions on the final confirmation of Rh proteins by determining the physicochemical profile of each protein based on analysis of hydrophobicity, EP, secondary structure and comparison of tertiary structures. Then, we created 3D models of RhD and RhCE to compare the protein regions that may explain the antigenic differences.

Homology modelling is a robust approach for comparing structural models, but it is directly associated with the accessibility of related protein templates. The PDBID:3HD6 was used to analyse some RHD variants. Srivastava et al. [26] created a structural model of RhD where they localized the positions of the DAU variants. de Brevern et al. [27] linked the structural changes in Phe223 to the risk of alloimmunization. In contrast to the other proposals, they suggested a specific two-dimensional (2D) structure for each change. Floch et al. [10] used comparative models to obtain 3D structural information about RhD variants associated with anti-D formation. However, like Srivastava, they mapped the positions of the different variants onto the structural model.

Although the sequences of Rh proteins are 91.71 ± 0.31% similar, they are distributed differently on the membrane because of the hydrophobicity of their AA, such that the hydrophobic thickness of RhCE is greater than RhD, which facilitates interaction with RhAG. The binding between RhD and RhAG would be less spatially stable because two of the AAs would be outside the membrane in the case of RhD. One of the questions at the forefront of Vallese's study is: why was RhCE only observed in complexes with RhAG? Our results suggest that the hydrophobic region of RHAG (34.2 Å) is very similar to that of the four RhCE forms, which average 33.83 Å. From a stoichiometric viewpoint, the hydrophobic region of RhD is 11 Å narrower; this explains that its exofacial loops are 3.89 times longer than those of the RhCE isoforms, and the coupling with RhAG would be unstable.

Several proteins form the macrocomplex of the erythrocyte membrane, whose quaternary structure results from their interactions. The cytoplasmic and transmembrane regions of the proteins analysed are conserved, possibly because of interactions with RhAG and ankyrin. The exofacial β and γ turns are the only 'free' regions defined by interactions, with the microenvironment being the most variable region of the five forms. In practice, these differences are indeed present because anti-RhD antibodies do not recognize RhCE forms, and there are differences between the latter that make them special.

It is true that the experimentally obtained structures are more accurate. However, it must be taken into account that they were obtained under controlled, even 'artificial' conditions, and the structure is the product of these 'stressed' zones. Even though the data from the computational models may not be as accurate, it is possible to theoretically control these changes. When we compared the RhCE models with the model obtained by Vallese, they were fitted to the RhCE structure, while RhD was fitted to RhAG, indicating that they are different proteins, confirming the models obtained by computational biology.

The structures of RhD and RhCE are different. EP values are specific for each protein, which means that organizes differently in the same solvent. Conformational differences of the loops in length and sequence were to be expected. However, they may also be a consequence of the conformational flexibility of the fully folded polypeptide.

The differences between RhD and RhCE are traditionally explained by changes in five AAs at positions 15, 59, 67, 102 and 225. Two changes stand out, the first at 102 because it is exofacial and distinguishes C/c. The second is 225 because it is the only change in E/e. However, our data suggest that these substitutions are largely transmembrane but have an effect on the exofacial conformation. Thus, when microdomains and secondary structures are analysed, the difference between C and c is in L1 (Ser_{36}-Leu_{37}) and L2 (Pro_{101}). The difference between antigens E and e is in L6 (Val_{351}-Met_{357}). The Rhce is the only one with the difference L4 (Arg_{228}-Pro_{230}).

It has been suggested that the G antigen is exposed in RhD, RhCe and RhCE, which is caused by the p.Ser102Pro variation. Our results suggest that the structural change is not due to the variant but is a consequence of interaction with other AA, not only with the lateral AA in the primary structure but also through physicochemical interactions with others in the tertiary structure, more so with AA of other proteins in the complex (quaternary structure). We suggest that this change is not necessarily in L2 (Phe_{100}-Ser_{102}), because the structural microdomains and the secondary structures predicted with IPBA and PBDsum are the same in that region in all five proteins.

When experimental data are not available (as in the case of RhD), it is difficult to validate a computational protein model in a meaningful way because the obtained model does not necessarily have the best geometry and conformational value close to the real model. Therefore, it is very important to balance the tools used to validate the models.

In conclusion, although they are homologous genes, the proteins of the five Rh forms differ because of their particular physicochemical properties. We believe that explaining the differences in these proteins by selectively replacing the model proposed by Wagner and Flegel is not correct because it does not allow us to demonstrate the structural differences between the Rh variants.

Using computational biology, we can model structures sufficiently precise and similar to those obtained experimentally, allowing us to assume the 'topological' differences that an antibody recognizes. The G+, RHCE*ceRT+ and RHCE*ceSL.02+ phenotypes, among others, prove that the variation in the AA sequence (primary structure) conformationally alters the tertiary structure. In addition, the interactions with the other proteins modify the electrostatic environment, which changes the spatial conformations (quaternary structure) and therefore the antigenic recognition.

Our proposal allows us to obtain a unique physicochemical profile that takes into account the theoretical differences and the possible consequences of antigen recognition.

ACKNOWLEDGEMENTS

R.T.-G. and H.E.-J. designed the research study, performed the research, acquired and analysed the data and wrote the paper; F.R.-M. and M.L.D.-L. wrote the first draft of the manuscript; H.A.B.-G. reviewed the manuscript.

CONFLICT OF INTEREST STATEMENT

All authors of this article declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available for RhCe: https://figshare.com/s/07dea47d376261626e16; RhCE: https://figshare.com/s/98ada4cf838e77a96d51; RhcE: https://figshare.com/s/5f15497e7b018c6c54d0; Rhce: https://figshare.com/s/09572b82b5808c403bf4; RhD https://figshare.com/s/17f41422446d5cee04eb.

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SHORT REPORT

Vox Sanguinis ISBT International Society of Blood Transfusion

Removal of UK-donor deferral for variant Creutzfeldt–Jakob disease: A large donation gain in Australia

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Abstract

Background and Objectives: Until 25 July 2022, people who spent more than 6 months in the United Kingdom during the variant Creutzfeldt–Jakob disease (vCJD) risk period 1980–1996 (UK donors) were deferred from blood donation in Australia. Regulatory approval to remove the deferral was underpinned by published mathematical modelling predicting negligible vCJD transmission risk increase with a gain of 58,000 donations.

Materials and Methods: The donor questionnaire retained the UK deferral screening question until a version update effective 12 February 2023, which enabled identification of the newly eligible cohort of UK donors. Their donations were tracked for a 6-month period (25 July 2022–24 January 2023) and compared with baseline Lifeblood donation metrics and predicted gains.

Results: A total of 38,462 UK donors attended to donate 78,762 times in the 6 months. Of these, 32,358 donors (females = 19,456, males = 12,902) successfully donated 67,914 times representing 8.4% of total collections.

Conclusion: Cessation of the UK deferral resulted in donation gains exceeding modelled predictions because of a higher than predicted number of donors who donated at a higher rate. Had these newly eligible donors not donated, overall donation numbers would have been 88% of target rather than the 96% achieved.

Keywords

blood donation, blood safety, donor eligibility, transfusion transmitted infections, vCJD

Highlights

- In Australia, from December 2000 until discontinuation in July 2022, Lifeblood indefinitely deferred people with prior residency in, or extended travel to, the United Kingdom during the variant Creutzfeldt–Jakob disease (vCJD) risk period, 1980–1996.
- Removing the vCJD UK geographical deferral has resulted in a large donation gain without any negative vCJD-risk impact to date.
- Other countries with a vCJD UK deferral could consider the risk–benefit of removing the deferral.

INTRODUCTION

Australian Red Cross Lifeblood (Lifeblood) collects a combined total of 1.6 million donations annually from around 515,000 donors [1]. From December 2000 until discontinuation in July 2022, Lifeblood indefinitely deferred people with prior residency in, or extended travel to, the United Kingdom during the variant Creutzfeldt–Jakob disease (vCJD) risk period 1980–1996 to mitigate the potential blood safety risk. Regulatory approval to discontinue the deferral was supported by published mathematical modelling that concluded cessation of the deferral would still result in a negligible blood safety risk [2]. The modelling also predicted that the increase in eligible donors associated with ceasing the deferral would result in an estimated 737,112 people in the Australian population becoming eligible to donate, translating into a predicted increase of over 17,000 donors and almost 58,000 donations annually, assuming these newly eligible donors donated at the same rate as the Australian population.

After receipt of the required regulatory and government approvals, the deferral was removed effective 25 July 2022. However, because additional government approvals are required to change the donor questionnaire (DQ), Lifeblood retained the UK-residency screening question until a version update on 12 February 2023. This permitted identification of the newly eligible donor cohort consisting of both returning (lapsed who have not donated in at least 2 years but have donated previously) and entirely new donors allowing metrics of these donors to be tracked and compared with baseline Lifeblood donor metrics and the modelled predictions.

MATERIALS AND METHODS

Identifying currently deferred and newly eligible donors

Lifeblood's national blood management system, ePROGESA (MAK-SYSTEM, France), has an electronic medical questionnaire used to record donors' responses and deferrals. From December 2000, Lifeblood's DQ Section A contained the following screening question, which is asked of new and lapsed donors only—*From 1 January 1980 through to 31 December 1996 inclusive, have you spent (visited or lived) a total time which adds up to 6 months or more in England, Scotland, Wales, Northern Ireland, the Channel Islands, the Isle of Man, or the Falkland Islands?* A 'Yes' answer resulted in indefinite deferral. These donors, previously deferred because of a geographical risk factor, are referred to as 'UK donors' in this paper. From 25 July 2022, a system enhancement resulted in no action from the question and donors were permitted to donate if they answered yes.

A report was developed to identify donors who answered 'Yes' to the UK screening question at their first donation during the period following cessation of the deferral, or for previously deferred donors who had a UK deferral. This included therapeutic donors, that is, donors with hereditary haemochromatosis who underwent phlebotomy for discard who were not required to complete Section A.

Donation metrics

From 25 July 2022 until 24 January 2023 (6-month study period), the number of donor attendances, successful donations and donation types from previously deferred (returned donors) and newly eligible (new donors) UK donors was tabulated. The collection success rate (number of successful collections per number of attendances) was calculated as well as the proportional contribution of UK donors to total collections and the deferral/plasma for fractionation restriction rate.

RESULTS

A total of 38,462 UK donors attended to donate 78,762 times; attendances peaked at 14.4% of total in the first week, stabilized at 8.57% after the first 3 weeks then increased slightly to 8.7% in the last week. Of these donors, 32,358 (females = 19,456, males = 12,902) successfully donated 67,914 times during the study period, representing 8.4% of the 804,830 total Lifeblood collections in the period, at an average collection success rate of 86.2%. New donors made up 84.5% of the total newly eligible UK-donor cohort (27,346 of 32,358) compared with 12.4% of total panel donors during 2022. The eligible UK-donor cohort was comparatively older, 51 years compared with 43 years for all donors, and the proportion over 60 was 22.5% versus 17.6% in all donors.

Of the 67,914 total donations (excluding discards and autologous, $n = 276$), 40,101 were transfusible component donations and 27,537 were plasma donations for further manufacture (Table 1). The trend of weekly donations by newly eligible UK donors showed an initial peak in the first 2 weeks (4020 and 3229 for weeks 1 and 2, respectively) with a subsequent stabilization, maintaining high donation rates with a weekly average of 2866 in the last 2 weeks of the study period (Figure 1).

During the same period, the collection success rate for all donors was 93.1%, higher than the UK donor rate of 86.2%. The most frequent single reasons for deferral or plasma restriction in the UK group (Table 2) were a transfusion in the United Kingdom, medical conditions such as high blood pressure and malignancy, and use of osteoporosis drugs that are associated with age.

TABLE 1 Donation modality by female and male UK donors.

Donation type	Female	Male	Total
Autologous	18	2	20
Clinical apheresis plasma	0	2868	2868
Plateletpheresis collection	0	597	597
Homologous whole blood	21,550	14,137	35,687
Source plasma for fractionation	16,567	10,970	27,537
Therapeutic: discard	72	184	256
Therapeutic: usable	297	652	949
Total	38,504	29,410	67,914

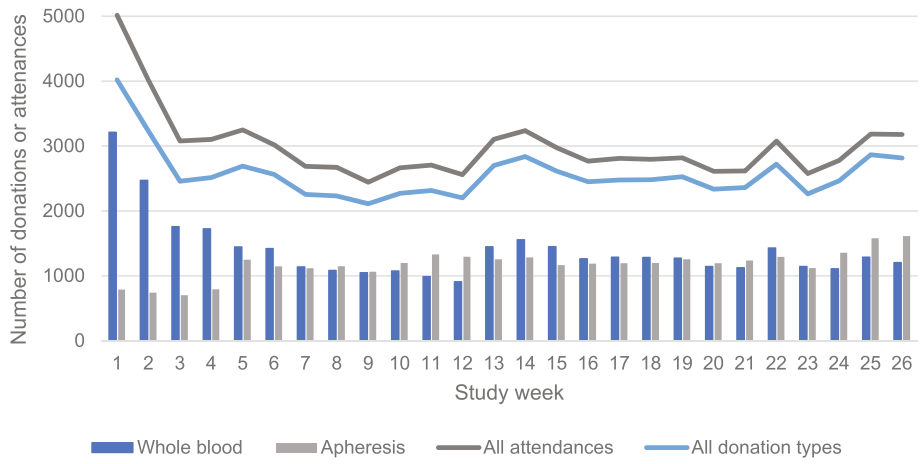

FIGURE 1 Weekly attendances and donation totals for the newly eligibly UK donors.

TABLE 2 Unique deferral/plasma restriction counts among UK donors.

Deferral/plasma for fractionation restriction reason[a]	Count	Proportion in period among all donors (%)
Transfusion in UK [variant Creutzfeldt–Jakob disease (vCJD) risk]	246	0.19
Medical condition under investigation	243	8.6
Untreated high blood pressure	189	1.12
Donor safety assessment required for condition	151	3.71
Veins unsuitable for whole blood	148	0.68
Therapeutic donor awaiting approval	80	1.05
Malignancy	78	0.36
Malaria	73	51.73
Medical Officer deferral	59	0.23
Denosumab	57	0.54
Other	2910	31.80

[a]Some reasons (e.g., 'Malaria') are not outright deferrals but a time-based restriction to donation for plasma for fractionation.

DISCUSSION

Cessation of the UK deferral in Australia has resulted in gains over the first 6 months of almost 68,000 donations, substantially exceeding modelled predictions. The additional newly eligible, successful UK donors made a substantial contribution to supporting local blood sufficiency at a time when donations were below Lifeblood's total internal targets due to multiple factors including COVID-19 pandemic impacts. Had these newly eligible donors not donated, total donations would have been 88% of total target in the second half of 2022, rather than the 96% achieved. Based on the donation frequency to date of 2.1 donations in 6 months versus 2.9 per annum for all panel donors, the cohort of UK donors appears highly committed. The additional 27,537 plasma donations made a valuable contribution towards Australian plasma supply.

The trend of weekly donations by newly eligible UK donors indicates an initial peak in the first 2 weeks with a subsequent stabilization that, importantly, was maintained throughout the study period. The initial high uptake likely reflected several factors, including pre-implementation marketing activity centred on direct invitation to 7000 existing 'deferred' donors, targeted media [3], promotional activity and possibly a higher level of motivation in donors deferred for over 20 years. The media response was unprecedented with over 2000 media stories in the first 2 days of the campaign alone (Lifeblood, unpublished).

A key Lifeblood donation metric is the proportion of successful collections (collection success rate). The average collection success from UK donors was 4.7% lower than the new donor panel (86.2% vs. 90.9%). However, it steadily increased over the study period, from 80% in the first week to 90% in the last week. The lower rate early in the study period probably reflected both a higher rate of first-time donors, who have a lower collection success, as well as a higher proportion of older, lapsed donors who had a higher rate of deferral than the general donor population (Table 2). The deferral for a UK transfusion that remains due to vCJD risk is overrepresented in this cohort. Lifeblood has submitted a request to our regulator to have this deferral removed, as like the geographical deferral it is considered a negligible risk.

There are a number of possible reasons for the underestimation of the predicted number of newly eligible donors. An important model assumption was that the newly eligible population would donate at the same rate as the general population, but our data demonstrate a higher donation rate. Travel data are incomplete because information on accrued durations of short-term travel in individuals was not directly available potentially leading to underestimation of the number of people affected by the UK deferral. People who had in fact spent less than 6 months may have answered 'yes' if they were unsure of the time in the United Kingdom. Blood donation [4] is associated with higher socio-economic status and the UK donor cohort may represent

a relatively high socio-economic group of targeted skilled migrants [5]. Another possible contributor is a higher level of initial donation commitment among the substantial proportion who had previously been regular donors and had been unexpectedly subject to a new indefinite deferral, which prematurely halted their eligibility to donate. Finally, other contributing factors likely include the efforts made to directly contact deferred donors and a robust media campaign.

In conclusion, the removal of the UK deferral has had a substantial positive impact on the Lifeblood donor base and contributing significantly to our ability to maintain blood product supply during a challenging period. Furthermore, the additional plasma donations support plasma for fractionation targets aiming to improve self-sufficiency for plasma-derived blood products. Given we previously estimated the post-deferral removal risk of transfusion-transmission of vCJD as less than 1 in 1.4 billion for a clinical case, any residual risk impact is unlikely ever to materialize. Other countries such as the United States have removed the deferral [6] and our demonstrated increase supports that removing this deferral in other countries will increase other countries' self-sufficiency for plasma [7].

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C.R.S. drafted the manuscript. V.C.H. did the data analysis. All authors contributed to manuscript review and finalization.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

Data not available-participant consent not obtained and aggregated data is presented in paper.

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SHORT REPORT

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Identification of a novel variant c.761C>T on *ABO*B.01* gene in ABO glycosyltransferases associated with B_{weak} phenotype

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Abstract

Background and Objectives: ABO antigens are produced from H antigen by the activity of glycosyltransferase enzyme encoded by the *ABO* gene. Variants in the *ABO* gene can produce a weak ABO phenotype. In this study, we identify a novel *ABO*BW* allele and investigate the underlying mechanism leading to the B_{weak} phenotype.

Materials and Methods: The ABO phenotype and genotype of the sample were determined using serological and direct DNA sequencing methods. We assessed the impact of the novel variant by three-dimensional modelling to predict protein stability changes ($\Delta\Delta G$), and carried out an in vitro expression assay. The total glycosyltransferase transfer capacity in the supernatant of transfected cells was also examined.

Results: Serological analysis confirmed the B_{weak} phenotype in the subject, and gene sequencing identified a novel variant c.761C>T (p.A254V) on the *ABO*B.01* allele, resulting in a *BW-var/O.01.02* genotype. In silico analysis suggested that the p.A254V variant on the *B* allele may reduce the stability of glycosyltransferase B (GTB), as indicated by the $\Delta\Delta G$ values. In vitro expression studies showed that the variant p.A254V impaired H to B antigen conversion, although it did not affect the expression of GTB.

Conclusion: We identified a novel *BW* allele and demonstrated that the variant c.761C>T (p.A254V) can cause the B_{weak} phenotype by reducing the stability of GTB.

Keywords

ABO, novel allele, subgroup, variant

Highlights

- Mutations in the glycosyltransferases encoded by the *ABO* gene can result in the weak ABO phenotype.
- A novel *BW* allele c.761C>T has been identified.
- The novel *BW* allele could reduce the stability of glycosyltransferase B to cause the B_{weak} phenotype.

Hang Lei and Hui Zhang contributed equally to this study.

INTRODUCTION

The ABO blood system plays a crucial role in transfusion medicine [1]. The *ABO* gene spans approximately 19.5 kb on chromosome 9q34.1–q34.2 and comprises a 1062 -bp coding region with seven exons and several regulatory elements [2]. The last two exons (exons 6 and 7) encode for the catalytic domain of ABO glycosyltransferases (GTs), representing 77% of the GT [3–5]. Most individuals have a standard ABO blood group phenotype, such as A, B, AB and O. Genetic alterations in the *ABO* gene, such as missense variant, insertions or deletions in coding regions, splicing sites or regulatory elements, can lead to weak expression of A or B antigens [6–8]. To date, more than 300 *ABO* subgroup alleles have been reported globally, including weak A, weak B, CisAB and B(A) subgroups. ABO subgroups have a low frequency of around 0.015% in China [9].

Weak B subgroups are rare and typically identified by apparent discrepancies between red cell typing and reverse grouping by serum or plasma. However, the molecular mechanism underlying weak B subgroups is still unclear. In this study, we identified a novel variant *ABO*B* c.761C>T (p.A254V) in a donor with the B_{weak} phenotype and investigated the possible mechanisms underlying this weak subgroup.

MATERIALS AND METHODS

Subjects and phenotype

Peripheral venous blood samples were collected from a blood donor. The ABO phenotype was determined using the IH1000 system (Bio-Rad, Roanne, France). On encountering a discrepancy, we followed up with a manual tube method and adsorption–elution test according to AABB technical manual [10]. In the tube method, monoclonal anti-A, anti-B, anti-A1, anti-H and ABO red blood cell kit (SHPBC, Shanghai, China) were used. Samples from 120 Chinese donors were used as normal controls.

ABO gene amplification and sequencing

Genomic DNA was extracted from the blood sample using a blood DNA kit (Tiangen, Beijing, China). All seven exons of the *ABO* gene were amplified using primers as previously described [11]. The polymerase chain reaction products were purified and sequenced using an ABI 377 sequencer (Applied Biosystems, Foster City, CA). The haplotypes were confirmed by cloning and sequencing the gel-purified products containing the variant site [5].

Modelling of the three-dimensional structure of GT

The x-ray structure of glycosyltransferase B (GTB; PDB code, 3SXE) was used as the template to construct the initial molecular model. The in silico variant was modelled using Chimera software (version 1.11.2, University of California, San Francisco, CA) [12]. The structural figures were generated using Chimera or prepared by visual molecular dynamics (VMD) [13].

Predicted protein stability changes in variants

The site directed mutator (SDM) field in DUET was used to calculate the difference in free energy of the variant: ddG ($\Delta\Delta G$) based on the GTB enzymes (PDB code, 3SXE) to examine the effect of the variant on protein stability. The predicted results are expressed as the variation in $\Delta\Delta G$ and the negative values denote destabilizing mutations [14]. The PolyPhen-2 was applied to predict impact of the variant on the structure and function of GTB. Two pairs of datasets, HumDiv and HumVar, were used to train and test PolyPhen-2 prediction models. The outcome can be one of probably damaging, possibly damaging or benign based on the scores.

In vitro expression of GTB variant p.A254V

The full-length *ABO*B.01* cDNA was cloned into expression vector pcDNA3.1 containing the 1X FLAG gene. Site-directed mutagenesis (QuikChange mutagenesis kit, Stratagene, Waldbronn, Germany) was used to introduce the variant p.A254V. HeLa cells were transfected with wild-type or variant-type vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The empty expression vector was transfected as a negative control. The total GTB transfer capacity in the supernatant of transfected HeLa cells was determined by standard serologic tube method [8]. To avoid the interference caused by the operation of different technicians, the test was done three times.

Western blot

Transfected cells were lysed, and the proteins in the cell lysates were measured by bicinchoninic acid method. Western blotting was done using rabbit anti-FLAG antibody (Sigma-Aldrich, St Louis, MO). The β-actin protein was used as a control. The protein bands were detected using chemiluminescence reagents (Thermo Fisher Scientific, Rockford, IL).

RESULTS

Characterization of blood group phenotype

The serologic results indicated a discrepancy between the forward and reverse typing (Table 1). The subject S777's red blood cells (RBCs) did not react with anti-A and anti-A1, but showed mix-field agglutination with anti-AB and anti-B in the tube tests. However, subject S777's serum strongly agglutinated A1 cells. Consequently, subject S777 was suspected to be B_{weak} subgroup (Table 1).

TABLE 1 ABO subgroup phenotype for the blood donor.

	Forward typing				Reverse typing			
No.	Anti-A	Anti-A_1	Anti-B	Anti-AB	Anti-H	A_1	B	O
S777	–	–	1 + mf	1 + mf	4+	4+	–	–

Note: A_1 indicates red blood cells with A_1 phenotype; B indicates red blood cells with B phenotype; O indicates red blood cells with O phenotype. '–' indicates negative reaction.

TABLE 2 The one novel ABO subgroup allele in this study.

No.	Allele	Critical nucleotide change	Corresponding amino acid change	Phenotype	Genotype	GenBank No.
S777	*BW-var*	c.761C>T	p.A254V	B_{weak}	*BW-var/O.01.02*	KF751861

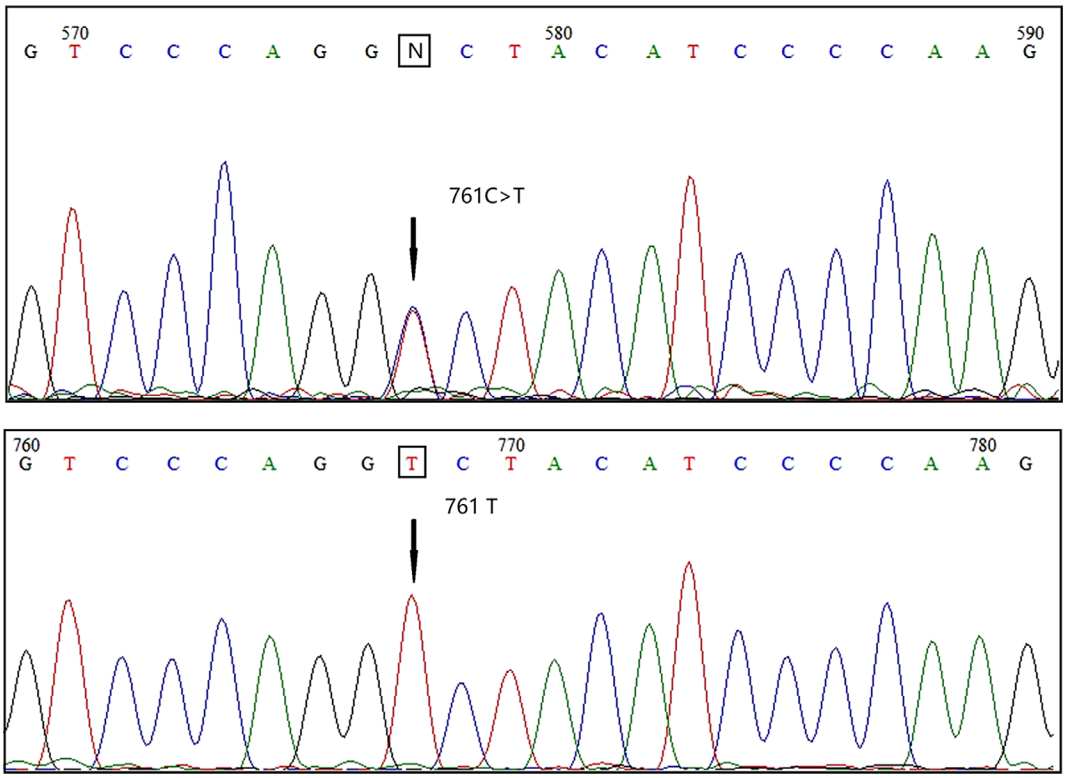

FIGURE 1 The DNA sequence of the region around nucleotide position c.761. The top chromatogram reveals a heterozygous status at c.761. The bottom chromatogram reveals the sequences of the isolated variant *B* allele after cloning. The nucleotide in box denotes the variant site.

ABO gene sequence analysis

Sequence analysis identified one novel variant (Table 2), which was not found in 120 random healthy Chinese individuals with common ABO blood groups. This novel variant c.761C>T (p.A254V) was detected in the *B.01* allele in subject S777 (GenBank No. KF751861) and was confirmed through cloning (Figure 1). It has not been found in other parts of the world according to the websites of PubMed, Erythrogene [15] and the International Society of Blood Transfusion (ISBT). We have submitted data on the novel variant to the ISBT for allele designation.

Three-dimensional structural analysis of the variant

The complete structure of the unliganded wild-type GTB and V254 variants was mainly built from the crystal structure 3SXE (Figure 2a,b), which is an unliganded x-ray structure of GTB enzyme. By comparing the static structure of the 3SXE wild-type and V254 variant, the root mean square deviation (RMSD) of the two structures was 0.855 Å, which represents the magnitude of the motion of each atom of the enzyme, indicating no significant change in the overall structure and charge of the proteins between the wild-type and variant. The results of Chimera analysis showed that the hydrogen bond structure around

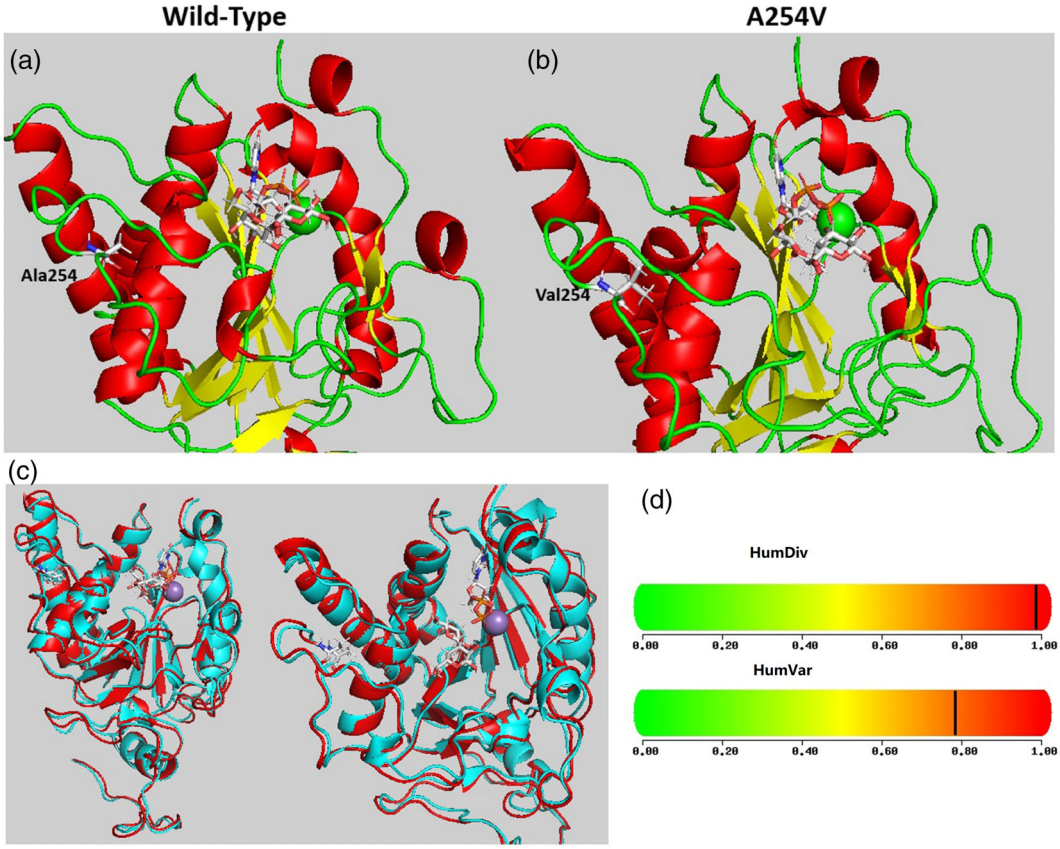

FIGURE 2 The different conformations in wild-type and variant glycosyltransferase B (GTB). The amino acids were coloured by elements in a ball and sticks method. O, N, C and H atoms represented by red, blue, long silver and short sticks. (a) The overall structure of the wild-type GTB and the side of A254 is coloured in blue. (b) The overall structure of the variant GTB and the side of V254 was coloured in blue. (c) The comparative structural compassion of wild-type and variant GTB. The wild-type was coloured in cyan and the variant was in red. (d) Impact of the variant on the structure and function of GTB. The top result was based on HumDiv, and the bottom was based on HumVar.

the 254 site did not change significantly between the wild-type and variant GTB (Figure 2c). The location of A254 was far away from the substrate binding site, and the variant of this site did not significantly shift or change the structure of helix in the His301-Arg312 region. Consequently, the variant did not have a direct effect on the substrate binding pocket. Thus, the weak phenotype was not caused by changing the local conformation of GTs.

Prediction of the stability of the GTB variant

Protein stability is affected by alterations in the number of hydrogen bonds, disruption of salt bridges or other changes in protein folding. Based on the three-dimensional (3D) structure analysis, the hydrogen bonds around the 254 site did not change significantly. Therefore, we hypothesized that GTB stability may be affected by the variant. To evaluate the effect of the variant on GT stability, we calculated the protein thermodynamic stability changes of the variant. We built homology models based on the 3SXE by DUET. The variant from ALA to VAL at position 254 resulted in a $\Delta\Delta G$ of -0.64 kcal/mol, implying that the variant can reduce protein stability. According to the PolyPhen-2 analysis, this variant was predicted to be probably damaging with a score of 0.986 based on HumDiv and possibly damaging with a score of 0.748 based on HumVar (Figure 2d). Thus, the p.A254V variant could impair the stability of GTB.

In vitro expression of GTB and variant p.A254V in HeLa cells

The supernatant from HeLa cells transfected with wild-type GTB showed a high dilution (1:2048) of catalytic ability to convert O type red cells into B type red cells, while the p.A254V variant GTB could only reach 1:16 (Figure 3a). The results of Western Blot showed that the synthesis of GTB protein was not significantly affected by the p.A254V variant (Figure 3b). Thus, the weak B antigen expression on RBCs may be caused by the dysfunction of the variant GTB.

DISCUSSION

The B_{weak} subtype of the B blood group is characterized by a low expression of B antigens on RBCs and is a general term of sub-phenotype to classify variant phenotypes of the B blood

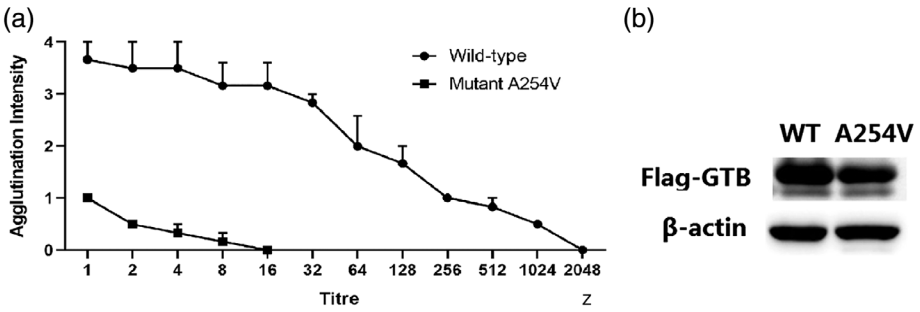

FIGURE 3 p.A254V did not affect glycosyltransferase B (GTB) expression but its function. (a) The dots in the plot represent the mean agglutination intensity ± SD of three independent experiments using a serial dilution of monoclonal anti-B antibody up to 2048-fold. $p < 0.01$ by a one-way analysis of variance (ANOVA). (b) Western blot analysis of GTs produced by HeLa cells transfected with wild-type (WT) and variant constructs. β-Actin was used as loading control.

group [16]. According to the ISBT, there are 34 such alleles known [7, 17–20]. In this study, we identified a novel *BW* allele, containing a variant (c.761C>T, p.A254V) on the *ABO*B.01* allele. The variant could result in the B_{weak} phenotype by impairing stability of the GTB protein and significantly downregulating the expression of B antigens on RBCs.

Subject S777's serologic result showed a mix-field aggregation with anti-B and anti-AB, but a strong aggregation of A1 cells, suggesting a B_{weak} subgroup. Gene sequencing confirmed the presence of the c.761C>T (p.A254V) variant, which significantly reduced the expression of B antigens on RBCs. However, Western blot analysis revealed that the variant did not affect the synthesis and expression of GTB protein in HeLa cells. Total GTB transfer capacity test showed that the variant GTB had lost most of its capability to convert O type into B type cells. Therefore, the low expression of B antigens on RBCs may be due to the dysfunctional variant GTB protein.

Different amino acids may play different roles in protein structure [21, 22]. Amino acid variants can affect protein structure, stability, substrate binding and specificity [23]. The substitution C>T at nucleotide position 761 has been reported in an *Am* allele named *A112* and this variant could lead to a decrease in the level of serum transferase activity and may be responsible for the serological observations of the Am [5]. We found the same effect of the c.761C>T on the *B* allele in this study. To gain insights into the potential mechanisms of the protein change caused by the p.A254V variant, we performed 3D structural modelling and analysed the variant changes in the structure of protein. The modelling studies revealed no significant structural changes in the overall protein structure, or charge caused by the variant. However, thermodynamic stability studies showed that the variant from ALA to VAL at position 254 results in a $\Delta\Delta G$ of −0.64 kcal/mol, indicating decreased protein stability. According to the PolyPhen analysis, the effect of the p.A254V variant was harmful to the GTB. These findings suggest that the GTB V254 variant can cause inadequate conversion of H antigen, resulting in a weak phenotype due to impaired stability of GTB.

In conclusion, we identified a novel *BW* allele with a variant (c.761C>T; p.A254V) on the *ABO*B.01* allele, leading to the B_{weak} phenotype. The GTB variant p.A254V reduces protein stability, resulting in the B_{weak} phenotype characterized by reduced expression of B antigens on RBCs.

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H.L. analysed the data and wrote the first draft of the manuscript. H.Z. and L.J.G. performed the research. D.X. and X.F.W. designed the research study. X.L. and X.H.C. supervised the research and reviewed and edited the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

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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SHORT REPORT

Vox Sanguinis ISBT International Society of Blood Transfusion

Simulated effects of ferritin screening on C-reactive protein levels in recruited blood donors

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Abstract

Background and Objectives: Ferritin is commonly measured to evaluate iron stores in the body. Some countries have added or considered adding ferritin lower bounds to donor eligibility criteria. Ferritin is also elevated by inflammation. The main goal of this study is to estimate how different ferritin cut-offs would affect the proportion of donors with a C-reactive protein (CRP) level over 3 mg/L, which is the decision limit of the highest chronic cardiovascular risk.

Materials and Methods: To simulate recruitment of new blood donors, we selected participants from two Finnish general population cohorts, namely FINRISK 1997 ($n = 5369$) and Health 2000 ($n = 3278$), that would likely fulfil the selection criteria of blood donation. We then calculated the proportion of individuals with high-sensitivity CRP values above 3 mg/L, over a range of ferritin values.

Results: We found that for several ferritin cut-offs the proportion of potential donors with CRP > 3 mg/L would rise by a statistically significant amount. The trend was significant and similar for all subgroups but weaker for non-menstruating women as well as men.

Conclusion: Our results show that screening a population of potential blood donors with ferritin cut-offs raises the number of people with CRP > 3 mg/L within the blood donor population.

Keywords

CRP, donor health, donor management, ferritin, ferritin screening

Highlights

- We demonstrate the use of well-characterized Finnish general population cohorts (FINRISK 1997 and Health 2000) for simulating the selection of new donors with a ferritin screening policy in addition to regular eligibility criteria.
- In our simulations, filtering the cohort data with rising cut-off ferritin levels results in a proportional increase of C-reactive protein (CRP) within the chronic inflammation range.
- The association between increasing CRP and ferritin levels was most prominent in menstruating females. This finding emphasizes the importance of thorough pre-donation interview and assessment in this group of whole blood donors.

INTRODUCTION

Ferritin is an intracellular protein that stores iron, protecting cells from its toxicity. It is found throughout the body and its synthesis and degradation align with cellular iron requirements. The iron-free form of ferritin, apoferritin, consists of H and L-subunits coded by two distinct, but similar genes. Ferritin is also secreted in blood by non-classical pathways mostly by macrophages [1], where it is composed primarily of iron poor L-subunits. Whether blood ferritin has an active regulatory role or just reflects cellular damage is debated, but it is not thought to have a role in iron uptake or transport [2].

A Cochrane library review recently concluded that there is low-certainty evidence that plasma or serum ferritin below 30 μg/L 'is reasonably sensitive and a very specific test for iron deficiency in people presenting for medical care' [3], while WHO recommends <15 μg/L to detect iron deficiency in apparently healthy adults. In contrast, in regular blood donors where blood ferritin levels are typically found to be below 50 μg/L and self-estimated health is on average good, no associations between self-estimated health and blood ferritin have been found so far [4, 5]. Some countries have considered adding ferritin lower bounds into donor eligibility criteria; for example, in the Netherlands this practice is already implemented. Although this can help in the management of donor iron stores, it can also introduce new challenges to donor health management and recruitment.

Ferritin levels are elevated in various diseases, from microbial infections to chronic metabolic diseases. This has been suggested to have a role in the modulation of immune responses and iron sequestration [1].

In general, the relationships between diseases and iron status appear to be complex. However, a meta-analysis concluded that ferritin levels are positively associated with metabolic syndrome [6].

C-reactive protein (CRP) is secreted by the liver as part of the acute-phase response to infection and inflammation. CRP has been used as a marker for bacterial infection, with 10 mg/L as a typical decision limit. However, CRP can be temporarily elevated by various environmental irritants and inflammatory responses, making the importance of minor CRP elevation difficult to interpret. Despite its unspecificity, high-sensitivity CRP (hs-CRP) at the levels beyond the acute-phase reaction (≤3 mg/L) has become a clinically useful marker for risk of cardiometabolic disease. A systematic review of 23 publications estimated that the relative risk of incident coronary heart disease was from 1.37 to 1.83 (95% confidence interval [CI]) higher in persons with CRP > 3 mg/L versus persons with CRP < 1 mg/L. The recent CANTOS trial on inflammation reduction using canakinumab provides strong evidence that inflammation, and in particular CRP, is causal for cardiovascular disease. Cardiovascular disease patients whose CRP was lowered by canakinumab to less than 2 mg/L had a hazard ratio of 0.66–0.85 (95% CI) of major adverse cardiovascular events, while those whose CRP was not lowered below 2 mg/L enjoyed no such benefit from the treatment [7].

Danish and Finnish regular blood donors have been found to have similar levels of minor CRP elevation and markedly lower levels than Finnish general population [8, 9]. This is likely due to the healthy donor effect: healthier individuals are selected to be donors and they can maintain the habit for years or even decades. Regardless, even in the healthy blood donor sub-population, low-grade inflammation (defined as a plasma CRP level between 3 and 10 mg/L) has been shown to be associated negatively with physical health-related quality of life [10].

As ferritin is both an iron and an inflammation marker, we hypothesized that adding ferritin lower bounds to blood donor selection policy might increase the level of inflammation in a blood donor population. As a surrogate for inflammation, we used CRP. With the help of Finnish representative general population cohorts, we here attempt to quantify the influence of ferritin cut-off levels to donor recruitment and donor health.

MATERIALS AND METHODS

We used data from two different Finnish health survey cohorts: FINRISK 1997 (FR97) and Health 2000 (H2000). Both FR97 and H2000 studies were approved by the Ethics Committee of the National Public Health Institute and carried out according to the recommendations of the Declaration of Helsinki. Permission to use the data for this project was granted by THL biobank (project THLBB2020_19).

Exact laboratory methods have been described previously for serum ferritin in FR97 and H2000 [11] and for serum hs-CRP in FR97 [11] and H2000 [12]. From these cohorts, we included individuals who had both a ferritin measurement and a measurement of hs-CRP. To improve the fit between the population cohorts and potential blood donors, we excluded individuals based on several donor eligibility criteria.

We assigned individuals into subgroups of men, menstruating women and non-menstruating women using their recorded sex and reported status of menstrual bleeding. In the H2000 cohort, women over the age of 55 were not asked about their menstruation status. We categorized these individuals as non-menstruating. The number of individuals in each subgroup for both cohorts is summarized in Table 1, along with the medians and interquartile ranges of CRP and ferritin.

To test whether ferritin filtering has the potential to increase the share of donors with an increase in inflammatory reaction in the otherwise eligible donor population, we examined the proportion of individuals over the commonly used threshold of 3 mg/L of serum hs-CRP for low-grade inflammation within sub-populations filtered using ferritin lower bounds ranging from 0 to 50 μg/L with a step size of 1 μg/L. We assign 95% CIs for the bootstrapped estimates (sample size 10,000) using normal approximation. We use these CIs to determine whether the proportion of possibly inflamed potential donors differs between the current eligibility criteria (no ferritin filtering) and an added ferritin lower bound requirement of 15, 30 and 50 μg/L in a statistically significant manner. The full R code of the analysis is available on Github (https://github.com/FRCBS/CRP_enrichment).

TABLE 1 The number of individuals, along with medians (mds) and interquartile ranges (IQRs) for ferritin and C-reactive protein (CRP) levels across all subgroups within both cohorts.

	Subgroup	N	Ferritin (μg/L) md | (IQR)	CRP (mg/L) md | (IQR)
FR97	Menstruating women	1912	23.91 | (12.38, 42.53)	0.77 | (0.39, 1.86)
	Non-menstruating women	876	55.78 | (31.27, 92.76)	1.27 | (0.61, 2.65)
	Men	2581	112.05 | (65.87, 181.79)	0.89 | (0.46, 1.89)
H2000	Menstruating women	938	27.9 | (15.12, 48.98)	0.62 | (0.27, 1.82)
	Non-menstruating women	651	55.9 | (32.8, 95.45)	1.04 | (0.38, 2.42)
	Men	1689	124.9 | (76.6, 194.2)	0.77 | (0.35, 1.75)

Abbreviations: FR97, FINRISK 1997; Health 2000, H2000.

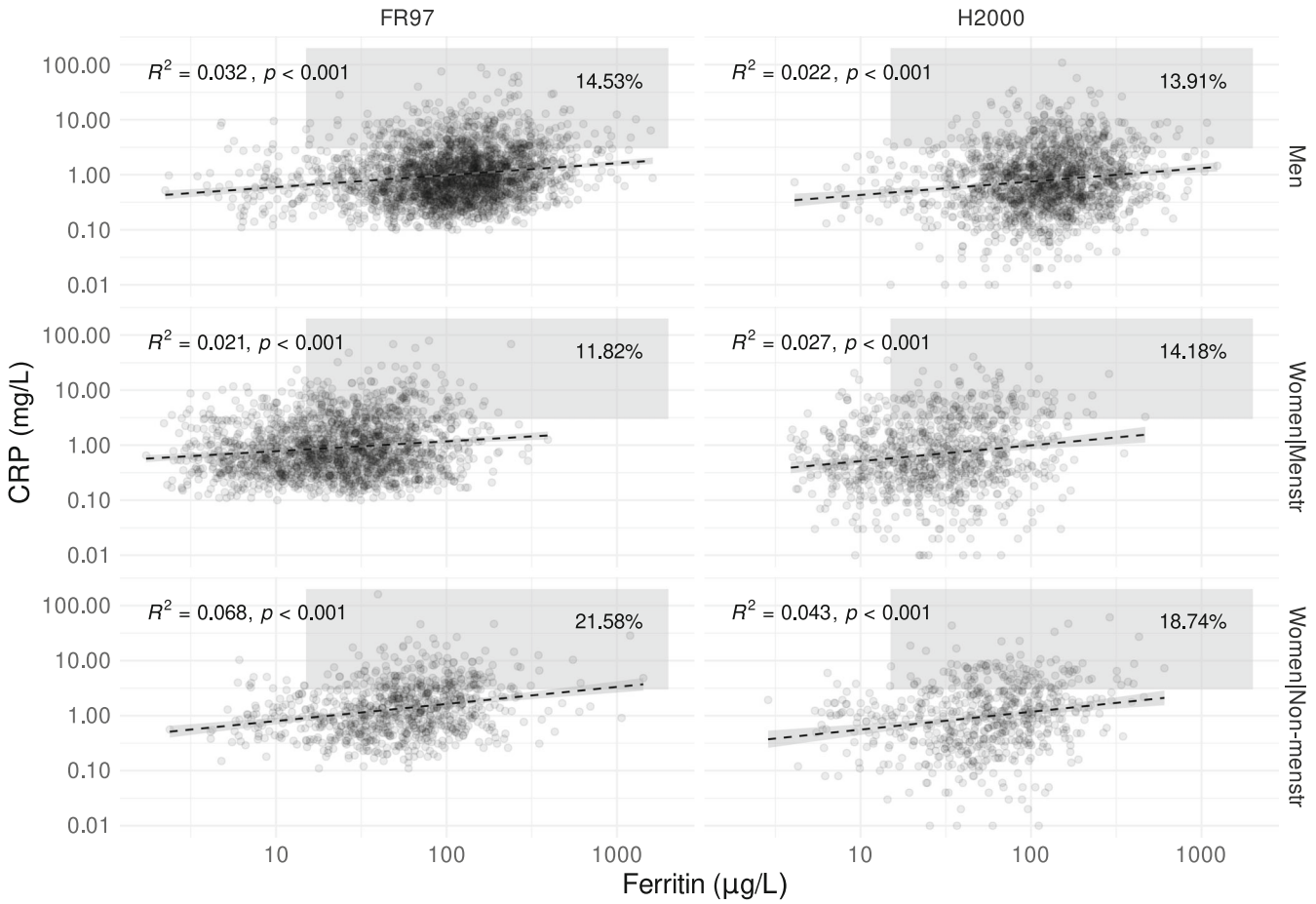

FIGURE 1 Scatter plots of C-reactive protein (CRP) and ferritin values in both cohorts and all subgroups. The grey boxes indicate the area where donors are both over 15 μg/L of ferritin and 3 mg/L of CRP. The proportion of people in the sample covered by the box is indicated with a percentage. FR97, FINRISK 1997; Health 2000, H2000.

RESULTS

The general relationship between CRP and ferritin within subgroups in the cohorts is shown as a scatterplot in Figure 1. The linear fits indicate a weak positive correlation between the variables for all subgroups (R^2 = 0.021–0.068, p < 0.001). Figure 2 presents the change in the proportion of people with CRP > 3 mg/L at different ferritin filtering levels. For example, if a ferritin cut-off of 30 were to be applied for menstruating women, the proportion of individuals >3 mg/L CRP among potential blood donors would rise by 2.3–6.3 percentage points based on the FR97 cohort. We find that for menstruating women in both cohorts, the proportion of people with CRP > 3 mg/L is significantly higher at ferritin filter levels of 15, 30 and 50 μg/L than without filtering. This holds for non-menstruating women

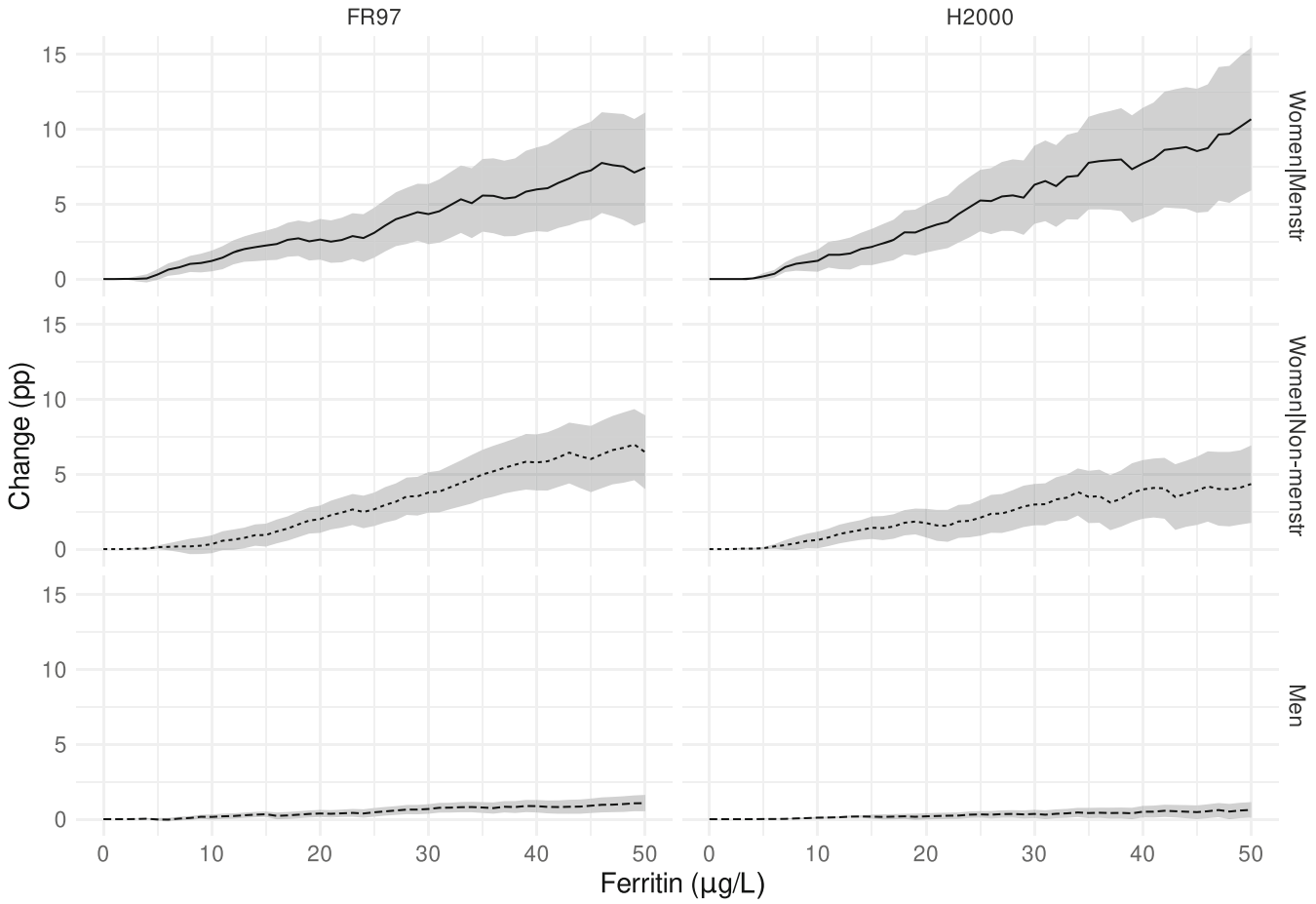

FIGURE 2 Line plots of the change in the proportion of people over 3 mg/L C-reactive protein at different ferritin filtering levels, with 95% confidence intervals. Change is presented in percentage points (pp). FR97, FINRISK 1997; Health 2000, H2000.

also. These differences are all significant in men, but the respective increases in proportions are much smaller.

DISCUSSION

As expected, a weak positive correlation between ferritin and CRP was found in the Finnish representative general population cohorts analysed (Figure 1). This is in line with the hypothesis that ferritin has a role in the modulation of immune responses and iron sequestration during microbial infection. For blood donation, it is important to note that the correlation of CRP and ferritin extends to the lower CRP range of ≤3 mg/L. Hence, even minor chronic inflammation will affect ferritin measurements.

Our simulation suggests that adding a ferritin threshold to the existing eligibility criteria when selecting blood donors from the general population will increase the percentage of donors with low-grade inflammation. These individuals might be less suited to tolerate the physiological stress of blood donation and to maintain long donation careers. Based on current research, high iron status could also be associated with higher disease risks, further aggravating this effect. Countries such as the Netherlands, where additional ferritin screening policies are in place, are well set to study this hypothesis and whether a ferritin measurement should be accompanied with a hs-CRP measurement to avoid these issues. Our results also provide further information on the effects on different ferritin cut-off levels, thus facilitating the selection of the most appropriate decision limit.

The effect of ferritin cut-off on CRP is highest in menstruating women. This suggests that a significant proportion of donors in this group may have ferritin levels in the expected range not because of adequate iron balance but because of an inflammatory response. Furthermore, ferritin filter levels of 15 and 30 µg/L exclude around 20% and 60% of otherwise eligible donors within this subgroup, respectively (Supplementary material). These considerations should be taken into account in designing the donor eligibility schemes.

Our approach suffers from the incomplete information available from the participants of the two cohort studies. Some of the people in question may have had conditions that would have been revealed in the pre-donation interview. Additionally, there were too few haemoglobin measurements in the FR97 cohort to include it in the eligibility criteria, while in reality haemoglobin is a very important criterion. However, as the results were similar in both cohorts, we believe that these challenges do not significantly undermine the statistical findings of our study.

Of Finnish regular premenopausal female blood donors, 18%–23% were found to have ferritin <15 μg/L in 2015–2017 [4]. The Finnish Red Cross Blood Service reacted to this finding by recommending the young female donors to donate only once a year. They were also informed in educational materials about the risk of iron deficiency by blood donation. The iron replacement policy for them was also revised by doubling the course of daily 50 mg iron tablets from 2 to 4 weeks. Such practices could provide an alternative approach to guarantee iron stores of blood donors to the costly combination of ferritin and CRP measurements.

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E.T. wrote the analysis code, analysed the data and compiled the results; E.T., M.A. and J.I. all wrote and reviewed the manuscript; J.I. instructed the analysis and writing; M.A. and J.I. conducted the literature review and M.A. supervised the research.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data used for the research was obtained from the THL Biobank in Finland. Researchers can apply for access by following the application process outlined at https://thl.fi/en/web/thl-biobank/for-researchers/application-process. All research code is available for use under GNU General Public License at https://github.com/FRCBS/CRP_enrichment.

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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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LETTER TO THE EDITOR

Tetanus antibodies in normal human immunoglobulin preparations

We would like to draw attention to the issue that anti-tetanus immunoglobulin (TIG), that is, human immunoglobulin (IG) with known antibody content against the neurotoxin, is essential medicine (WHO) for post-exposure prophylaxis (PEP) and therapy of tetanus [1–3]; yet, TIG is not available in all countries [1] or has been difficult to obtain in recent years [4]. In the absence of TIG, PEP with human IG for intramuscular immunoglobulin (IMIG) or subcutaneous immunoglobulin (SCIG) use is an alternative [4]. For tetanus treatment, intravenous immunoglobulin (IVIG) at a dose of 200–400 mg/kg is recommended in the United States [1], whereas in the United Kingdom, a fixed dosage with selected IVIG preparations was defined [4]. As IG is not routinely tested for tetanus antibody concentrations, little information is available on lot-to-lot variability and dosage required to administer the recommended 250–500 IU of tetanus antibody for PEP or 3000–10,000 IU for therapy [1–4]. In this context, we received requests on tetanus antibody levels in 10% immunoglobulin for infusion (IGI) from practitioners recently, which reflects the need for further information.

We, therefore, investigated tetanus antibody concentrations of current 10% IGI preparations manufactured in 2021 from plasma collected in the United States and European Union, by apheresis (source plasma) as well as from blood donations (recovered plasma), which can be administered as IVIG (Gammagard Liquid/KIOVIG) as well as SCIG (HyQvia), using the Tetanus IgG enzyme-linked immunosorbent assay (ELISA) (Virotech Diagnostics GmbH, Rüsselsheim, Germany) and methodology validated according to ICH principles, reporting results against the second WHO International Standard for Anti-Tetanus Immunoglobulin Human (NIBSC code: 13/240).

We determined a mean ± standard deviation (SD) anti-tetanus potency of 29.2 ± 2.0 IU/mL for recent IGI, 10% lots (Table 1), where lots fractionated from US plasma had significantly ($p < 0.0001$) higher tetanus toxin antibody content (30.8 ± 1.2 IU/mL) than lots fractionated from EU plasma (27.5 ± 1.2 IU/mL). The lowest antibody concentration determined in any lot was 26.0 IU/mL (Table 1). There was no relevant difference in tetanus toxin antibody content for IGI lots fractionated from source or recovered plasma. These data were brought into context with tetanus toxin antibody concentrations reported for other IGI preparations by the National Institute for Biological Standards and Control (NIBSC) [4], where a selection for more recent test date (i.e., tested in 2016–2019) was made and values were normalized to 10% protein content. When tetanus toxin antibody concentrations of 39 lots of 13 different commercial IGI products of six different manufacturers [4] were normalized for protein content, a mean potency of 30.5 ± 2.5 IU/mL was calculated (Table 1), very similar to the antibody concentration determined in the current IGI, 10% preparations.

Our investigation showed that normal human IG preparations contain tetanus toxin antibodies at remarkably similar concentrations. This is true for lots of the same manufacturer, as well as for different commercial preparations of several manufacturers (Table 1) [4]. A difference in tetanus potency was seen for the 10% IGI preparations analysed here depending on the origin of plasma that was used for fractionation, that is, higher potency was seen in US plasma-derived IGI than in EU plasma-derived IGI. The geographic difference in tetanus potency was already reported previously and had been related to differences in tetanus vaccination policy and vaccine potency [5]. As the slightly lower tetanus toxin antibody content in EU plasma-derived IGI still provides for dosages well within PEP and therapy recommendations (see below), this potential lot-to-lot difference does not appear clinically relevant. A serum tetanus toxin antibody concentration of ≥0.1 IU/mL is indicative of immune protection.

TABLE 1 Summary of tetanus anti-toxin potency in recent immunoglobulin for infusion (IGI) preparations, normalized for 10% protein content.

Year of test	Number of different IGI products (number of manufacturers)	Number of lots analysed	Protein content (%)	ELISA IU/mL (mean ± SD) [min]	Reference
2021	2 (1)	36	10	29.2 ± 2.0 [26.0]	n.a.
2016–2019[a]	13 (6)	39	5, 10, 16, 16.5	30.5 ± 2.5 [24.0]	Lejtenyi and Mazer [5]

Abbreviation: n.a., not applicable.

[a]Protein content of IGI preparations prior to normalization to 10%.

The 10% IGI preparations tested here are suitable for subcutaneous as well as intravenous application, making them amendable for tetanus PEP as well as tetanus therapy. With respect to PEP of tetanus, 10–20 mL of 10% IGI should suffice to administer the recommended 250–500 IU of tetanus antibody, assuming the lowest tetanus potency that was determined, that is, 26.0 IU/mL (Table 1). This corresponds well with the recommendation to manage tetanus-prone wounds with 4.5–9 mL of 20% SCIG [4], a product that is manufactured by the same pathway as the 10% IGI lots tested here, with a final adjustment of protein content. For tetanus therapy with 10% IVIG, a person with 80 kg of body weight would receive 4160 IU of tetanus antibodies, assuming treatment with the lowest recommended dosage of 200 mg/kg [1] and the lowest tetanus potency that was determined for a lot here (Table 1), that is, a dosage well within the recommended 3000–10,000 IU for therapy [1–4].

In conclusion, we provide comprehensive evidence that current 10% IGI preparations consistently contain tetanus antibodies at levels that allow for tetanus PEP as well as tetanus therapy, with negligible lot-to-lot variability. Although TIG does have the benefit of smaller volumes being required for treatment, the information of consistent anti-tetanus potency in 10% IGI aids to alleviate concern about the uncertain availability of specific TIGs and opens further options for tetanus PEP and treatment.

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M.R.F and T.R.K conceived the study; A.S. supervised the study; M.R.F and C.L. analysed the data and wrote the manuscript. All the authors revised the manuscript and contributed significantly to the final version of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors are employees of Takeda Manufacturing Austria AG, Vienna, Austria. M.R.F and T.R.K have Takeda stock interests.

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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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REVIEW

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Expansion strategies for umbilical cord blood haematopoietic stem cells in vitro

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Abstract

Haematopoietic stem cell transplantation (HSCT) is considered an effective treatment for some haematopoietic malignancies, haematopoietic failure and immunodeficiency. Compared with bone marrow and mobilized peripheral blood, cord blood has the advantages of easy access, being harmless to donors and low requirement for HLA matching. In addition, umbilical cord blood transplantation (UCBT) has achieved remarkable clinical success in the past 30 years due to the low recurrence rate of malignancies treated by UCBT, mild degree of chronic graft-versus-host disease (GVHD) and good quality of life for patients after transplantation. However, the number of cells in a single cord blood is too small for rapid bone marrow implantation. We summarize the various factors involved that need to be considered in the expansion of haematopoietic stem cells (HSCs) in vitro, which all avoid complex operations, such as vector construction and virus transfection. We also found it necessary to identify a new molecule as the carrier of HSCs cultured in vitro, which not only would provide a three-dimensional structure conducive to the self-renewal of HSCs but also prevent their differentiation.

Keywords

cytokines, HSC expansion, in vitro, small molecule compounds, umbilical cord blood

Highlights

- Compared with bone marrow and mobilized peripheral blood, cord blood has the advantages of easy access, being harmless to donors and having a low requirement for HLA matching.
- This review summarizes the factors that should be considered in developing strategies for effective in vitro expansion of haematopoietic stem cells (HSCs).
- Current tools offer powerful means by which to track how stem cells reconstruct haematopoiesis, which is also of great significance to further explore the molecular mechanism of HSC maintenance and expansion.

INTRODUCTION

Haematopoietic stem cells (HSCs) are a population of cells in the haematopoietic system that can self-renew and differentiate into various mature blood cells. They are particularly important for maintaining the haematopoietic homeostasis and immune function. At present, haematopoietic stem cell transplantation (HSCT) is considered an effective treatment for some haematopoietic malignancies, haematopoietic failure and immunodeficiency. Depending on the source of stem cells, HSCT can be classified into bone marrow transplantation (BMT), umbilical cord blood transplantation (CBT) and peripheral blood stem cell transplantation (PBSCT). Compared with bone marrow

and mobilized peripheral blood, cord blood has the advantages of easy access, being harmless to donors and low requirement for HLA matching. In addition, CBT has achieved remarkable clinical success in the past 30 years due to the low recurrence rate of primary malignancies treated with CBT, mild chronic graft-versus-host disease and good quality of life for patients after transplantation. However, a single cord blood contains very few cells, such as total nucleated cells and CD34+ cells. Despite the improvements in transplantation technology, the implantation is still slow and sometimes insufficient to meet the needs of haematopoietic reconstruction. Therefore, effective expansion of HSCs ex vivo is the most direct and effective way to solve this dilemma [1, 2].

Expansion of HSCs increases the total nucleated cells and CD34+ cells, and more importantly, the expanded offspring can stably reconstruct multi-lineage haematopoiesis in the recipient for a long time after transplantation. According to the reconstruction kinetics of HSCs after primary and secondary recipient transplantation, HSCs can be divided into long-term haematopoietic stem cells (LT-HSCs) and short-term ones (ST-HSCs). LT-HSCs are a group of primitive cells in the haematopoietic system in a resting or circulating state, which can produce ST-HSCs, multipotent progenitor cells (MPPs), lineage-specific progenitor cells and terminally differentiated haematopoietic cells [3]. After transplantation of LT-HSCs, recipients showed high donor chimerism in both myeloid and lymphoid cells, reaching the threshold 4 weeks after transplantation, and maintained it for at least 24 weeks. On the other hand, ST-HSCs did not provide lasting chimerism for any lineage after transplantation [4–6]. Therefore, the proportion and absolute number of LT-HSCs in the expanded cell population are very important for the long-term and stable reconstruction of multi-lineage haematopoiesis. Optimal growth conditions are critical to promote self-renewal, maintain the differentiation potential of HSCs and prevent them from differentiating into lineage-specific progenitor cells.

Application of cytokines in maintenance and amplification of HSCs ex vivo

In 1978, Schofield first proposed the concept of bone marrow haematopoietic microenvironment—'niche'. Since then, the exploration of maintenance elements for HSCs has been vigorous. Through the construction of a gene-modified mouse model, researchers confirmed that the microenvironment signal plays an important role in regulating the self-renewal and differentiation of HSCs [7]. The haematopoietic microenvironment is composed of various cells and cytokines, which interact with HSCs to maintain the haematopoietic homeostasis. These cells and factors include mesenchymal stem cells (MSCs), osteoblasts, leptin receptor (LEPR)-expressing perivascular stromal cells, CXCL12-abundant reticular (CAR) cells, osteopontin (OPN), thrombopoietin (TPO), granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF) and CXC chemokine ligand 12 (CXCL12 or SDF-1) [7, 8]. In view of this, in order to promote the maintenance and expansion of HSCs, researchers often co-culture HSCs with MSCs or endothelial cells, and add supplement cytokines (such as SCF, TPO, FLT3-ligand [FLT-3L], interleukin [IL]-3, IL-6) to the medium to simulate the haematopoietic microenvironment [9–16].

SCF can activate and promote dormant stem cells to enter the cell cycle and provide favourable growth conditions for stem cells by regulating the haematopoietic microenvironment in vivo. Comazzetto et al. proved that SCF produced by endothelial cells, LEPR+ stromal cells and adipocytes in bone marrow plays an important role in promoting HSCs' proliferation and the haematopoietic reconstitution after transplantation [17]. Interaction between SCF and its receptor (c-kit) mediates rapid expansion of HSCs. At present, SCF is widely used in HSC culture, and it promotes the expansion of HSCs either alone or in combination with other cytokines [11, 12, 18].

TPO is an important growth factor that regulates the production of megakaryocytes and platelets. The blocking of the TPO signalling pathway not only damages megakaryopoiesis but also reduces the generation of erythroid and myeloid progenitor cells, especially pluripotent progenitor cells and functional HSCs. This suggests that TPO may participate in maintaining HSCs or promoting the expansion of HSCs after transplantation. This was verified by assay of colony-forming units (CFUs) and SCID-SRC in 2005 [19]. Wilkinson et al. and Marx-Blümel et al. used SCF and TPO in an in vitro culture experiment and observed significant expansion of HSCs [12, 13].

Like SCF and TPO, the FLT-3L is one of the most commonly used cytokines for in vitro expansion of HSCs. It can promote the proliferation of HSCs and improve the engraftment of recipients [20]. Petzer et al. analysed the role of 16 cytokines in CD34+CD38− cell culture and showed that FLT-3L produced nearly 30-fold amplification of the long-term culture-initiating cells (LTC-IC) within 10 days [21]. However, the transgenic mouse model showed that the persistent overexpression of FLT-3L not only led to expansion of haematopoietic progenitor cells but also promoted their migration to peripheral lymphoid organs and further differentiation, thereby increasing the number of mature lymphocytes and myeloid cells [22].

Leary et al. showed that IL-3 could maintain or even enhance the activity of primitive stem cells [23]. Other reports have pointed out the role of IL-3 in promoting differentiation. These contrasting effects may be related to supplementary substances such as serum or its substitutes in the medium [24]. The activity evaluation of the original HSC after amplification still relies on transplantation experiments in immunodeficient mice. A study by Nitsche et al. suggested that IL-3 enhances short-term haematopoietic reconstruction but does not improve long-term implantation in recipients [15], which indicates that IL-3 is more inclined to promote the proliferation of ST-HSCs (Figure 1).

IL-6 is a non-specific haematopoietic growth factor that can induce HSCs into the cell cycle and promote haematopoietic recovery. Ariyama et al. studied the synergistic effects of SCF in combination with IL-6, IL-11, IL-3, IL-1α and interferon-gamma (IFN-γ) on the expansion of murine haematopoietic progenitor cells (HPCs) in a short-term liquid suspension culture. Combinations of SCF with IL-11, IL-6 or IL-1α markedly increased the numbers of total colony-forming cells (CFCs), mixed-colony forming units (CFU-Mix) and high-proliferative potential

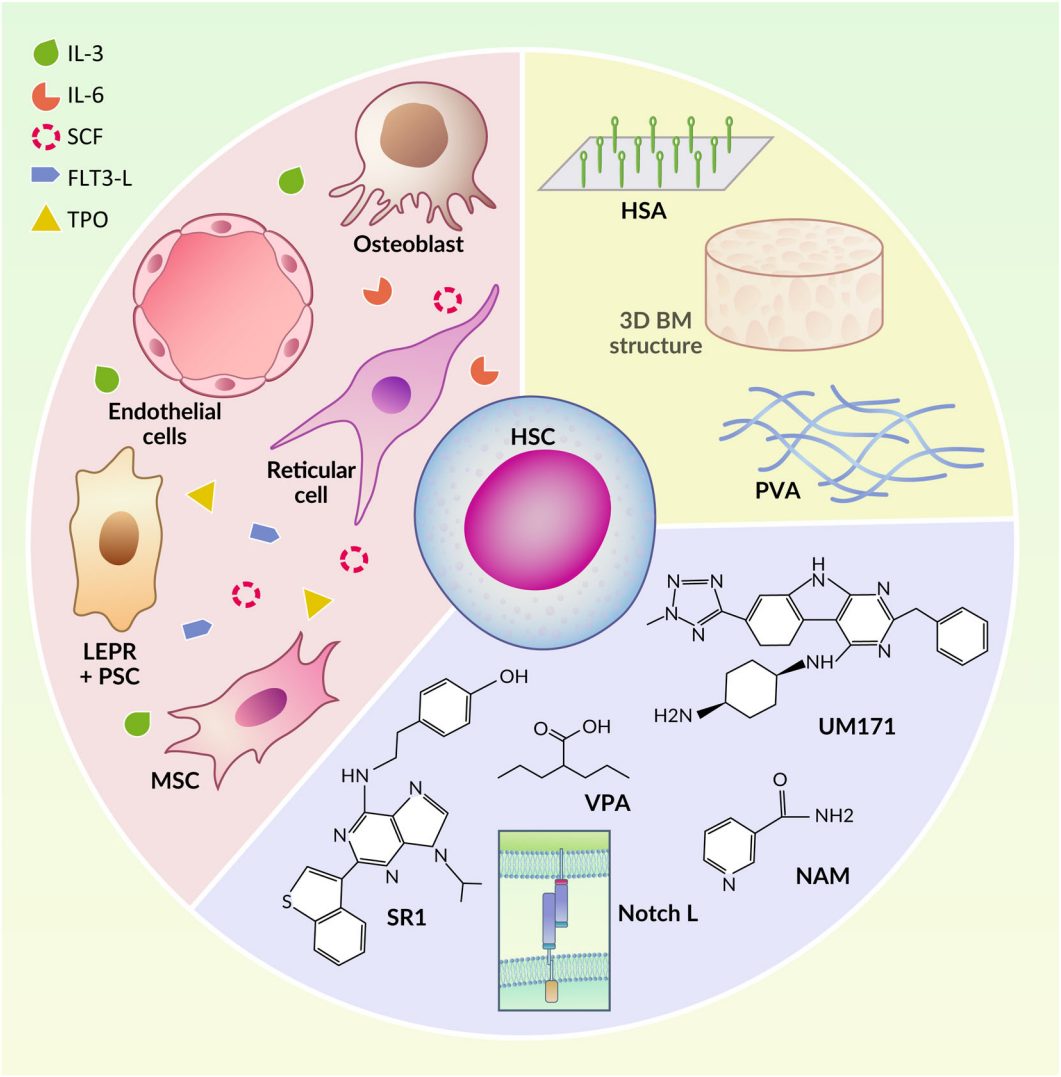

FIGURE 1 Endogenous and exogenous factors that regulate self-renewal, differentiation and homing of haematopoietic stem cells in the bone marrow and in culture. BM, bone marrow; FLT3-L, FLT3-ligand; HSA, human serum albumin; HSC, haematopoietic stem cell; IL, interleukin; LEPR, leptin receptor; MSC, mesenchymal stem cell; NAM, nicotinamide; PSC, perivascular stromal cell; PVA, polyvinyl alcohol; SCF, stem cell factor; SR1, StemRegenin 1; TPO, thrombopoietin; VPA, valproate.

colony-forming cells (HPP-CFC). These results indicated that the combination of SCF with either IL-6 or IL-11 expands murine HPCs [25].

The role of small molecular compounds in the expansion of HSCs in vitro

Optimized culture conditions for HSCs (serum-free media supplemented with SCF, TPO, FLT-3L, IL-6 and IL-3) result in robust proliferation accompanied by differentiation, leading to loss of stem cell activity. This differentiation goes along with the loss of the cell-surface proteins CD34 and CD133, which are expressed on HSCs and HPCs [26–28]. Accordingly, to identify molecules that promote HSC expansion, Boitano et al. evaluated the expression of CD34 and CD133 on the surface of cultured HSCs using confocal microscopy, screened a library of 100,000 heterocyclic compounds and ultimately identified a purine derivative (StemRegenin 1 [SR1]), which significantly increased the number of CD34+ cells after 5–7 days [29, 30]. Transcriptional profiling of SR1 revealed two genes that are significantly repressed by SR1 treatment: cytochrome P450 1B1 (*CYP1B1*) and the aryl hydrocarbon receptor repressor (*AHRR*). Both genes are transcriptionally regulated by the aryl hydrocarbon receptor (AHR), which suggested that SR1 may increase the implantation potential of CD34+ cells by antagonizing the AHR signal [31].

Fares et al. screened a library of 5280 low-molecular-weight compounds to evaluate their ability to expand human CD34+CD45RA− cells, enriched with LT-HSCs. They eventually discovered that UM729 helps in actively expanding CD34+CD45RA− cells. UM171 is a newly synthesized UM729 analogue that has 10–20 times the ability of UM729 to stimulate the expansion of CD34+CD45RA− cells [32]. In

TABLE 1 Study on small molecular compounds in the expansion of haematopoietic stem cells in vitro.

		Pre-clinical studies			Clinical studies			
Cytokine/ molecule	Input cells	Expansion period (days)	Result	Animal experiment	ST-engraftment ANC/platelets (median days)	LT-engraftment	Possible mechanism	Reference
TPO, SCF, FLT-3L, IL-6/ SR1	mPB HSC CD34+	21	A 73-fold increase in CD34+ cells	–	–		Inhibition of the AHR	Boitano et al. (2010) [30]
	CB HSC CD34+	35	A 47-fold increase in CD34+ cells	Enhancement of early and late engraftment of human cells in NSG mice				
TPO, SCF, FLT-3L, IL-6/ SR1	HSC CD34+	15	A 330-fold increase in CD34+ cells	–	15/49	The CD15/33 populations in 65% patients and CD3 populations in 35% patients are entirely derived from the manipulated unit	Inhibition of the AHR	Wagner et al. (2016) [47]
TPO, SCF, FLT-3L/UM171	CB HSC CD34+	12	A 60–75-fold increase in CD34+ cells	The capability of LT-HSC to expand was preserved in primary recipients	–		Suppression of transcripts associated with erythroid and megakaryocytic differentiation	Fares et al. (2014) [32]
TPO, SCF, FLT-3L/UM171	CB HSC CD34+	7	A 28.1-fold increase in CD34+ cells	–	18/42	UM171-expanded cord blood unit/a benefit in 12 month transplant-related mortality, progression-free survival and overall survival	Suppression of transcripts associated with erythroid and megakaryocytic differentiation	Cohen et al. (2020) [48]
TPO, SCF, FLT-3L/LSD1 inhibition	CB HSC CD34+	6	Around 7-fold increase in CD34+ cells	The LT engraftment levels measured 16 weeks after transplantation were very high	–		LSD1 and CoREST are principal targets of UM171-mediated HSC expansion	Subramaniam et al. (2020) [33]
TPO, SCF, FLT-3L, IL-3, IL-6/Notch ligand	CB HSC CD34+	17–21	A 164–222-fold increase in CD34+ cells	Repopulating ability and SRC frequency was significantly higher in mice	16/–	Contribution almost entirely from the non-manipulated CB unit	Stimulating notch signal and inducing self-renewal	Delaney et al. (2010) [34]
TPO, SCF, FLT-3L, IL-3, IL-6/Notch ligand	CB HSC CD34+	14–16	A 141-fold increase in CD34+ cells	–	19/35	Cells derived from the expansion product were no longer detected at day 14 in 82% patients	Stimulating Notch signal and inducing self-renewal	Dahlberg et al. (2015) [49]

TABLE 1 (Continued)

Cytokine/ molecule	Input cells	Pre-clinical studies			Clinical studies		Possible mechanism	Reference
		Expansion period (days)	Result	Animal experiment	ST-engraftment ANC/platelets (median days)	LT-engraftment		
TPO, SCF, FLT-3L, IL-3/ VPA	CB HSC CD34+	10	More than 10-fold increase in CD34+ cells	Exposure to valproic acid increased the long-term potential of HSCs	–		Induction histone acetylation as well as DNA demethylation or inhibition of GSK3β	Bug et al. (2005) [37]
TPO, SCF, FLT-3L, IL-3/ VPA	CB HSC CD34+	7	The degree of expansion was substantially greater	The number of SCID-repopulating cells were increased in primary and secondary immune–deficient recipient mice	–		VPA is a histone deacetylase inhibitor	Chaurasia et al. (2014) [50]
TPO SCF FLT-3L IL-6/ NAM	CB HSC CD34+	21	An 80-fold increase in CD34+ cells	The homing to murine BM was increased by threefold and the incidence of ST-SRC increased by 7.6-fold compared to cultures without NAM	–		Inhibition of the SIRT1 and differentiation of HSC	Peled et al. (2012) [38]
TPO SCF FLT-3L IL-6/ NAM	CB HSC CD133+	21	A 72-fold increase in CD34+ cells	–	13/33	An expanded graft is capable of outcompeting an unmanipulated unit and of providing both rapid engraftment and multi-lineage haematopoiesis	Inhibition of the SIRT1 and differentiation of HSC	Horwitz et al. (2014) [51]

Abbreviations: AHR, aryl hydrocarbon receptor; BM, bone marrow; CB, cord blood; FLT3-L, FLT3-ligand; HSC, haematopoietic stem cell; IL, interleukin; LSD1, lysine-specific histone demethylase 1A; LT-HSC, long-term haematopoietic stem cell; mPB, mobilized peripheral blood; NAM, nicotinamide; NSG, NOD-scid-gamma; SCF, stem cell factor; SCID, severe combined immune-deficiency mice; SR1, StemRegenin 1; ST, short-term; TPO, thrombopoietin; VPA, valproate.

order to elucidate the mechanism of UM171, RNA sequencing (RNA-seq) and expression profile analysis showed that, in contrast to SR1, UM171 did not down-regulate the AHR target genes such as *CYP1B1*, *CYP1A1* and *AhRR*. This suggests that the mechanism of UM171 in enhancing the self-renewal of human LT-HSCs may be independent of AHR inhibition. Last year, Subramaniam reported that inhibition of the epigenetic regulator lysine-specific histone demethylase 1A (LSD1) induced a phenotype and molecular characteristics very much like cells treated with UM171. The knockout model constructed by CRISPR/Cas9 suggested that LSD1 might be the target of UM171 [33].

Delaney's laboratory studied the role of the Notch signalling pathway in regulating the expansion of HSPCs in vitro. They used serum-free medium with the immobilized, engineered Notch ligand (delta1ext IgG) to culture CD34+ cells isolated from umbilical cord blood (UCB). This increased the absolute number of HSPCs, including functional HSCs that reconstruct recipient haematopoiesis [34, 35].

Valproate (VPA), a histone deacetylase inhibitor (HDACi), is an epigenetic modifier. Papa et al. found that VPA improved the maintenance of stem cells and the proliferation of CD34+ cells [36]. Cells incubated with VPA also had increased aldehyde dehydrogenase activity and decreased mitochondrial membrane potential, both being functional markers of HSCs. In addition, VPA inhibits glycogen synthase kinase 3β (Gsk3β) by inducing its phosphorylation on ser9, thereby activating the expression of *Wnt* and *HOXB4* genes, both of which are direct stimulators of self-renewal of HSCs [37].

Nicotinamide (NAM), a water-soluble vitamin B-3, is a well-established inhibitor of enzymes that rely on nicotinamide adenine dinucleotide (NAD+) activity, such as SIRT1. Peled et al. demonstrated that NAM increases the proportion of CD34+CD38− cells, inhibits stem cell differentiation and enhances homing and engraftment [38]. Horwitz et al. performed a phase III clinical trial in 125 patients and reported that omidubicel (an expanded UCB product with NAM) shortened the recovery time of neutrophils and platelets by more than 10 days compared with the standard umbilical cord blood transplantation group, and reduced implantation time and hospital stay [39]. In particular, the graft was from a single-unit UCB, which included the CD133+ part amplified by nicotinamide and the CD133− non-cultured fraction containing immunocompetent lymphoid cells.

Some of the above small molecular compounds have achieved remarkable results in clinical trials of UCB amplification. We have summarized them in Table 1.

The role of three-dimensional bone marrow structure in the expansion of haematopoietic stem progenitor cells

In addition to the above soluble factors, the bone marrow microenvironment provides a specific three-dimensional (3D) spatial structure for HSPCs. In order to understand the role of the 3D structure in the maintenance and expansion of HSPCs, many researchers choose different materials to simulate the spatial structure of the microenvironment in vitro. Compared with common plate culture, 3D culture systems (such as non-woven porous carrier, macroporous collagen carrier and porous microspheres) increase the self-renewal of human HSCs, and this effect is further enhanced by supplementing with cytokines. Among them, 3D scaffolds coated with fibronectin can enhance the expansion of HSCs up to 100-fold [40, 41].

We are inspired by the work of Marx-Blümel et al. and Bai et al. who have shown that the topology of the stem cell niche and the elasticity and surface hydrophobicity of the cell culture matrix are important factors affecting cell differentiation and stem cell potential maintenance [42, 43]. Human serum albumin (HSA) may have multiple functions in the culture of HSCs, its main role being that of a 'carrier molecule'. However, it seems to be the main source of biological pollution in HSC culture, so we must find a substitute for HSA. Wilkinson and colleagues found that polyvinyl alcohol (PVA) was functional and economical in this role, which could expand functional mouse HSCs by 236–899 times in 1 month. The system also supports the survival and expansion of CD34+ cells derived from human UCB ex vivo. However, the expansion of human LT-HSCs is not so sensitive to the amphiphilic molecule PVA [12]. Accordingly, we speculate that there may be better albumin substitutes as carriers for the amplification of human LT-HSCs.

DISCUSSION

The use of cytokines promotes the expansion of HSPCs in vitro, but at the same time differentiation is inevitable and the types and concentrations of cytokines used in different studies are highly heterogeneous. Although small molecular supplements can promote the self-renewal of HSCs and inhibit their differentiation, these expanded cells have less colony-forming activity than untreated cells. Researchers have analysed the haematopoietic reconstitution ability of expanded cells by transplanting fresh or expanded cells into NOD-scid-gamma (NSG) mice. SR1 treatment seems to impair the proliferative potential of lymphomyeloid HSCs in vivo, because SR1 in a UM171-treated culture slightly impedes the proliferative potential of the expanded cells. Moreover, the activity of AhR inhibitors seems restricted to the production of cells with less durable self-renewal ability. UM171 treatment may produce LT-HSCs with the lymphoid deficiency phenotype [31–33]. We need to develop an HSC expansion system in which various cytokines and small molecules can, to the greatest extent possible, stimulate the expansion of HSCs with multi-lineage differentiation potential. So far, no ideal carrier has been found for this. Such an ideal 'carrier molecule' should not only provide the 3D space structure conducive to the maintenance and self-renewal of HSCs but also prevent differentiation and facilitate the maintenance of LT-HSCs. In fact, the mainstream view is that the haematopoietic microenvironment is divided into the duality of intra-bone and perivascular, while the new view is that it is divided according to the imaging of living animals after transplantation. Both concepts point out that HSCs are located in the hypoxic part of the

bone marrow [44]. Unlike differentiated progenies, HSCs rely on glycolysis as the major energy source to adapt to the hypnoxic microenvironment. This metabolic balance promotes the maintenance of HSCs by limiting the production of reactive oxygen species (ROS). HSCs are exceedingly sensitive to the redox state of cells and show a low level of ROS closely related to cell metabolic activities. The increase of ROS level can damage the function of HSCs, and slight fluctuation may change their fate [3, 45].

In recent years, as the main source of ROS, mitochondria have become one of the main research objects in the expansion of HSCs. Mitochondrial dynamics and quality control are related to the differentiation, self-renewal and regeneration potential of HSCs. LT-HSCs may positively maintain low mitochondrial activity as a part of their intrinsic characteristics [45]. In addition, the mRNA and protein of hypoxia inducible factor-1 (*HIF-1*) are highly expressed in LT-HSCs, which is a crucial transcription factor for cells to adapt to the hypoxic environment. Under hypoxia, the degradation of HIF-1α is inhibited and it forms heterodimers with HIF-1β. Then, activated transcription of downstream genes mediates the transition from oxidative metabolism to glycolysis [46]. The ideal 'carrier molecule' serves as a 'redox agent' to maintain and improve the function of HSCs by regulating the level of ROS.

In summary, a variety of factors need to be considered in the expansion of HSCs in vitro, to avoid complex operations such as vector construction and virus transfection. If such factors are combined to develop an optimal system for the expansion of HSCs, the advantages of CBT will benefit more patients. In addition, with the development of single-cell sequencing technology, the Gene Expression Omnibus (GEO) database collects a large quantity of sequencing data about HSCs. The transcriptome-based classification of HSCs and their progenies by scRNA-seq offers a comprehensive yet powerful means to track how stem cells better reconstruct haematopoiesis. This is also of great significance to further explore the molecular mechanism of the maintenance and expansion of HSCs and to improve existing expansion strategy.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

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'Ironing out the risk': Assessing the effect of plateletpheresis donation frequency on iron stores in South-Asian male donors

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Abstract

Background and Objectives: Repeated blood donation is a well-known cause of iron deficiency among donors. However, present scientific literature lacks comprehensive evidence regarding the impact of regular plateletpheresis procedures on body iron reserves. In this study, we aimed to detect and correlate iron deficiency (using iron indices) with the frequency of platelet donations. Additionally, we also analysed the correlation between other iron and haematological indices with serum ferritin to determine cost-effective parameters that may serve as an initial screening approach to determine which donors should be subjected to serum ferritin testing.

Materials and Methods: A total of 180 male participants from our platelet donor registry were enrolled in this observational cross-sectional study. Enrolment questionnaires were administered to eligible donors, and biological samples were collected during plateletpheresis donation. Biological tests such as complete blood count, reticulocyte indices, iron indices, vitamin B12 and folate were performed.

Results: Donors with ≥12 donations per year showed the highest prevalence of low ferritin (serum ferritin: 15–30 ng/mL) and absent iron stores (serum ferritin <15 ng/mL) (41.3% and 26.7%, respectively). Ferritin showed a significant negative correlation with recent ($r = -0.346$) and lifetime donations ($r = -0.196$). The efficacy of other indices for identifying iron depletion was much better using a serum ferritin value <15 ng/mL.

Conclusion: Regular plateletpheresis donations can lead to varying severities of non-anaemic iron deficiency. Blood centres must regularly monitor frequent plateletpheresis donors (especially donors with more than 11 donations in a calendar year) and ideally maintain their serum ferritin above 30 ng/mL.

Keywords

donor, ferritin, frequency, iron deficiency, plateletpheresis

Highlights

- Higher prevalence of low ferritin/absent iron stores is observed with increasing frequency of plateletpheresis.
- Haematological indices of donors were within normal limits, which would allow these individuals to continue donating in the non-anaemic iron deficiency state.
- Plateletpheresis donors may benefit from a lower frequency of donation in order to maintain their body iron reserves.

INTRODUCTION

Donation-induced iron deficiency (ID) is an established phenomenon among blood donors, with a growing recognition in poor resource settings. Published literature confirms that regular blood donors experience a progressive decline in iron reserves as the frequency of donation increases [1, 2]. This results in the depletion of the body ferritin first, followed by red blood cells (RBCs), ultimately resulting in decreased haemoglobin (Hb) only in the last stage of ID. Systematic serum ferritin (SF) measurements can detect iron depletion earlier, resulting in timely prevention of ID and ensuing donor deferral [3]. Furthermore, cut-offs for low Hb or low ferritin (LF) vary across countries, especially with regard to the duration of deferral and repeat testing policy for such donors [4]. Various strategies have been suggested to reduce the risk of ID among blood donors, such as lengthening the inter-donation interval, ferritin testing and iron supplementation [5].

Although much of the ID research is focused on whole blood donors, comprehensive evidence is lacking among regular apheresis donors. Although apheresis donors lose fewer RBCs than whole blood donors, they may be at risk for iron depletion because of the higher donation frequency and chronic, small-volume red cell losses occurring with each procedure [6].

ID is estimated to account for more than 60% of all anaemia globally [7]. India's data from the National Family Health Survey (NFHS) proves that this is of particular importance in developing countries because of the high prevalence of anaemia, which has further increased between NFHS-4 and NFHS-5 from 53% in 2015–2016 to 57% in 2019–2021 among women and from 23% in 2015–2016 to 25% in 2019–2021 among men [8].

The scarcity of data related to body iron status among Asian platelet donors makes this a crucial research avenue in order to ensure donor welfare in this geographic region.

The objective of this study was to investigate the variation in haematological and biochemical parameters among repeat plateletpheresis donors, particularly concerning their iron reserves. Furthermore, we sought to evaluate the correlation between donor iron status and the frequency of donations (recent and lifetime). We compared the different indices using ferritin as a reference standard (which was the best assay available at our institution) to ascertain economical alternatives for identifying iron depletion via receiver operating characteristic (ROC) curve analysis. Lastly, we wanted to assess other haematinic dietary nutrients, such as vitamin B12 and folate levels, to obtain insight into the nutritional status of donors.

MATERIALS AND METHODS

Study design

This was a single-centre, prospective observational study performed in the Department of Transfusion Medicine, Advanced Centre for Treatment, Research and Education in Cancer, Navi Mumbai. Institutional ethics committee approval and Clinical Trials Registry-India registration were obtained prior to starting the study.

Donor recruitment

A total of 150 repeat donors and 30 first-time donors (FTD) were recruited in the study from our platelet donor registry when they presented for platelet donation. These voluntary donors are recruited exclusively for plateletpheresis donations, and we do not perform RBC apheresis or plasmapheresis at our centre. Donor eligibility criteria in accordance with national regulations were ensured prior to enrolment (minimum Hb >12.5 g/dL, platelet count >150,000/μL, minimum interval between successive platelet donations of 2 weeks and donor health assessment questionnaire). Of the 204 invited eligible donors, 180 consented to participate in the study. The repeat donors were enrolled into two equal groups: repeat infrequent donors (RID; 6–11 platelet donations in last 1 year) and repeat frequent donors (RFD; ≥12 donations in last 1 year). In light of the scarcity of plateletpheresis donors, especially female donors, the study focused exclusively on male participants and utilized a purposive sampling technique to ensure that the recruitment target in each study group was met.

Consent

Informed consent was obtained and a structured enrolment questionnaire was administered to eligible donors, which collected data related to platelet donation history as well as smoking, dietary and medication/supplement history.

Sampling

Blood samples were collected from the integrated sample pouch attached to the apheresis kit during the procedure itself. Complete blood count (CBC), reticulocyte indices, biochemical assays (iron indices: serum iron (Fe), SF, total iron binding capacity (TIBC), vitamin B12 and folate) were performed by the hospital laboratory. Transferrin saturation (TSAT) was calculated as ([Fe/TIBC] × 100). This laboratory takes part in regular external quality assessment exercises.

Sample logistics

Samples for CBC and reticulocyte indices were maintained at room temperature and analysed within 6 h of collection. The serum samples were centrifuged, separated, appropriately labelled and frozen at −20°C in Eppendorf tubes for batch testing (10–20 samples). Serum samples were thawed and sera were tested for biochemical parameters.

Equipment and assays

Analysis of CBC and reticulocyte indices were performed on the ADVIA haematology analyser according to manufacturer's instructions, while the biochemical assays were performed on a Siemens biochemistry analyser using appropriate reagents and diluents.

Study participants were stratified based on their iron status, as depicted in Table 1 [9, 10]. As donors are typically healthy individuals who donate altruistically, we prioritized their safety by opting for an SF cut-off value of 30 ng/mL as the criterion for defining reduced iron stores.

Statistical analyses were carried out using IBM SPSS v22. Descriptive data are presented in the form of means and standard deviation. Chi-square and one-way analysis of variance (ANOVA) tests were used for inter-group comparisons, as applicable. Following the one-way ANOVA tests, pair-wise comparison of the means was done using Tukey honestly significant difference post hoc analysis. ROC curve analysis was used to test various haematological and biochemical parameters against SF values (15 and 30 ng/mL) and to derive optimal cut-off values with their sensitivity and specificity. Statistical significance was established at $p \leq 0.05$.

RESULTS

Donor demographics

The final enrolment in our study was 180 donors: 30 FTD, 75 RID and 75 RFD. We saw a younger population (18–45 years) enrolled in the FTD and RID groups (86.7% and 74.7%, respectively). However, the majority of the donors in the RFD group were in the 46–60-year age group (54.7%) followed by the 31–45-year age group (36%).

Donor demographics and their relevant medical history are summarized in Table 2.

Donation history and deferrals among repeat donors

Apheresis donation history and deferral data among the repeat donor groups are summarized in Table 3.

The most significant finding was that deferral due to low Hb was higher in the RFD group (29.3%) compared with the RID group (4%). While a majority of the donors in the RFD group had been deferred between 1 and 4 times (17.3%), about 4% of donors had been deferred >10 times, with one donor being deferred as many as 14 times.

TABLE 1 Criteria for stratification of donors based on their iron reserves.

Criteria	Donor strata	Iron status
Hb ≥ 13 g/dL and SF > 30 ng/mL	Donors with iron-replete status	Iron-replete/normal
Hb ≥ 13 g/dL and SF 15–30 ng/mL	Donors with low ferritin	Negative iron balance/low ferritin
Hb ≥ 13 g/dL and SF < 15 ng/mL	Donors with absent iron stores	Absent iron stores
Hb < 13 g/dL	Donors with iron-deficiency anaemia	Iron-deficiency anaemia

Abbreviations: Hb, haemoglobin; SF, serum ferritin.

Donor stratification based on ferritin levels

SF-based stratification is summarized in Table 4. The proportion of iron-replete donors was highest among FTD (66.7%) and lowest among RFD (21.3%). In contrast, the frequent donor group had the highest proportion of donors with low ferritin (DLF) and donors with absent iron stores (DAIS) (41.3% and 26.7%, respectively). The proportion of DLF and DAIS in the infrequent group was 20% and 12%, respectively, and even among FTD, there were a small proportion of donors who showed LF and absent iron stores (AIS). Of the donors enrolled, 9.4% had iron-deficiency anaemia (IDA) as per the WHO anaemia classification.

TABLE 2 Donor demographics and medical history.

	FTD ($N = 30$)	RID ($N = 75$)	RFD ($N = 75$)
A. Demographics			
Age (years)			
18–30	8 (26.7)	20 (26.7)	7 (9.3)
31–45	18 (60)	36 (48)	27 (36)
46–60	4 (13.3)	19 (25.3)	41 (54.7)
BMI class (kg/m^2)			
Underweight (<18.5)	0	0	1 (1.3)
Normal weight (18.5–24.9)	9 (30)	27 (36)	21 (28)
Pre-obesity (25–29.9)	14 (46.7)	32 (42.7)	38 (50.7)
Obesity class I (30–34.9)	5 (16.7)	16 (21.3)	13 (17.3)
Obesity class II (35–39.9)	1 (3.3)	0	2 (2.7)
Obesity class III (>40)	1 (3.3)	0	0
Dietary preferences			
Non-vegetarian	17 (56.7)	47 (62.7)	32 (42.7)
Vegetarian	13 (43.3)	28 (37.3)	43 (57.3)
B. Medical history			
Smoking history			
Current smoker	5 (16.7)	23 (30.7)	14 (18.7)
Non-smoker	25 (83.3)	52 (69.3)	61 (81.3)
Acidity symptoms			
Yes	6 (20)	17 (22.7)	5 (6.7)
No	24 (80)	58 (77.3)	70 (93.3)
Acidity medication			
Yes	3 (10)	7 (9.3)	3 (4)
No	27 (90)	68 (90.7)	72 (96)
Iron supplementation			
Yes	0 (0)	1 (1.3)	1 (1.3)
No	30 (100)	74 (98.7)	74 (98.7)
Vitamin supplementation			
Yes	1 (3.3)	6 (8)	5 (6.7)
No	29 (96.7)	69 (92)	70 (93.3)

Note: Data presented as *n* (%).
Abbreviations: BMI, body mass index; FTD, first-time donors; RFD, repeat frequent donors; RID, repeat infrequent donors.

TABLE 3 Repeat donor groups: Donation and deferral history.

	RID ($N = 75$)	RFD ($N = 75$)
A. Donation history		
Lifetime donations		
1–25	62 (82.7)	20 (26.7)
26–50	7 (9.3)	16 (21.3)
51–150	6 (8)	33 (44)
>150	0	6 (8)
Donations in last 12 months		
6–11	75 (100)	NA
12–18	NA	52 (69.3)
19–24	NA	23 (30.7)
B. Lifetime deferral history		
Deferral due to low Hb		
Yes	3 (4)	22 (29.3)
No	72 (96)	53 (70.7)
If yes, number of times deferred[a]		
1–4 times	2 (2.7)	13 (17.3)
5–9 times	1 (1.3)	5 (6.7)
>10 times	0	3 (4)
Lowest historical Hb (g/dL)[a]		
11–12	3 (4)	14 (18.7)
12.1–12.5	0	6 (8)
NA	72 (96)	53 (70.7)
Lowest historical Hct (%)[a]		
33–35	0	1 (1.3)
35.1–37	0	11 (14.7)
37–40	1 (1.3)	8 (10.7)
NA	72 (96)	53 (70.7)

Note: Data presented as *n* (%).
Abbreviations: Hb, haemoglobin; Hct, haematocrit; NA, not applicable; RFD, repeat frequent donors; RID, repeat infrequent donors.
[a]Missing values; if yes, number of times deferred, RFD = 1; lowest historical Hb, RFD = 2; lowest historical Hct, RID = 2, RFD = 2.

Dietary intake preferences

The prevalence of LF/AIS/IDA was observed to be ≈8%–10% higher in donors who preferred a vegetarian diet, while the prevalence of iron-replete status was higher among non-vegetarians (52.1%) as compared to vegetarians (38.1%) (Table S1).

Distribution of haematological and biochemical parameters

In inter-group comparisons, ferritin, TIBC and TSAT were significantly different between the first-time and frequent donor groups, whereas they were not significantly different between the first-time and infrequent donor groups. Additionally, paired comparisons between the frequent and infrequent donor groups showed significantly higher red cell distribution width and lower Hb, ferritin, iron and TSAT among the frequent donor group (Tables S2–S4).

TABLE 4 Serum-ferritin-based stratification of the donor groups.

Donor stratification					
	N	DIR	DLF	DAIS	DIDA
FTD	30	20 (66.7)	4 (13.3)	2 (6.7)	4 (13.3)
RID	75	46 (61.3)	15 (20)	9 (12)	5 (6.7)
RFD	75	16 (21.3)	31 (41.3)	20 (26.7)	8 (10.7)
Total	180	82 (45.6)	50 (27.8)	31 (17.2)	17 (9.4)

Note: Data presented as *n* (%).
Abbreviations: DAIS, donors with absent iron stores; DIDA, donors with iron-deficiency anaemia; DIR, donors with iron-replete; DLF, donors with low ferritin; FTD, first-time donors; RFD, repeat frequent donors; RID, repeat infrequent donors.

TABLE 5 Pearson correlation coefficients (haematological and iron indices with lifetime and recent donations).

	Lifetime donations		Recent donations (<1 year)	
Indices	*r*	*p*-value	*r*	*p*-value
Haematological				
Hct	−0.204	0.01	−0.208	0.005
RDW	0.149	0.05	0.013[a]	0.858
PLT	0.115[a]	0.12	0.162	0.030
CHr	0.077[a]	0.30	0.171	0.022
CHm	0.093[a]	0.21	0.203	0.006
Reticulocyte %	−0.042[a]	0.58	−0.082[a]	0.275
Iron				
Serum iron	−0.169	0.02	−0.195	0.009
SF	−0.196	0.01	−0.346	0.000
TIBC	0.209	0.01	0.235	0.002
TSAT	−0.225	0.00	−0.265	0.000

Abbreviations: CHm, mature red blood cell cellular haemoglobin content; CHr, mean haemoglobin content—reticulocytes; Hct, haematocrit; PLT, platelet count; SF, serum ferritin; RDW, red cell distribution width; TIBC, total iron binding capacity; TSAT, transferrin saturation.
[a]Non-significant results.

Correlations between donor indices and donation frequency

The main objective of our study was to examine the relationship between haematological and iron indices with recent donations (within last 12 months), as well as lifetime donations to ascertain their effect on donor indices. In our analysis, ferritin showed a significant negative correlation with recent ($r = -0.346$) and lifetime donations ($r = -0.196$). We also evaluated a previously established correlation between platelet count and ferritin but did not find any statistically significant correlation (Table 5).

ROC analysis using ferritin as a reference standard

Table 6 provides a summary of the area under curve values, optimal cut-off values, sensitivity and specificity obtained from the ROC analysis for the different indices. Figures 1 and 2 show the ROC curves for the top-performing parameters at SF levels less than 15 ng/mL.

ROC curve analysis also allowed us to suggest the threshold number of apheresis donations in a year after which individuals might experience iron depletion. We found that donors with more than 11 donations in a calendar year have a higher probability of having a ferritin level below 30 ng/mL, with a sensitivity and specificity of about 62% and 82%, respectively (Figure 3).

TABLE 6 Performance metrics using ferritin as a standard reference: Area under curve (AUC), cut-offs, sensitivity and specificity of different indices tested.

	Ferritin cut-off							
	<15 ng/mL				<30 ng/mL			
Indices tested	AUC	Cut-off	Sensitivity	Specificity	AUC	Cut-off	Sensitivity	Specificity
Hb (g/dL)	0.744	14.1	71.8	73.0	0.693	14.1	53.7	82.4
Hct (%)	0.732	43.8	66.7	75.9	0.660	45.3	64.2	63.5
CH (pg)	0.743	27.5	71.8	75.2	0.689	29.4	76.8	51.8
RDW[a] (%)	0.667	14.6	48.7	79.4	0.644	13.9	66.3	60.0
%HYPO[a] (%)	0.685	5.6	87.2	45.4	0.646	4.7	81.1	44.7
Iron (ng/mL)	0.786	76.5	82.1	65.2	0.692	89.5	77.9	50.6
TSAT (%)	0.862	20.6	94.9	68.8	0.765	20.9	69.5	78.8
CHr (pg)	0.667	27.3	74.4	63.1	0.590	27.7	58.9	61.2
CHm (pg)	0.690	25.7	82.1	61.7	0.619	26.1	68.4	60.0
TIBC[a] (μg/dL)	0.837	392.8	82.1	73.8	0.776	383.6	62.1	83.5

Note: Youden's index was used for obtaining the optimal cut-off point.
Abbreviations: CH, cellular haemoglobin content; CHm, mature red blood cell cellular haemoglobin content; CHr, mean haemoglobin content—reticulocytes; Hb, haemoglobin; Hct, haematocrit; TSAT, transferrin saturation.
[a]Red cell distribution width (RDW), percentage of hypochromic cells (%HYPO), total iron binding capacity (TIBC) were inversely related to ferritin.

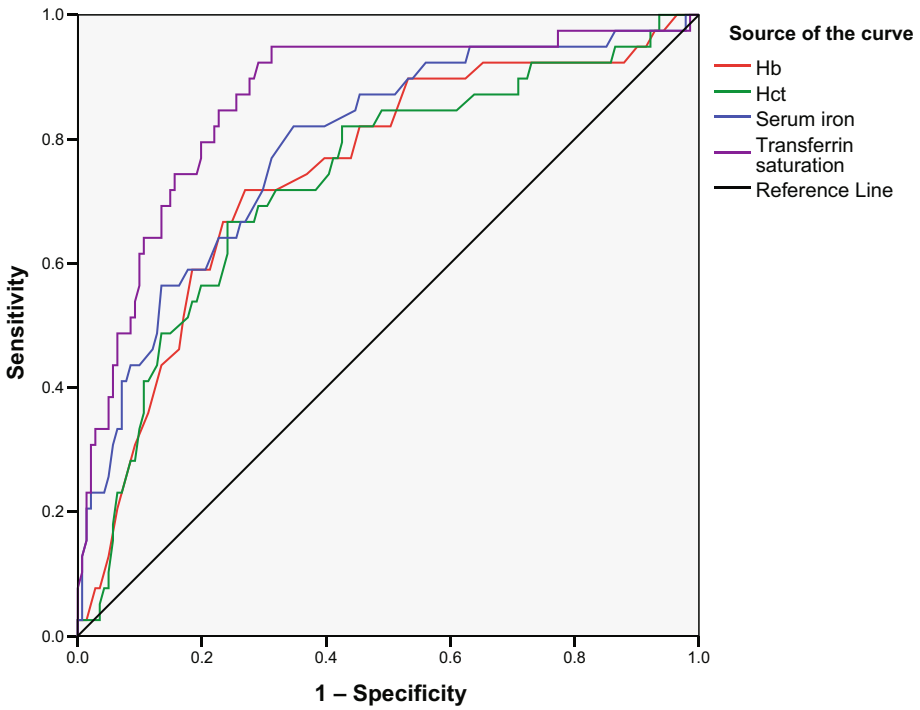

FIGURE 1 Receiver operating characteristic curves for haemoglobin (Hb), haematocrit (Hct), serum iron and transferrin saturation using a serum ferritin cut-off of <15 ng/mL (indices with positive correlation).

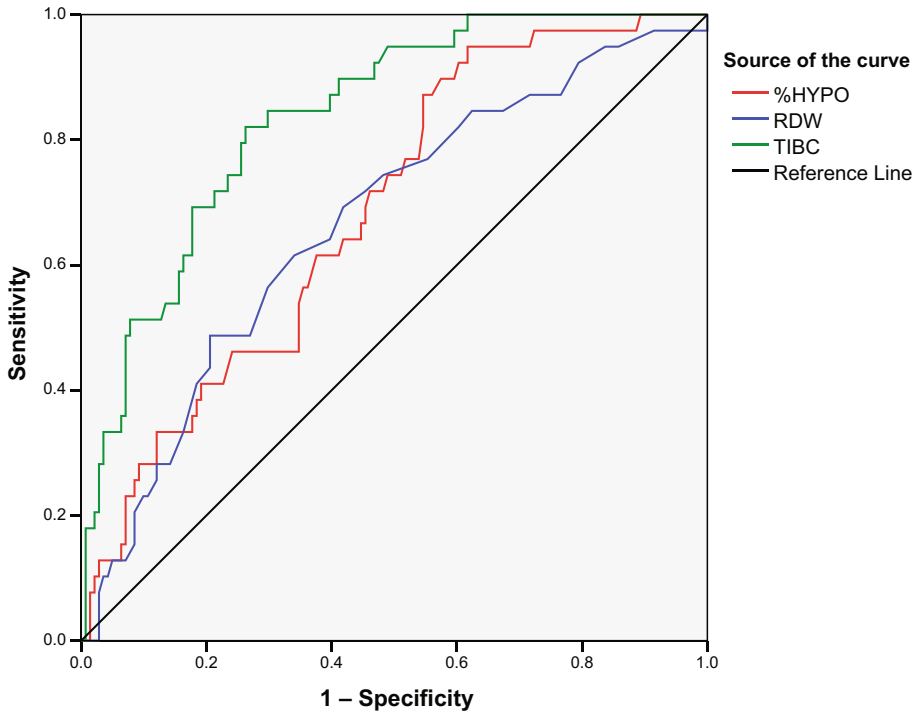

FIGURE 2 Receiver operating characteristic curves for percentage of hypochromic cells (%HYPO), red cell distribution width (RDW) and total iron binding capacity (TIBC) using a serum ferritin cut-off of <15 ng/mL (indices with inverse correlation).

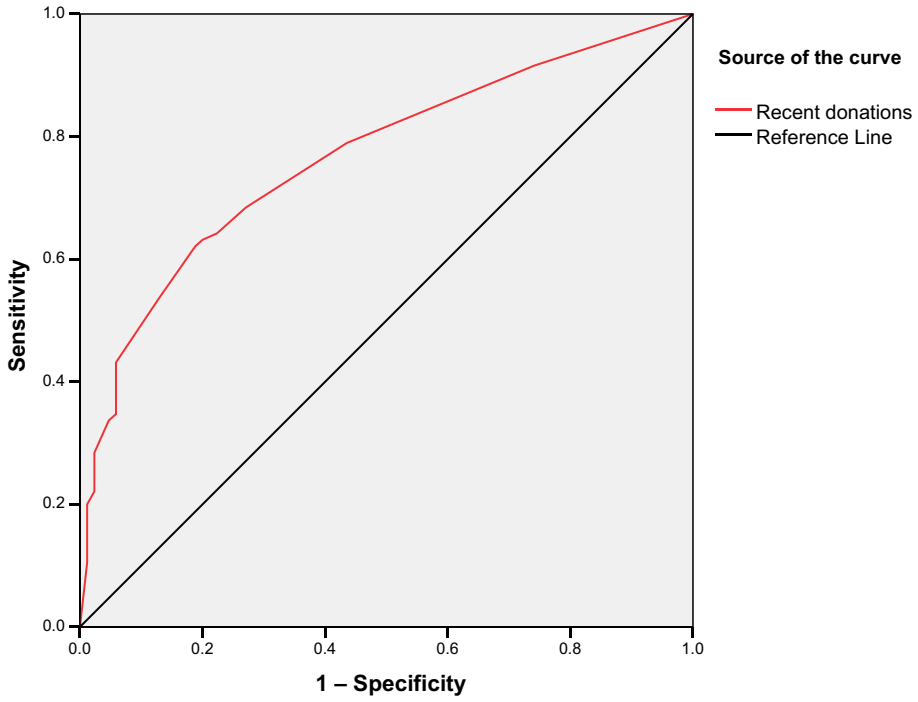

FIGURE 3 Receiver operating characteristic curve for the number of recent donations (last 12 months) using a serum ferritin cut-off of <30 ng/mL.

DISCUSSION

Eligibility for blood donation is usually tested using Hb as a marker since it is available as a point-of-care test. However, it has been established in several studies that Hb is a poor indicator of iron status and may not show a decline till the iron stores have been completely depleted [11–13]. The present minimum Hb requirement of 12.5 g/dL serves to prevent the development of anaemia but, on the contrary,

does not accurately mitigate the development of ID [14]. Additionally, AIS, which is the more common form of ID among repeat donors, may not always progress to IDA [5], and published data suggest several non-specific symptoms among individuals with AIS [15]. Therefore, it is crucial to implement early interventions to prevent the development of ID among altruistic blood donors.

Plateletpheresis donors are eligible to donate up to 24 times/year in several countries like United States and India, since it is thought that the red cell losses were significantly lower than in whole blood donation [3]. Platelet donors could experience an annual whole blood loss of ≈1900–2400 mL if they are to donate at the maximum allowable frequency, which would be much higher than for a blood donor donating at the maximum frequency allowed for whole blood donation [16].

According to our data, about 53% of total participants showed either low or absent iron stores, with a greater prevalence among repeat donors (especially in the frequent donor group). These findings align with previous studies, which showed that repeated plateletpheresis donations could result in decreased ferritin levels and ID [3, 17]. Another intriguing finding was that there were 16.7% and 10% of donors showing LF and AIS, respectively, among the FTD. This indicates that they already had low iron stores at the start of their donation career although their Hb levels conferred eligibility for donation. Our findings regarding the prevalence of ID depending on the frequency of donation are also consistent with Page et al.'s study [18]. Our study revealed that the frequent donor group had the highest prevalence of LF/AIS, in 88% (59/75) of total participants, while, in contrast, the prevalence within the infrequent group was lower, at 37% (28/75). Overall, we found a higher prevalence of ID compared with previously published data, which probably represented Western donor characteristics. Donors in the study were informed about their SF level if it was <30 ng/mL and were advised dietary changes (iron-rich foods) and a physician visit if they wished to consider iron supplementation. We also recommended halting platelet donations for 2–3 months and a re-testing of their ferritin levels before they returned for donation.

The history of deferral for low Hb among our participants was another interesting finding of our study. We found a higher proportion of such donors in the RFD group, with the majority of donors being deferred between 1 and 4 times during their donor career.

Our study found a lower proportion of iron-replete status in vegetarians (32/84), and even non-vegetarians showed some degree of LF and AIS, suggesting that genetic and environmental factors may be a contributory factor for developing ID. This finding is in accordance with established evidence that nutritional anaemias like IDA are inherently more prevalent in the Asian sub-continent [19].

We observed a significant negative correlation of ferritin with both recent and lifetime donations, consistent with other studies reporting increased ID with higher frequency of donation [16, 17]. However, a previous study had reported no increase in likelihood of ID in donors who only donated apheresis components, regardless of frequency [20]. In our study, the correlation between ferritin and platelet count was insignificant, in contrast to another study that reported a significant negative correlation [16]. Other studies have found that blood donors with ID have a lower prevalence of elevated platelet counts compared to individuals with IDA, suggesting an exaggerated response in anaemic individuals that was probably not replicated to the same extent in individuals with ID [21–23].

Our results from the paired inter-group comparisons were similar to those of previous studies which reported generally lower levels of ferritin and other iron indices among repeat plateletpheresis donors [16, 17]. Lastly, we analysed the haematological, reticulocyte and other iron assays for their efficacy in identifying ID among regular apheresis donors. Using detailed ROC analysis, we derived the optimal cut-off values for different parameters along with their sensitivity and specificity [24]. Since a ferritin-based monitoring strategy for the entire donor pool is prohibitively expensive, these cost-effective assays could be of utility as a screening technique in resource-poor settings for identifying donors who may be benefitted by targeted SF testing.

In the absence of a consensus ferritin level for defining ID and variation in the sensitivity of assays used, we believe that a ferritin level of <30 ng/mL can be considered to maintain the health of altruistic donors. Our study showed that the optimal cut-off values for the tested parameters were achieved at 15 ng/mL, with increased sensitivity and specificity. Similarly, another study had found that a ferritin level of <15 ng/mL has a high likelihood of detecting ID [25]. Our study showed that iron indices such as TSAT have the best diagnostic probability (including higher sensitivity and specificity), followed by serum iron and TIBC. Use of these three parameters may be a cost-effective alternative to SF testing given that the combined cost of serum iron and TIBC is much lower (10 times lower than SF at our centre).

A study by Kiss et al. had also performed a similar analysis in blood donors using selective RBC indices. Our findings show the highest sensitivity and specificity using a mean haemoglobin content—reticulocytes (CHr) cut-off of 27.3 pg as compared with the study by Kiss et al. which found better results at a CHr cut-off of 32.6 pg. We hypothesize that this difference in CHr results could be due to the poor availability of dietary iron in our population. Therefore, the concurrent blood losses occurring during frequent plateletpheresis may outpace the body's capacity to adequately replenish the reticulocyte Hb compartment [14]. Another Swiss study also showed good diagnostic probabilities for TSAT, percentage of hypochromic cells (% HYPO) and CHr, consistent with our findings [26].

Lastly, we present ROC analysis that suggests the threshold of apheresis donations in a calendar year which would predispose to iron depletion. We propose that any donor donating >11 times in a given year must be subjected to iron studies to safeguard their health.

The strength of our study is the uniform enrolment of donors based on recent donations, allowing for accurate correlation between iron status and donation frequency. Furthermore, our study shows good evidence that cost-effective tests could be used as an alternative to SF, and we provide the optimal cut-off values for these tests.

Blood centres in resource-poor regions may be benefitted from this data to develop a cost-effective intervention model which could even be extended for blood donors. Additionally, we studied vitamin B12 and folate levels, but they were found to be normal in most donors.

However, the limitations of our study include the donor heterogeneity based on their lifetime donation history and lack of prospective monitoring for further depletion or recovery of iron stores. We were limited by the total pool of registered voluntary platelet donors, which meant that matching between the groups with regard to age, body mass index and medical history was not feasible during study enrolment. Lastly, the unavailability of a centralized blood donor registry in our country made it unfeasible to acquire any whole blood donation history prior to registration as a platelet donor at our centre.

To summarize, repeat plateletpheresis donors may often sustain their Hb, haematocrit and RBC count to remain eligible for donation but are usually unaware of their diminished body iron reserves [17]. Nominal blood loss during mandatory infectious disease testing and in disposable plastic systems, as well as haemolysis during apheresis, seems to be the primary cause of decreased Hb and ferritin levels in apheresis donors [27].

In conclusion, regular apheresis donation can lead to varying severities of ID. As a result, the allowable frequency of donation (24 times/year) may not be able to maintain body iron stores as a result of genetic, environmental and dietary factors in our population. Blood centres must regularly monitor frequent plateletpheresis donors (especially donors with >11 donations in a calendar year) and ideally maintain their SF levels above 30 ng/mL. Feasibility and cost effectiveness of other iron indices need to be assessed further, especially among donors. Larger prospective studies among apheresis donors are recommended to better understand the iron depletion kinetics.

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D.M. conceptualized the research study with the help of mentors from the I TRY IT Program and performed the research study (recruitment, data compilation, analysis and manuscript compilation). S.H. sought Ethics Committee approval and supervised budget utilization and participant enrolment. P.C. supervised lab investigations. J.M. performed statistical analysis and assisted in clinical correlation of findings. J.M. and S.S. contributed to analysis, interpretation and compilation of the manuscript. All authors have discussed the results, commented on the manuscript and approved its publication.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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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

Nutrient supplementation of culture media improves the detection of *Cutibacterium acnes* in platelet components by an automated culture system

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Abstract

Background and Objectives: Platelet concentrates (PCs) contaminated with *Cutibacterium acnes* are often transfused prior to detection by the BACT/ALERT system. Though *C. acnes* is implicated in mild transfusion reactions, delayed clinical effects are unknown. This study assessed the ability to enhance *C. acnes* detection by supplementing culture media with Tween 80 (T80, an oleic acid source) and a commercial nutrient supplement.

Materials and Methods: Anaerobic culture bottles (BPN) were supplemented with T80 or oleic acid. T80-supplemented BPN bottles were inoculated with four *C. acnes* isolates (10 or 100 colony-forming units [CFU]/bottle) or other transfusion-relevant bacteria (10 CFU/bottle). Samples of plasma containing SSP+ (platelet additive solution [PAS]) (PAS-plasma) at different concentrations, plasma-PCs and PAS-PCs, spiked with two *C. acnes* isolates (10 CFU/bottle), were inoculated into T80-supplemented BPN bottles. Furthermore, plasma-PCs were spiked with *C. acnes* and tested in BPN bottles supplemented with the BD Difco Supplement VX (BDVx). Bottles were incubated in the BACT/ALERT system and times to detection (TtoD) were compared ($N = 3$).

Results: A reduction in TtoD of ≤3.5 days was observed for *C. acnes* in T80-supplemented BPN, while other species did not show the same effect. However, false positives were observed when T80-supplemented BPN was inoculated with PAS-plasma (except for 70% PAS:30% plasma), plasma-PCs or PAS-PCs. Oleic acid supplementation also resulted in false positives. Interestingly, BDVx-supplemented BPN reduced the TtoD of *C. acnes* in PCs by ≤1.2 days ($p < 0.05$), with no false-positive results.

Conclusion: BDVx supplementation for detection of *C. acnes* from PCs could result in timely unit retrieval, preventing the transfusion of contaminated products. In clinical settings, T80 supplementation could significantly enhance *C. acnes* detection from non-blood-derived samples.

Keywords
automated culture system, culture media supplementation, *Cutibacterium acnes*, platelet concentrates

Highlights

- *Cutibacterium acnes* can be detected from platelet concentrates up to 1.2 days earlier in the BACT/ALERT system by addition of the BD Difco Supplement VX to anaerobic culture bottles (BPN media).
- Tween 80 can be used to significantly enhance the growth and improve detection of *C. acnes* by up to 3.5 days from non-blood-derived samples; furthermore, the growth of other species is not affected by supplementation of BPN media with Tween 80.
- Plasma and platelet components (likely lipases) trigger false-positive results in the BACT/ALERT system in BPN media supplemented with Tween 80.

INTRODUCTION

Platelet concentrates (PCs) consist of platelets that are either suspended in plasma or a combination of plasma and platelet additive solution (PAS) and are used to treat individuals experiencing low platelet counts or acute trauma [1]. Bacteria are mainly introduced into donated blood from the donor's skin at the time of venipuncture [2], while donor bacteraemia [3] or sterility breaches during manufacturing contribute to a lesser extent [4]. To mitigate the risk associated with the transfusion of contaminated blood products, Canadian Blood Services have implemented several strategies, which include donor screening, donor skin disinfection, first aliquot diversion and bacterial screening of PCs with both aerobic (BPA) and anaerobic (BPN) culture bottles using the automated BACT/ALERT 3D culture system (bioMérieux, Saint-Laurent, QC, Canada) [5]. Despite these measures, bacterial contamination continues to occur, with *Cutibacterium acnes*, an anaerobic, aerotolerant member of the skin microflora, accounting for approximately 70% of all bacteria isolated from contaminated PCs [5].

Canadian Blood Services use a large volume, delayed sampling testing algorithm that stipulates that bacterial screening takes place at least 36 h after collection, followed by a minimum 6-h post-sampling quarantine period prior to release into inventory [5]. Briefly, sampling is performed by inoculating an 8–10 mL PC sample into a BPA bottle and a BPN bottle respectively, after which the bottles are incubated in the BACT/ALERT 3D system for 7 days at 36 ± 1°C. Data gathered at Canadian Blood Services indicate that approximately 36% of contaminated PCs are issued to hospitals and presumably transfused, and that *C. acnes* contamination accounts for 95% of these units [5]. One of the major challenges attributed to this trend is the inherent slow-growing nature of *C. acnes* in culture media, which results in observed detection times ranging from 3 to 7 days in the BACT/ALERT system and the inability to retrieve contaminated PCs in a timely manner [5]. Fortunately, as this anaerobe is incapable of proliferating in the aerobic PC environment [6], *C. acnes* has only been implicated in a few mild transfusion reactions [7, 8]. It should be noted, however, that very little is known about the long-term impacts of transfusing *C. acnes*-contaminated PCs into vulnerable patients. This is especially pertinent since *C. acnes* has been implicated in serious, slow-developing, chronic infections [9]. Therefore, the early detection of *C. acnes* in the BACT/ALERT system could potentially prevent the transfusion of contaminated PCs into susceptible patients.

Several studies have investigated the nutritional requirements of *C. acnes* in vitro and have shown that its growth requires a host of amino acids and vitamins supplemented into culture media [10], and that it can be enhanced with the addition of oleic acid, a skin sebum component [11]. Furthermore, there have been multiple reports that have used Tween 80 (T80) as a source of oleic acid to enhance the growth of other bacterial species in vitro [12, 13]. In this study, we evaluated the ability of pure oleic acid, T80 and the BD Difco Supplement VX (BDVx) to improve *C. acnes* detection. The BDVx supplement consists of essential growth factors including vitamins and amino acids used to promote the growth of fastidious bacteria. Enhancing the detection of *C. acnes* may prevent the release and transfusion of PCs contaminated with this bacterium.

MATERIALS AND METHODS

PC and plasma units

Leukocyte-reduced PC pools were prepared by the buffy coat method and suspended in 100% plasma. PC and plasma units were manufactured according to standard procedures at the Canadian Blood Services netCAD Blood4Research Facility (netCAD, Vancouver, BC, Canada) and were shipped to the Canadian Blood Services Microbiology Laboratory in Ottawa, Canada. For experiments where PCs were re-suspended in PAS, double dose apheresis units were collected according to standard procedures at Canadian Blood Services, and then diluted with PAS SSP+ (Macopharma, Tourcoing, France) to obtain PC samples containing approximately 1.5×10^9 platelets/mL corresponding to the minimum platelet yield requirement for apheresis units. Ethical approval for this study was granted by the Canadian Blood Services Research Ethical Board.

Bacterial strains

Four *C. acnes* strains, BPNBT-19195, BPNBT-19153, BPNBT-19322 and BPNBT-19422, and one isolate each of *Staphylococcus epidermidis* (BPNBT-18090), *Serratia marcescens* (CBS 07/05) and *Staphylococcus saccharolyticus* (BPNBT-20617), isolated from positive culture bottles obtained during routine bacterial screening of PCs at Canadian Blood Services, were used for this study. Enumerated bacterial suspensions corresponding to approximately 1×10^8 colony-forming units (CFU)/mL were prepared in brain heart infusion (BHI) medium supplemented with 15% (v/v) glycerol and verified by plating serial dilutions on tryptic soy agar supplemented with 5% sheep blood (BA).

Supplements

Pure oleic acid and T80 (Sigma-Aldrich, Oakville, ON, Canada) were assessed in this study. A stock solution consisting of T80 (20%, v/v) in BPN media extricated from BPN bottles was prepared (BPNt). The solution was autoclaved and mixed when warm to produce a homogenous solution and stored at room temperature until use. The BDVx supplement (VWR, Mont-Royal, QC, Canada) was reconstituted and employed as per the manufacturer's recommendations.

Effect of the direct supplementation of oleic acid in BPN media bottles

Pure oleic acid was used to supplement BPN bottles with different final concentrations, 100, 200, 300 and 400 µg/mL. Each concentration was tested two independent times in the absence of bacterial inoculum or PCs. Briefly, oleic acid was added to 1-mL aliquots of BPN media, vortexed and quickly aspirated into 1-mL syringes, and used to inoculate BPN bottles. An additional 9 mL of BPN media was then added to the BPN bottles to obtain a total inoculum volume of 10 mL. The bottles were incubated in the BACT/ALERT 3D system (36 ± 1°C) for 7 days or until a positive flag was obtained.

Assessment of T80 concentrations used for BPN media supplementation in the BACT/ALERT 3D system

Two bacterial loads of *C. acnes* and five T80 concentrations derived from serial dilutions of BPNt in BPN media were tested in BPN bottles as described in Figure 1. Enumerated stocks of *C. acnes* isolates were serially diluted in unsupplemented BPN media and the spiking suspensions were prepared in the appropriate T80-supplemented BPN or unsupplemented BPN media. BPN

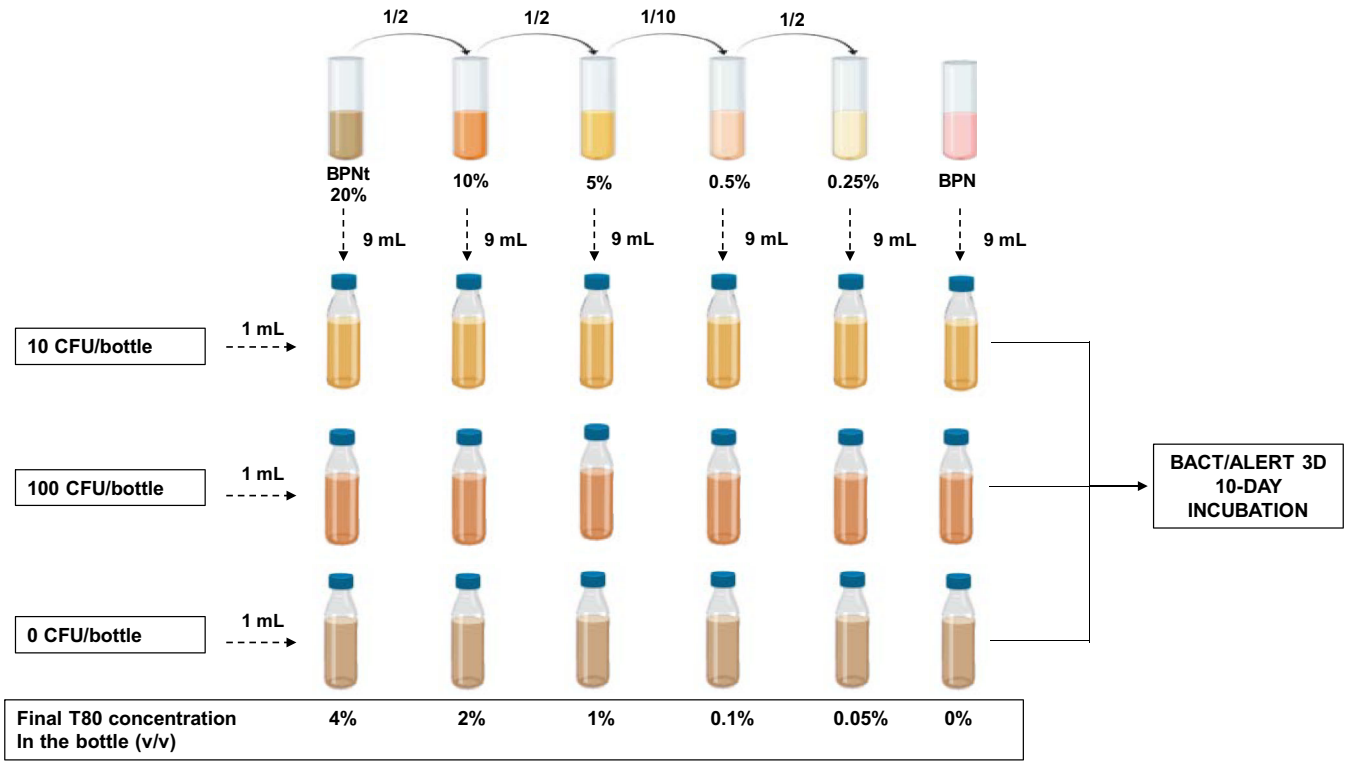

FIGURE 1 Supplementation of BPN media bottles with Tween 80 (T80). BPN bottles were supplemented with T80 to varying final concentrations (0.05%–4%, v/v) and were assessed for their ability to enhance the detection of four *Cutibacterium acnes* platelet concentrate isolates tested at two bacterial loads (10 and 100 colony forming units [CFU]/bottle). Controls included media and media supplemented with T80 in the absence of bacteria, and the comparative control included unsupplemented media inoculated with bacteria. Bottles were incubated for 10 days in the BACT/ALERT 3D system and experiments were performed three independent times. Images created on Biorender.com.

culture bottles were then inoculated with 9 mL of the appropriate media and 1 mL of the spiking suspension. Bottles inoculated with 9 mL of BPN media and 1 mL of spiking suspension prepared in BPN media served as the unsupplemented control. Bottles inoculated with 10 mL of supplemented or unsupplemented BPN served as the unspiked controls. The bottles were incubated in the BACT/ALERT 3D system (36 ± 1°C) until a positive flag was obtained for spiked bottles or for a maximum of 10 days. An extended incubation period of 10 days was used since the isolate BPNBT-19195 is a slow grower in media and may not have triggered a positive flag within the 7-day incubation period used for routine bacterial screening of PCs. Each concentration was tested at least three independent times per bacterial isolate and T80 concentration tested.

Impact of T80 supplementation on the growth of *C. acnes* in media

T80 supplemented (4%, v/v) or unsupplemented BPN media bottles were inoculated with 10 CFU/mL of *C. acnes* BPNBT-19195 and BPNBT-19153 as previously described. Bottles were incubated for up to 7 days (36 ± 1°C) in the BACT/ALERT 3D system. Bottles were sampled every 24 h and immediately returned to the BACT/ALERT system. The samples were serially diluted in BPN media and plated on BA to determine bacterial counts. Experiments were repeated three independent times per species.

Effect of T80 supplementation on the detection of other transfusion-relevant bacteria in media

Spiking bacterial suspension of *S. saccharolyticus*, *S. marcescens* and *S. epidermidis* corresponding to 10 CFU/mL was prepared in BPNt or BPN as previously described. BPN media bottles were inoculated with 1 mL of the bacterial suspension (~10 CFU/mL) and 9 mL of either BPN or BPNt. BPN bottles inoculated with media (supplemented and unsupplemented) served as controls. Bottles were incubated in the BACT/ALERT 3D system (36 ± 1°C) until a positive flag was obtained or for a maximum of 10 days. Each species was tested three independent times.

Effect of T80 and BDVx supplementation on the detection of *C. acnes* in PCs

BPN media bottles were supplemented to a final concentration of 4% (v/v) of T80 and *C. acnes* isolates BPNBT-19153 and BPNBT-19195 were chosen for further testing in PCs. These two isolates were selected due to their characteristic slow growth in the BACT/ALERT system, making them ideal model organisms for worst-case scenarios of detection of *C. acnes*. Before BPN bottles were supplemented with T80, 10 mL of BPN media was aseptically extricated from the bottles, after which 10 mL of PCs containing approximately 10 CFU of bacteria and 10 mL of BPNt were inoculated into the bottles. BDVx supplementation was assessed by inoculating BPN bottles with 9.5 mL of PCs containing 10 CFU of bacteria and 500 µL of reconstituted BDVx. BPN bottles inoculated with an unspiked sample of the same PC unit served as the sterility control, bottles inoculated with PCs containing bacteria served as the unsupplemented control, and supplemented bottles inoculated with PCs in the absence of bacteria served as the unspiked controls. The bottles were then incubated in the BACT/ALERT 3D system (36 ± 1°C) until a positive flag was obtained or for a maximum of 10 days. Each supplement was tested with at least three PC units. Furthermore, to assess bacterial growth in PCs, 10 mL of PC samples containing 10 CFU of BPNBT-19195 were inoculated into BPN bottles with or without supplementation with T80 and incubated for 7 days in the BACT/ALERT system at 36 ± 1°C. These bottles were sampled every 24 h and returned to the BACT/ALERT system. Samples were serially diluted and plated for bacterial enumeration. The experiments were repeated three times.

Impact of reducing plasma content in the detection of *C. acnes* in T80 supplemented bottles

Plasma samples were diluted in SSP+ PAS (PAS-plasma) to obtain a range of suspensions consisting of 100% plasma to 30% plasma in PAS (v/v). BPN media was extricated as described previously, and 10 mL of the plasma suspensions and 10 mL of BPNt were used to inoculate the bottles. Plasma dilutions that did not give rise to false positive with T80 supplementation were used in successive experiments. Bottles were inoculated with BPNt and the appropriate PAS-plasma suspensions or PCs prepared in PAS (PAS-PCs) containing ~10 CFU of *C. acnes* BPNBT-19153 or BPNBT-19195. Bottles inoculated with PAS-plasma or PAS-PCs suspensions spiked with bacteria served as unsupplemented controls. For all experiments, bottles inoculated with unspiked PAS-plasma and PAS-PCs, with and without BPNt, served as supplement and sterility controls. The bottles were then incubated in the BACT/ALERT 3D system until a positive flag was obtained or for a maximum of 10 days. Experiments were performed three independent times.

Statistical analyses

Statistical tests were performed to determine the difference of time to detection between supplemented and unsupplemented samples. The time to detection across technical replicates was used to calculate the standard deviation. For growth curve comparisons, the differences in bacterial counts were assessed for each time point tested. p values were determined using the Student's t-test (unpaired, two-tailed, unequal variance) using Excel. Differences were deemed significant if the p value obtained was ≤0.05 (95% confidence interval).

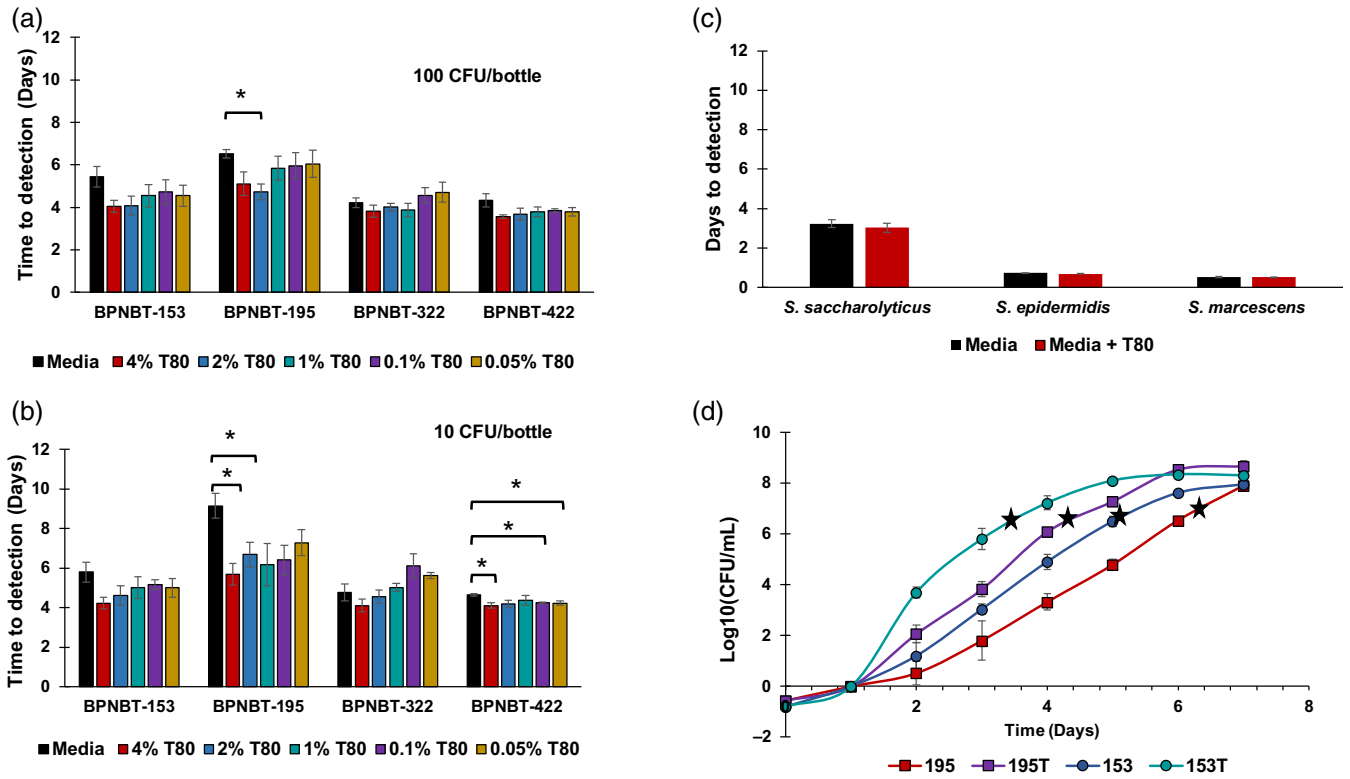

FIGURE 2 Time to detection of transfusion-relevant bacteria from media in the BACT/ALERT system with Tween 80 (T80)-supplemented BPN bottles. Time to detection of *Cutibacterium acnes* platelet concentrate isolates with varying concentrations of T80 supplementation, compared with unsupplemented BPN media when inoculated with (a) 100 colony-forming units [CFU]/bottle and (b) 10 CFU/bottle. (c) The time to detection of *Staphylococcus saccharolyticus*, *Staphylococcus epidermidis* and *Serratia marcescens* with (red bars), and without (black bars) T80 supplementation (4%, v/v). (d) Comparison of growth curves of *C. acnes* isolates BPNBT-19195 and BPNBT-19153 in the BACT/ALERT system with T80-supplemented (195—purple square, 153—green circle) and -unsupplemented (195—red square, 153—blue circle) BPN bottles. Time to detection in the BACT/ALERT 3D system has been indicated by black stars. Student's *t*-test analysis, **p* value ≤0.05. $N = 3$.

RESULTS

T80 supplementation of BACT/ALERT BPN media enhances the growth and decreases the time to detection of *C. acnes*

The direct addition of oleic acid to BPN bottles resulted in false-positive flags in the BACT/ALERT system (data not shown); however, supplementation with BPNt (stock solution of T80 [20%, v/v] in BPN media) did not yield false-positive results and was used in further assessments. Figure 2a,b shows that the slowest detection times in media were observed for isolates BPNBT-19195 (100 CFU/bottle: 6.52 ± 0.19 days, 10 CFU/bottle: 9.15 ± 0.63 days) and BPNBT-19153 (100 CFU/bottle: 5.44 ± 0.49 days, 10 CFU/bottle: 5.79 ± 0.50 days). Two concentrations of T80 (4% and 2%, v/v) reduced detection times in all four isolates at both bacterial loads tested. However, T80 concentrations lower than 1% resulted in delayed detection of isolate BPNBT-19322 at both bacterial loads assessed (Figure 2a,b). Though all concentrations of T80 tested reduced time to detection of isolates BPNBT-19153, BPNBT-195 and BPNBT-19422, significant reductions were observed for *C. acnes* BPNBT-19195 tested at 10 CFU/bottle with T80 4% ($p = 0.015$) and 2% ($p = 0.049$) (Figure 2b), and at 100 CFU/bottle with T80 2% ($p = 0.030$). Similarly, significantly lower times to detection were observed for isolate BPNBT-19422 tested at 10 CFU/bottle with T80 4% ($p = 0.044$), 0.1% ($p = 0.007$) and 0.05% ($p = 0.044$) (Figure 2b). All bottles that were not inoculated with bacteria remained negative during the 10-day incubation period in the BACT/ALERT system.

Notably, T80 supplementation (4%, v/v) did not significantly change the time to detection of the other transfusion-relevant bacteria tested, *S. saccharolyticus*, *S. marcescens* and *S. epidermidis* (Figure 2c).

Enhanced time to detection in the BACT/ALERT system was directly related to growth promotion in T80-supplemented media as demonstrated for isolates BPNBT-19195 and BPNBT-19153 (Figure 2d). Increased bacterial loads in supplemented bottles were observed with a maximum difference of ~2.8 ± 0.23 Log on day 4 and ~2.8 ± 0.23 Log on day 3 detected for isolates BPNBT-19195 and BPNBT-19153, respectively, after which differences in bacterial load between supplemented and unsupplemented bottles decrease, until on day 7 similar bacterial loads were obtained. At all time points, bacterial counts in T80-supplemented BPN were significantly higher than those obtained in unsupplemented media ($p < 0.05$), except on day 7 for BPNBT-19153, when the difference was not significant.

Plasma- and platelet-derived factors contribute to the false-positive results obtained with T80 supplementation of BPN bottles

T80 supplementation gave rise to false-positive flags in the BACT/ALERT 3D system in every instance where PCs or plasma were inoculated into BPN bottles in the absence of bacteria. Times to detection of false-positive results were 1.10 ± 0.28 days and 1.24 ± 0.22 days for unspiked PCs and plasma, respectively. Importantly, supplementation of BPN bottles with T80 did not inhibit the growth of *C. acnes* from samples of spiked PCs as exemplified with isolate BPNBT-19195 (Figure 3). Furthermore, all but one dilution of plasma in PAS (70% PAS + 30% plasma) resulted in false-positive flags. Significant reduction in times to detection was observed for BPNBT-153 of 2.23 days ($p < 0.005$) and BPNBT-195 of 1.26 days ($p < 0.005$) from diluted plasma (70% PAS + 30% plasma) in T80-supplemented BPN bottles (Figure 4a). However, when PCs were prepared in the same plasma to PAS ratios, it led to false positives in the BACT/ALERT system with times to detection of 0.83 ± 0.05 days.

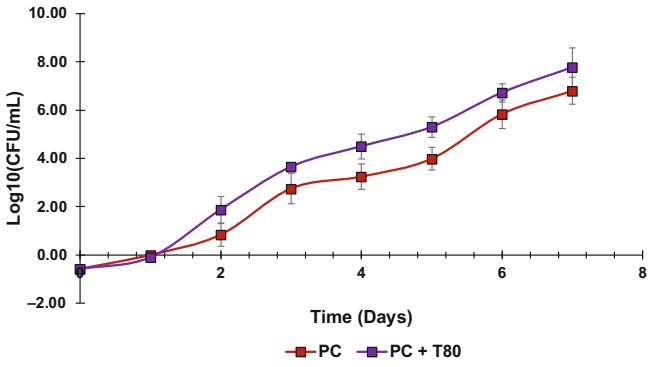

FIGURE 3 Growth curves of *Cutibacterium acnes* in platelet concentrates (PCs) in the BACT/ALERT system. Comparison of growth curves of *C. acnes* BPNBT-19195 inoculated into PCs in the BACT/ALERT system with Tween 80 (T80) supplemented (purple square) and unsupplemented (195—red square) BPN bottles. $N = 3$. CFU, colony-forming units.

BDVx supplementation reduces the time to detection of *C. acnes* in PCs

Supplementation of BPN media with BDVx supplement resulted in a reduction in the time to detection of the two *C. acnes* isolates tested BPNBT-19153 (0.62 ± 0.26 days, $p = 0.048$) and BPNBT-19195 (1.15 ± 0.29 days, $p = 0.030$) in PCs suspended in 100% plasma (Figure 4b) with no false-positives results.

DISCUSSION

A major obstacle faced in the transfusion setting that culminates in the transfusion of *C. acnes*-contaminated PCs is the inability to detect *C. acnes* early during bacterial screening. This study aimed at assessing two supplements, T80 and BDVx, to enhance the growth and therefore the detection of *C. acnes* in BPN bottles by the BACT/ALERT system used at Canadian Blood Services.

T80 has been used to enhance the growth of other bacteria like *Lactobacillus casei* [12], *S. aureus* [13] and *Corynebacterium accolens* [14] in vitro. It has been suggested that T80 enhances growth in a species-dependent manner by providing essential nutrients needed for growth, by changing membrane permeability and by enhancing nutrient availability [13]. The work described in this study demonstrates that

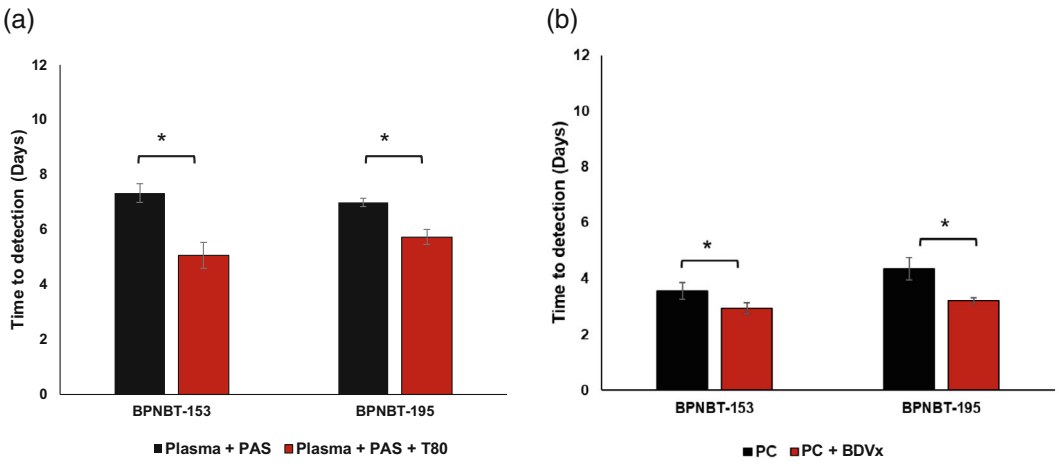

FIGURE 4 Time to detection of *Cutibacterium acnes* in platelet concentrates (PCs) in the BACT/ALERT system with Tween 80 (T80)- and nutrient-supplemented BPN bottles. *C. acnes* isolates BPNBT-19153 and BPNBT-19195 were inoculated into BPN bottles at a concentration of 10 colony-forming units/bottle, (a) from PCs prepared in a combination of platelet additive solution (PAS) and plasma (70% PAS, 30% plasma) with T80 (4%, v/v) supplementation; (b) from PCs with BD Difco Supplement VX (BDVx) supplementation. Student's *t*-test analysis, *p value ≤0.05. $N = 3$.

supplementation of BPN media with T80 (4%, v/v) can also significantly enhance the detection of *C. acnes* from media in the BACT/ALERT system. Furthermore, our growth curve analysis indicated that T80 supplementation promoted the growth of *C. acnes*, resulting in bacterial loads that trigger an earlier positive flag in the BACT/ALERT. Unfortunately, T80 supplementation triggered false-positive flags when PCs prepared in plasma or PAS were tested. These results indicate that the false-positive triggers are caused by plasma and platelet factors such as plasma- and platelet-derived lipases [15, 16]. The lipases likely degrade T80, releasing oleic acid, which causes changes in the gel of the bottle sensor leading to positive flags. Though this could hinder the detection of *C. acnes* in blood products, supplementation of BPN media with T80 could potentially be used in the clinical setting for non-blood samples where incubation periods can be long, allowing for timely intervention of *C. acnes* infections [17]. However, since clinical samples can be derived from various sources, this strategy should be tested to ensure that these samples do not contain factors that may lead to false positives in the presence of T80 supplementation. Importantly, our data indicate that T80 supplementation (4%, v/v) did not hinder the detection of other bacterial species from media as evidenced by similar detection times in supplemented and unsupplemented bottles. Therefore, T80 supplementation (4%, v/v) will not inadvertently prevent or delay the detection of other bacterial contaminants.

Interestingly, the addition of the commercial supplement BDVx resulted in the early detection of *C. acnes* from contaminated PCs by up to 24 h in the BACT/ALERT system when supplemented at the vendor's recommended concentrations. The BDVx supplement is used in clinical laboratories to enhance the growth and isolation of *Haemophilus* and *Neisseria* species by providing growth-promoting factors including vitamins and cofactors like nicotinamide adenine dinucleotide essential for bacterial metabolism and enzymatic processes [18]. It is possible that *C. acnes* growth is promoted in a similar fashion although the exact component of the BDVx supplement involved in the observed enhanced detection of *C. acnes* requires further investigation.

Our study involved supplementation of commercial BPN bottles, providing a proof of principle, that could serve as a basis for the development of enhanced media by vendors for application in blood product screening with automated culture systems. Although our study could be complemented by testing more *C. acnes* isolates, we have provided strong evidence that growth and detection of *C. acnes* can be improved during PC screening with the BACT/ALERT system. Reduction in detection times by 24 h could provide blood suppliers with the ability to recall *C. acnes*-contaminated products before they are transfused into vulnerable patients, thereby improving the safety of the blood supply.

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D.K. and S.R.-A. designed the study. D.K. performed the experimental work and data analyses, and wrote the manuscript, which was reviewed by S.R.-A.

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

Vox Sanguinis ISBT International Society of Blood Transfusion

Home transfusions are implemented using diverse approaches in Japan

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Ministry of Health, Labour and Welfare

Abstract

Background and Objectives: Japan's ageing society has increased the need for home healthcare, including home transfusions. We hence aimed to elucidate the purpose and utilization of home transfusions in Japan, which has not been clarified to date.

Materials and Methods: Clinics throughout Japan that provide home care and have experience in performing blood transfusions were surveyed. The study period was February to December 2019, and information of patients receiving home red blood cell transfusions, including patient background, pre-transfusion laboratory data and the purpose of the transfusions, was collected.

Results: Haematological malignancies and solid tumours accounted for 70% of the patients' underlying diseases, with the former being significantly more common in urban areas. Regarding the purpose of the home transfusions, haematologists

focused on symptom improvement, whereas gastroenterology surgeons focused on life support. Furthermore, maintenance of life was more likely to be the aim in the group of patients with the lowest level of activities of daily living. The main items that were significantly associated with a low haemoglobin level before transfusion included age ≥90 years and a gastroenterologist being the physician in charge.

Conclusion: Home transfusions were found to be performed in a restrictive and diverse manner in Japan. Life support is the second most common purpose of home transfusion in Japan, and optimizing effective home transfusion remains a challenge.

Keywords

Hb level, home transfusion, specialty, transfusion purpose

Highlights

- In urban areas, haematological malignancies were the more common underlying diseases of patients receiving home transfusions.
- 'Maintenance of life' was the second most common purpose of home transfusions and was particularly emphasized by gastrointestinal surgeons.
- The haemoglobin concentration threshold was lower in patients aged ≥90 years and in those with a gastroenterologist as the physician in charge.

INTRODUCTION

In Japan, the number of home transfusions has not increased substantially in the previous 8 years, owing to problems with the implementation of multidisciplinary cooperation and safety concerns regarding the management of transfusion reactions [1]. We previously investigated the safety and availability of home transfusions in Japan; in the present study, we focus on the purpose and utilization of home transfusions in Japan. There is a Japanese government policy to provide patients with medical care that they desire, and at the time of their choice. Therefore, home transfusions should be designed to complement outpatient care, depending on each patient's wishes and the local healthcare system. Although in Japan it is not appropriate for hospitals to provide home transfusions owing to the way the roles of facilities are divided, it is important for hospitals and clinics to cooperate in pre-transfusion testing and in making decisions on indications.

The system of home transfusion services in Japan differs in many aspects from those of Western countries. First, doctors in clinics must manage the entire home transfusion process. Second, clinics usually only have doctors and nurses as medical personnel, and pre-transfusion testing is outsourced to a registered laboratory. Third, blood centres are mainly in charge of blood collection as well as the production and supply of blood products. Finally, transfusion specialists are rarely involved in home transfusions because they work in hospitals.

As the system of the family physician as a specialist was not established in Japan until 2018, clinic physicians have a variety of specialties. Traditionally, approximately 30% of physicians switched from hospital-based specialists to clinic physicians in the middle of their careers [2]. Therefore, it can be inferred that their expertise and backgrounds affected their awareness of home transfusions.

In Japan, there is still a substantial controversy regarding home transfusions, and regional and inter-clinic differences in home transfusion practices are observed. Unlike the general practitioner clinic system in the United Kingdom and other countries, there is no close collaboration between hospitals and clinics in Japan because of the free accessibility of medical care. Furthermore, primary care has not been traditionally emphasized in Japan, and hence the quality of primary care is not standardized. In addition, the availability and implementation of home transfusions is dependent on the policy of each clinic physician.

The Japanese guidelines named 'Red Blood Cell Transfusion at Home' was released in 2017. These guidelines focused on patient safety. Target diseases were limited to end-stage chronic diseases, and the conditions of implementation were also strict. The conditions included the following: the patient must be awake and cooperative; the patient must have a history of blood transfusions and no history of serious transfusion reactions; and the patient must be accompanied by an attendant. In a previous paper, we reported that nurses are involved in 83% of all transfusions as attendants monitoring transfusion reactions, while family members were involved in 77% of them [1]. Of those, multiple attendants were involved in 61% of all transfusions. Regarding the observation period, 10% of patients were observed for 30 min or less, 37% were observed until the end of the transfusion and 41% were observed until the next morning. Thus, we conducted a survey of home transfusions in Japan to analyse the characteristics of patients undergoing home transfusion, including the purpose of the transfusion, regional differences of patient characteristics and factors associated with haemoglobin (Hb) trigger levels. By analysing these items, we aimed to clarify the differences between home transfusion in Japan and Western countries, and to facilitate future improvements.

MATERIALS AND METHODS

Study background

This study was conducted as a part of the 2019 Health and Labour Science Research Project, entitled 'Comprehensive management system of blood transfusions in the community'. The study group consisted of transfusion specialists from teaching hospitals and clinics, experts from blood centres and the director of a public health centre. The survey and questionnaire were designed and reviewed by these experts to clarify the problems and to improve the efficacy of home transfusion in the local healthcare setting.

TABLE 2 Activities of daily living scale.

ADL level	Physical status
J	Has some disabilities, but is nearly independent in ADL and can go out alone
A	Nearly independent in indoor activities, but cannot go out without assistance
B	Needs some assistance with indoor life, mostly remains in bed during the daytime, but can maintain a seated position when required
C	Stays in bed all day, and needs assistance for hygiene, eating and changing clothes

Abbreviation: ADL, activities of daily living.

TABLE 1 Characteristics of the patients in the different region types of Japan.

Item	Urban regions ($n = 79$)	Satellite and rural regions ($n = 65$)	Total[a] ($n = 150$)	p-Value
Underlying disease[b]				
Blood disease (benign)	22 (28%)	11 (17%)	37 (25%)	0.1415
Blood disease (malignant)	35 (44%)	15 (23%)	51 (34%)	0.0107
Solid tumour	23 (29%)	32 (49%)	56 (37%)	0.0103
Other diseases	2 (3%)	8 (12%)	10 (7%)	0.0197
Primary reason for home transfusion[c]				
Anaemia caused by underlying disease	65 (82%)	42 (65%)	111 (74%)	0.016
Bleeding from tumour lesion	9 (11%)	14 (22%)	24 (16%)	0.098
Gastrointestinal bleeding	6 (8%)	8 (12%)	14 (9%)	0.296
Renal anaemia	2 (3%)	6 (9%)	9 (6%)	0.081
Anaemia of chronic inflammation	3 (4%)	4 (6%)	7 (5%)	0.513
Others	3 (4%)	7 (11%)	10 (7%)	0.200
Age group				
≥90 years	10 (13%)	11 (17%)	22 (15%)	0.471
80–89 years	30 (38%)	19 (29%)	51 (34%)	0.270
70–79 years	22 (28%)	19 (29%)	44 (29%)	0.855
60–69 years	11 (14%)	10 (15%)	21 (14%)	0.805
<60 years	6 (8%)	6 (9%)	12 (8%)	0.724
Specialty of the physician in charge of the patient				
Haematologist	42 (53%)	7 (11%)	49 (33%)	<0.001
Cardiologist	6 (8%)	9 (14%)	21 (14%)	0.245
Gastroenterologist	14 (18%)	16 (25%)	35 (23%)	0.354
Gastroenterology surgeon	26 (33%)	35 (54%)	66 (44%)	0.017
Number of home transfusions				
1–2 times	40 (51%)	46 (71%)	89 (59%)	0.023
3–5 times	8 (10%)	10 (15%)	18 (12%)	0.376
6 times or more	31 (39%)	9 (14%)	43 (29%)	<0.001
Reason for home transfusion				
Referral from an acute care hospital	52 (66%)	24 (37%)	77 (51%)	<0.001
Deterioration of disease condition in the outpatient clinic	16 (20%)	14 (22%)	34 (23%)	0.842
Referral of patient in home care	4 (5%)	21 (32%)	25 (17%)	<0.001

Note: Data for one patient was missing for activities of daily living level, haemoglobin level and age group, and data for two patients were missing for the reason for home transfusions. There were a significant number of patients with more than one specialist as their primary physician.

[a]There were six patients with an unknown geographic location.

[b]Three patients in the urban regions and one patient in satellite and rural regions had multiple underlying diseases.

[c]Twenty patients had multiple reasons.

Study design

A total of 856 clinics were selected from the database of the Japanese Red Cross Society, which is the sole manufacturer of blood products in Japan. The database did not include information on whether the clinics performed transfusions on an outpatient basis or at home. Therefore, clinics that provide home healthcare were determined from the clinics' websites. Clinics in all 47 prefectures of Japan were included in the survey.

This survey was conducted from February to November 2019, using the same method as a previously reported study [1]. Red blood cells (RBCs) were the only target blood product. The survey forms were sent out prior to the survey period to enable the respondents to prepare for the questions. The full text of the survey is shown in the Supplementary Data (https://figshare.com/s/77a1537dfcd20f32c9d8). Survey items were created based on the Japanese transfusion guidelines, a literature review and background factors of transfusion practice in Japan. The survey was prepared in a systematic manner. The primary evaluation item was factors associated with the purpose of transfusion. Secondary evaluation items were the percentage of each underlying disease and variables affecting Hb trigger levels. The survey consisted of sections A and B. In this study, data from section B, which contained the patients' characteristics (such as age group, underlying diseases, reasons for transfusion and physical condition), the specialty of the physician in charge, the purpose of the transfusion, the number of transfusions received and the process of home transfusions, were analysed. We focused on these section B data to analyse regional differences in patient background, factors affecting transfusion objectives and Hb trigger levels.

Ethics statement

This study was approved by the Institutional Review Board of Tokyo Medical University (study approval no.: T2018-0048).

Statistical analysis

Numerical data were analysed using the Mann–Whitney *U* test, and categorical data were analysed by the Fisher exact test and the chi-square test. A *p*-value of less than 0.05 was considered to indicate a statistically significant difference between groups. SPSS Statistics 28 software (IBM Corporation) was used for statistical analyses.

RESULTS

A total of 290 clinics responded to the questionnaire, resulting in a response rate of 33.9%. Of these, 51 clinics answered that they provided a home transfusion service. Detailed information on 150 patients receiving home transfusions was obtained. The total number of home transfusions was approximately 435, and the median number of transfusions in individual patients was 2.

Patient characteristics by region

The surveyed areas of Japan were classified into three regions, namely urban regions (4 large prefectures, i.e., Tokyo, Aichi, Osaka and Fukuoka), satellite regions (7 prefectures neighbouring the prefectures containing large cities, i.e., Saitama, Chiba, Kanagawa, Gifu, Shizuoka, Kyoto and Hyogo) and rural regions (the 36 other prefectures). The most common underlying diseases were solid tumours (39%), haematological malignancies (34%) and benign haematological diseases (25%) (Table 1). Patients from urban regions had a significantly higher frequency of haematological malignancies, whereas patients from satellite and rural regions had a higher frequency of solid tumours. The most common reason for transfusion was anaemia caused by the underlying disease (74%), followed by chronic bleeding, such as bleeding from a tumour lesion or gastrointestinal bleeding (25%). Regarding patient age, 78% of the patients were older than 70 years. The classification of activities of daily living (ADL) levels is shown in Table 2 [3]. In urban regions, haematologists were significantly more likely to be the patient's primary physician, whereas in satellite and rural areas, gastroenterological surgeons were significantly more likely to be the patient's primary physician. In terms of the process leading to home transfusions, urban areas had significantly more referrals from acute care hospitals, whereas satellite and rural areas had significantly more referrals of patients already receiving home healthcare.

Purpose of transfusion

The most common purposes of home blood transfusions were, in descending order, 'improvement of symptoms' (85%), 'maintenance of life' (53%) and 'maintenance of quality of life (QOL)' (49%). The common combinations of objectives are summarized in Table 3. The items that showed significant differences between 'maintenance of life' and 'improvement of symptoms' are shown in Table 4. The items that showed significant differences both between the above objectives and between 'maintenance of life' and 'maintenance of

TABLE 3 Purpose of home transfusion of the patients.

Item	Number of patients[a] (total: 150)	Percentage
1. Maintenance of life	80 (17)	53.3
2. Maintenance of the quality of life	73 (4)	48.7
3. Improvement of physical function	12 (0)	8.0
4. Improvement of symptoms	128 (38)	85.3
Main combinations of the above items		
1 + 2 + 4	41	27.3
2 + 4	27	18.0
1 + 4	22	14.7

Note: Numbers in parentheses indicate the number of patients who chose only that item.
[a]Multiple choices were allowed for items 1–4.

TABLE 4 Patient profiles by purpose of transfusion.

Item	Maintenance of life only ($n = 17$)	Maintenance of life and maintenance of QOL and/or improvement of symptoms ($n = 63$)	p-Value	Improvement of symptoms only ($n = 38$)	p-Value	Other purpose ($n = 32$)	Total* ($n = 150$)
Underlying disease							
Blood disease (benign)	4 (23%)	15 (24%)	0.981	8 (21%)	0.837	10 (31%)	37 (25%)
Blood disease (malignant)	4 (23%)	21 (33%)	0.512	18 (47%)	0.095	8 (25%)	51 (34%)
Solid tumour	9 (53%)	23 (37%)	0.220	9 (24%)	0.033	15 (47%)	56 (37%)
Primary reason for home transfusion[a]							
Anaemia caused by underlying disease	9 (53%)	46 (73%)	0.113	30 (79%)	0.049	26 (81%)	111 (74%)
Bleeding from tumour lesion	6 (35%)	10 (16%)	0.076	3 (8%)	0.011	5 (16%)	24 (16%)
Gastrointestinal bleeding	4 (24%)	7 (11%)	0.187	1 (3%)	0.013	2 (6%)	14 (9%)
Activity of daily living level							
Level J	2 (12%)	7 (11%)	0.974	6 (16%)	0.696	5 (16%)	20 (13%)
Level A	4 (23%)	19 (30%)	0.542	19 (50%)	0.066	15 (47%)	57 (38%)
Level B	1 (6%)	26 (41%)	0.005	5 (13%)	0.424	8 (25%)	40 (27%)
Level C	10 (59%)	10 (16%)	<0.001	8 (21%)	0.006	4 (12%)	32 (21%)
Haemoglobin level							
9.0 g/dL or more	0	3 (5%)	0.351	3 (8%)	0.233	2 (6%)	8 (5%)
8.0–8.9 g/dL	3 (18%)	2 (3%)	0.032	4 (11%)	0.464	3 (9%)	12 (8%)
7.0–7.9 g/dL	2 (12%)	16 (25%)	0.211	15 (39%)	0.040	11 (34%)	44 (29%)
6.0–6.9 g/dL	3 (18%)	17 (27%)	0.393	9 (24%)	0.616	8 (25%)	37 (25%)
Less than 5.9 g/dL	9 (53%)	23 (37%)	0.259	7 (18%)	0.009	8 (25%)	47 (31%)
Specialty of the physician in charge of the patient							
Haematologist	0	14 (22%)	0.016	25 (66%)	<0.001	10 (31%)	49 (33%)
Cardiologist	0	16 (25%)	0.020	4 (11%)	0.165	1 (3%)	21 (14%)
Gastroenterologist	6 (35%)	11 (17%)	0.111	4 (11%)	0.028	14 (44%)	35 (23%)
Gastroenterology surgeon	15 (88%)	38 (60%)	0.031	5 (13%)	<0.001	8 (25%)	66 (44%)
Number of home transfusions							
1–2 times	14 (82%)	37 (59%)	0.072	20 (53%)	0.036	19 (59%)	90 (60%)
3–5 times	1 (6%)	7 (11%)	0.524	7 (18%)	0.219	3 (9%)	18 (12%)
6 times or more	2 (12%)	19 (30%)	0.126	11 (29%)	0.166	10 (31%)	42 (28%)
Reason for home transfusions							
Referral from an acute care hospital	4 (24%)	26 (41%)	0.152	30 (79%)	<0.001	17 (53%)	77 (51%)
Deterioration of disease condition in the outpatient clinic	13 (76%)	15 (24%)	<0.001	3 (8%)	<0.001	3 (9%)	34 (23%)
Referral of patient in home care	0	14 (22%)	0.029	1 (3%)	0.500	10 (31%)	25 (17%)

Note: Analysis of statistical significance was performed between the maintenance-of-life-only group and the maintenance of life and maintenance of quality of life (QOL) and/or improvement of symptoms group, as well as between the former and the improvement-of-symptoms-only group. There were a significant number of patients with more than one specialist as their primary physician.

*Significant differences.

[a]Three patients in the maintenance-of-life-only group and one patient in the other-purpose group had multiple primary reasons.

life and maintenance of QOL and/or improvement of symptoms' were the following: ADL level C, a haematologist or gastroenterological surgeon being the physician in charge and the process to home blood transfusion was deterioration of the disease condition in outpatients at the clinic. Even when the target was limited to solid tumours, the trend remained the same, with haematologists emphasizing symptom improvement and gastroenterological surgeons emphasizing sustaining life.

TABLE 5 Factors associated with a low pre-transfusion haemoglobin level.

Factor	Hb level (≤6.9 g/dL)	Hb level (≥7.0 g/dL)	Total	*p*-Value
Age (≥90 years old)	18	4	22	0.009
ADL level A	24	33	57	0.002
Blood disease (malignant)	16	34	50	<0.001
Solid tumour	39	16	55	0.008
Anaemia caused by underlying disease	56	54	110	0.015
Anaemia caused by chronic bleeding	24	9	33	0.036
Transfusion performed by a haematologist	21	28	49	0.016
Transfusion performed by a gastroenterologist	27	8	35	0.005
Maintenance of life	52	26	78	0.010
Two transfusions or less	59	30	89	0.003
Referral from an acute care hospital	31	45	76	<0.001
Deterioration of disease condition in the outpatient clinic	26	8	34	0.007

Note: Numbers in the table represent the number of patients.
Abbreviations: ADL, activities of daily living; Hb, haemoglobin.

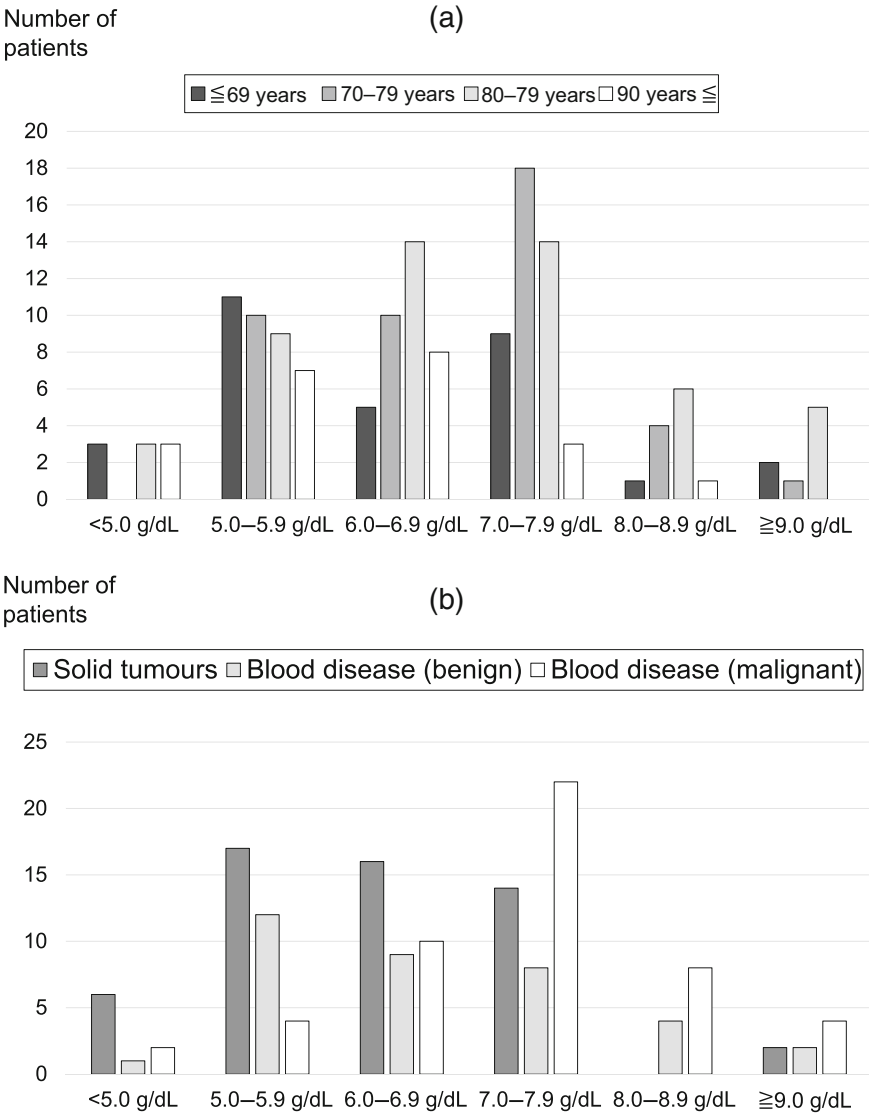

FIGURE 1 Frequency distribution of various parameters in patients with different haemoglobin (Hb) levels. Distribution of age group (a) and underlying diseases (b) in patients with different Hb levels. Patients over 90 years of age tended to have low Hb levels. On the other hand, patients with malignant blood diseases tended to have high Hb levels.

Factors associated with pre-transfusion Hb level

The most common pre-transfusion Hb level of the 150 patients who received home transfusions was in the 7 g/dL range, followed by the 6 and 5 g/dL ranges.

To identify factors associated with a low pre-transfusion Hb level (≤6.9 g/dL), we performed the chi-square test. Significant factors were age ≥90 years, a gastroenterologist being the physician in charge and the purpose of transfusion being maintenance of life and others, as shown in Table 5. The frequency distributions of patient Hb levels by age group and underlying disease are shown in Figure 1.

DISCUSSION

This study was conducted as a Health and Labor Science Study, and the survey forms were distributed prior to the survey period with the purpose of future utilization. Therefore, this cohort was considered to be representative of the actual situation of home blood transfusion in Japan. In addition, the response rate for this survey was 34%, and hence the actual number of home transfusion patients may be approximately three times higher than the reported number of patients.

Patient characteristics by region

In our previous paper, we reported that in rural areas, there is a lack of close cooperation between hospitals and clinics, resulting in an insufficient monitoring system of transfusion reactions, compared with urban areas. Therefore, we assumed that there would be regional differences in patient characteristics as well. Blood diseases and solid tumours were found to be the common underlying diseases in the transfusion patients, as reported previously [4]. Haematological malignancies were significantly more common in urban areas, most likely associated with the larger number of haematologists. Advanced treatments for haematologic diseases, such as haematopoietic stem cell transplantation, are often performed in urban areas, resulting in the uneven distribution of haematologists [5]. Furthermore, there were some physicians among haematologists in urban areas who actively performed home transfusions [6]. On the other hand, the fact that there were more gastrointestinal surgeons performing transfusions in satellite and rural areas may be associated with their previous experience of transfusions. With persistent concerns about the safety of home transfusions in Japan, gastrointestinal surgeons were assumed to be the main physicians performing home transfusions in satellite and rural areas, for two possible reasons: Firstly, gastrointestinal surgeons may often be confident in their own responses, and secondly, they may often underestimate transfusion reactions (because of their limited experience with serious reactions).

Patient background by purpose of transfusion

The survey revealed that maintenance of life is one of the primary purposes of home transfusions in Japan. According to the Japanese Guidelines for the use of RBCs, the use of RBCs in terminally ill patients is classified as inappropriate [7]. Therefore, it is assumed that the indication for home transfusions is carefully evaluated, taking into account the minimum transfusions required based on the patient's physical condition. This is supported by the data that in the group in which the only purpose of transfusion was for life support, many patients had Hb levels below 5.9 g/dL, ADL level C or less than two transfusions. However, the above guidelines state that 'The opinion that providing life support against the patient's wishes should be avoided is becoming increasingly accepted', and hence not all transfusions in the terminal phase are inappropriate as long as the patient's wishes are respected. It may hence be necessary to distinguish the stage of impending death from situations in which some period of survival can be expected, without uniformly interpreting the terminal phase. In addition, the Hb level that should be maintained for life support is not clear [8, 9]. The purpose of home transfusions in patients near the terminal stages should be clearly defined in the above guidelines to help physicians in their decision making.

Factors associated with pre-transfusion Hb level

Recently, many studies on RBC transfusion with high evidence levels have been published, leading to restrictive transfusions, with a Hb concentration threshold of 7–8 g/dL becoming the mainstream [10–12]. However, the subjects in these previous reports were acutely ill inpatients with stable haemodynamics, and whether this threshold can also be applied to chronically ill older patients being treated outside hospital environments is unclear. In a meta-analysis of randomized controlled trials on individuals aged ≥65 years [13], the mortality rate was significantly higher in the restrictive transfusion group than in the liberal transfusion group. Another randomized study of frail patients with femoral neck fracture living in elderly care facilities also showed similar results [14]. These findings suggest that liberal transfusion leads to a more favourable prognosis in older individuals [15]. The lower threshold for Hb concentration in the older patients in our survey could be explained by differences in patient backgrounds and adherence to Japanese guidelines [7].

The preferred threshold pre-transfusion Hb level of patients receiving palliative care is 8.0–10.0 g/dL, as previously reported [16–18]. Another small randomized controlled study performed in 38 outpatients with myelodysplastic syndromes indicated that clinically significant improvements were observed in the physical functions and QOL of patients in the liberal transfusion group [19]. Furthermore, although evidence is not clearly presented, the threshold Hb level for transfusion in palliative care patients may be more liberal than in other patients [20, 21]. On the other hand, determining a transfusion Hb threshold level for life support in palliative care is even more difficult. There was a recommendation that RBC transfusions should be considered for patients with Hb levels of less than 5 g/dL for the management of chronic anaemia owing to occult blood loss [22]; however, the threshold should be carefully determined depending on the patient's background.

Patients receiving palliative care are considered to have various complications, physical frailties, as well as personal values, and hence it is crucial to individualize the indications for transfusion. For this purpose, for home transfusions, it is necessary to establish an assessment system for post-transfusion effects, including QOL and symptom improvement, as part of the standard practice of transfusion therapy [23].

Strengths and limitations

The strength of this study is that it is the first in-depth study of home transfusions in Japan. We believe that the findings of this study will be useful for promoting clinical research and revising the guidelines for home transfusions. However, it is a retrospective study with a small number of patients, and its weakness is that the objective of the transfusions is determined based on the subjectivity of the physicians. The key uncertainties are the clinical heterogeneity of the patients as well as of the transfusion method.

The advantages of home transfusion are that it reduces the physical and emotional burden on the patient and family and helps to maintain the patient's QOL. The disadvantages are the risk of potentially serious complications occurring as a result of the reduced availability of emergency care and transfusion testing and the increased burden on healthcare providers. Therefore, the implementation of home transfusion should be considered after careful review of the balance of these factors. The negative aspects of home transfusions have been highlighted, and there is concern that actual transfusions are limited to a minimum owing to the fact that only patients in the terminal phase are indicated for home transfusions in the guidelines in Japan. Moreover, approaches to maximize the efficacy of blood transfusions are still lacking. The shortcomings of Japan's healthcare policy, which has not emphasized the development of family physicians, may be a reason for this situation. Therefore, the ideal home transfusion system for each type of region in Japan should be investigated and improved according to the medical resources available.

Whereas it is possible to choose not to perform home transfusions and to perform transfusions only in the hospital or on an outpatient basis, it is desirable to promote the development of home transfusion providers in the future. To improve home transfusion, it is necessary to revise its guidelines, promote clinical research and improve the cooperation between hospitals and clinics according to the local healthcare setting.

In conclusion, home transfusions are found to be performed in a restrictive and diverse manner in Japan. Further accumulation of data is necessary to provide more effective transfusion therapies in Japan together with safety assurance.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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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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Exploring the potential harm of varied blood storage on patients undergoing cardiovascular surgery

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Abstract

Background and Objectives: Debate exists surrounding the optimal duration of red blood cell (RBC) storage. A hypothesis emerging from previous research suggests that exposure to fresh blood may be harmful to patients undergoing cardiac surgery. This study uses a large transfusion medicine database to explore the association between in-hospital mortality and red cell storage duration.

Materials and Methods: This is an exploratory retrospective cohort study of all adult patients at Hamilton, Canada, over a 14-year period that received at least one allogeneic red cell transfusion during their hospitalization for cardiac surgery requiring bypass. The primary outcome for the study was in-hospital death. Analysis was performed using multivariate Cox regression modelling with time-dependent and time-independent covariates and stratification variables. Five models with varying definitions for short, intermediate and prolonged duration of RBC storage were tested.

Results: From March 2004 to December 2017, 11,205 patients met the inclusion criteria and were included in the regression analyses. No significant effect of short-duration red storage on patient mortality was observed in all statistical models, with the red cells stored for the longest duration as the reference group. When patients who received exclusively fresh (hazard ratio [HR] 1.040, 95% confidence interval [CI] 0.588–1.841, p-value = 0.893) and older aged (HR 1.038, 95% CI 0.769–1.1.402, p-value = 0.0801) RBCs were compared with those who received exclusively mid-age red cells as the reference, statistical significance was similarly not reached.

Conclusion: Red cells stored for the shortest duration are not associated with increased risk of mortality among cardiac surgery patients.

Keywords

blood donors, blood transfusion, cardiac surgery, red blood cells, storage

Highlights

- Previous research suggests that exposure to fresh red blood cell concentrates (RBCCs) may be harmful to patients undergoing cardiac surgery.
- We have explored the effect of storage of RBCCs in a retrospective cohort study where 11,205 patients were included.

Shuoyan Ning and Akash Gupta contributed equally to the development of the manuscript and share co-first authorship.

- RBCCs stored for the shortest duration are not associated with increased risk of mortality among cardiac surgery patients.

INTRODUCTION

The identification of progressive structural, biochemical and functional changes in red blood cells (RBCs) during storage has led to the hypothesis that longer duration of RBC storage before transfusion is potentially harmful. Over the past 20 years, this hypothesis has resulted in observational studies and randomized controlled trials (RCTs) designed to determine whether RBCs stored for a shorter period of time improve patient clinical outcomes compared with RBCs stored for longer duration. In a meta-analysis published in 2018 [1], which included 16 RCTs (randomizing a total of 31,359 patients), the authors concluded that transfusion of fresher, compared with RBCs stored for longer durations, was not associated with a lower risk of death (relative risk 1.04, 95% confidence interval [CI] 0.98–1.09). However, a trend towards increased mortality when patients were exposed to fresh blood has also been observed—although one limitation to this literature is the variable definitions of fresh (less than 3 days–less than 21 days) versus old (21 ± 4 days—standard issue) between studies [1]. The three largest RCTs [2–4] using mortality as an outcome all had a trend of higher mortality in patients receiving fresher RBCs, a finding that was not statistically significant but was reflected in the overall results of the 16 study meta-analysis. It has also been suggested that the freshest and oldest RBCs may both be harmful to patients [5] compared with mid-aged RBCs, but to the authors' knowledge, this has never been studied.

In a subgroup analysis of cardiac surgery patients from the INFORM study, there was a trend of increased mortality with the transfusion of blood stored for shorter duration compared with longer duration of storage (odds ratio 1.13; 95% CI 0.99–1.29; $p = 0.07$); this trend was not observed in subgroups of intensive care and haematology/oncology patients [3]. This trend, as well as the complex pro-inflammatory state of cardiovascular surgery patients [6], led us to hypothesize that exposure to fresh blood may be harmful to patients undergoing cardiac surgery.

In this study, we used the Transfusion Research, Utilization, Surveillance and Tracking (TRUST) database to undertake an exploratory retrospective cohort study to address the research question: for patients requiring at least one allogeneic RBC transfusion during their hospitalization for cardiac surgery requiring bypass, is there an association between in-hospital mortality and RBCs stored for a short-duration when compared with RBCs of intermediate or prolonged storage duration?

MATERIALS AND METHODS

Study design and participants

This was a retrospective exploratory cohort study spanning a 14-year period from March 2004 to December 2017. Patients were eligible for inclusion if they met the following criteria: adults (≥18 years); undergoing cardiac surgery requiring bypass (using Canadian Classification of Health Interventions [CCI] codes 1.LZ.37.GP-GB and 1.LZ.37.LA-GB); and receiving at least one allogeneic RBC transfusion during their hospitalization. Patients were excluded if they received autologous or directed donations; these patient populations were excluded to account for the inherent differences in transfusion indication/thresholds, and potential inflammatory effects of transfusion (with autologous from self and directed often from relatives) between this population and those receiving allogeneic transfusions. Only data from a patient's first admission for cardiac surgery during the study period were included in the analysis to allow for independent data to be used. The study was approved by the Hamilton Integrated Research Ethics Board (no. 4212).

Clinical and laboratory data were extracted from the TRUST database, developed by the Michael DeGroot Centre for Transfusion Research that captures demographic, clinical, laboratory and blood product information from medical records and the laboratory information system [7]. The following information was extracted on all eligible patients at Hamilton hospitals: demographic information (age, sex, ABO blood group, admission category and diagnosis); duration of hospital stay, daily haemoglobin and creatinine values, and information on transfused RBCs, platelets, plasma and cryoprecipitate including number of units, date of transfusion and blood product ABO group. Information pertaining to relevant donor and product information for each transfused RBC unit, including the collection and donation date, the processing method, and the product manufacturing and processing times, were obtained from the blood supplier. The transfusions may have occurred in the pre-operative, intraoperative or post-operative setting. The processing method refers to the method by which red cells are manufactured in Canada (buffy coat collection or whole blood filtration); buffy coat collection was introduced by the blood supplier during the time frame of this study.

Duration of maximum red cell storage was 42 days throughout the study period. International Classification of Diseases and Related Health Problems 10th Revision (ICD-10-CA) codes were used to extract data from the TRUST database for the following patient comorbidities: diabetes, peripheral arterial disease, ischaemic heart disease, heart failure, pulmonary hypertension, endocarditis and sepsis.

Statistical analysis

The primary outcome was in-hospital death. The observation period started at the time of the first RBC transfusion and ended at the time of hospital discharge or death. Statistical analysis was based on multivariate Cox regression analysis with fixed covariates, time-dependent and time-independent covariates, and stratification

variables. Exposure groups were updated with each transfused RBC unit. Fixed covariates included patient age, ABO blood group and the pre-specified comorbidities (diabetes, peripheral arterial disease, ischaemic heart disease, heart failure, pulmonary hypertension and endocarditis). The time-dependent covariates included: whether the patient received ABO non-identical RBCs (yes or no); daily haemoglobin and creatinine results and whether the patient received additional blood products (platelets, plasma and/or cryoprecipitate) over their hospital stay. Variables that did not satisfy the proportional hazards assumption were used as stratification variables. Fixed stratification variables were year of hospital admission, patient sex and admission category (urgent or elective). Time-dependent stratification variables were cumulative number of RBCs transfused and processing method of RBC units. Time-dependent variables were updated daily in the analysis as new information became available (e.g., additional RBCs transfused or new haemoglobin or creatinine test result).

In the first four models, individual patient exposure to short-term stored RBCs was defined by the minimum age of RBCs transfused and summarized in four different ways: age of transfused RBCs as a continuous variable (Model 1); age of transfused RBCs categorized by weeks of storage (1 week: 1–7 days, 2 weeks: 8–14 days and so on) (Model 2); categorizing age of transfused RBCs using quartiles of the overall distribution as cut points (Model 3); and categorizing age of transfused RBCs into three groups: fresh (1–7 days), mid-age (8–35 days) and old (36–42 days) (Model 4a), with other varying three group cut-offs (Models 4b-c). The category representing the transfused RBCs stored for the longest duration was the reference group for Models 2 through 4 and compared against RBCs of short as well as intermediate storage ages.

An additional model (Model 5) categorized the age of transfused RBCs by four mutual exclusive exposure groups: fresh, exclusive mid-age only, old and a combination of fresh and old; with fresh defined as either 1–7 days, 1–10 days or 1–14 days; and old defined as either 36–42 days, 33–42 days or 29–42 days (Models 5a, 5b and 5c, respectively). The 'fresh' exposure group was defined by exposure to fresh RBCs with permissible exposure to mid-age RBCs; the 'exclusive mid-age' group defined exposures to only mid-age RBCs; the 'old' RBC group was defined by exposure to old RBCs, with permissible exposure to mid-age RBCs; and, the 'fresh and old' group defined exposures to both fresh and old blood, with permissible exposure to mid-age RBCs. Exposures are dynamic, and thus a patient can move exposure groups during their hospital stay (e.g., from exclusive mid-age to fresh as they receive more RBC transfusions). These models were designed to evaluate whether there was a relationship between patient mortality based on exposure to short-term and long-term stored products compared with the reference group of products stored in the mid-age group.

All approaches for summarizing exposure to short-term stored RBCs were defined a priori. A single post hoc analysis was run on Schoenfeld residuals and associated tests of the proportional hazard assumptions were conducted for all models. All analyses were done using SAS 9.4 (Cary, NC).

RESULTS

Patient characteristics and outcomes

During the study period, 19,684 adult patients underwent cardiac surgery requiring bypass. Of these patients, 11,364 received at least one allogeneic RBC transfusion during their hospital stay. Patients with missing data (haemoglobin or creatinine values, or donation information) were excluded from the regression analysis ($n = 159$), leaving 11,205 patients who were included in the analyses (Figure 1).

In this study, 4356 (38.9%) of the patients were female, the median age was 72 years (interquartile range [IQR] 64–78), the median duration of hospital stay was 10 days (IQR 8–15) and the median number of RBCs transfused per patient was 3 units (IQR 2–5). There were 607 (5.4%) patients who died in hospital: mortality was 3.2% in patients who received 1–9 RBC units and 28.5% in patients who received ≥10 RBC units. The documented comorbidities included ischaemic heart disease (49.0%), sepsis (12.4%), diabetes (11.1%) and heart failure (9.3%) (Table 1). In addition to RBC transfusions, 52.4% of patients received a platelet transfusion, 47.0% received a plasma transfusion and 12.3% received cryoprecipitate.

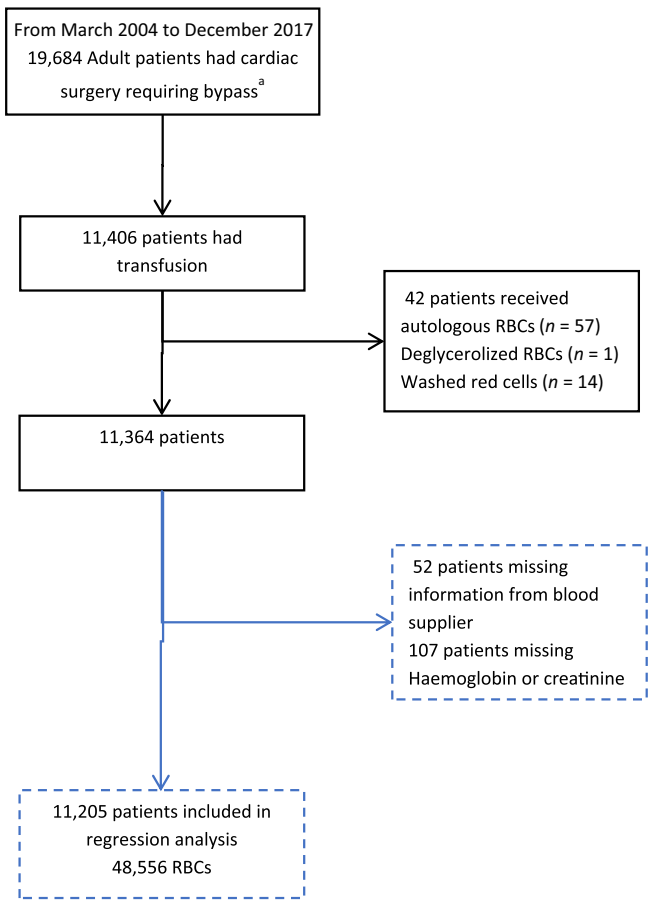

FIGURE 1 Flow chart demonstrating patient inclusion and exclusion. [a]Bypass surgery ICD-10 codes: CCI 1.LZ.37.GP-GB and 1.LZ.37.LA-GB. RBCs, red blood cells.

TABLE 1 Patient characteristics and blood product information.

Variables	Patients included in regression analysis (n = 11,205)
Female, *n* (%)	4356 (38.9)
Age (years), mean/SD; median (Q1, Q3) (min, max)	69.9/11.0; 72 (64, 78) (18, 97)
Blood group, *n* (%)	
A	4503 (40.2)
AB	489 (4.4)
B	1265 (11.3)
O	4948 (44.2)
Admission category	
Elective	6580 (58.7)
Urgent	4625 (41.3)
Haemoglobin (g/L) at first transfusion, mean/SD; median (Q1, Q3)	75.6/9.2; 75 (70, 81)
Creatinine (μmol/L) at first transfusion, mean/SD; median (Q1, Q3)	106.1/83.0; 84 (68, 111)
Diabetes, *n* (%)	1239 (11.1)
Peripheral arterial disease, *n* (%)	270 (2.4)
Ischaemic heart disease, *n* (%)	5496 (49.0)
Heart failure, *n* (%)	1045 (9.3)
Pulmonary hypertension, *n* (%)	88 (0.8)
Endocarditis, *n* (%)	63 (0.6)
Sepsis[a], *n* (%)	1386 (12.4)
Duration of hospital stay (days), mean/SD; median (Q1, Q3)	14.1/13.7; 10 (8, 15)
Number of RBC transfusions, mean/SD; median (Q1, Q3)	4.3/4.7; 3 (2, 5)
In-hospital death, *n* (%)	607 (5.4)
Patients receiving 1–9 RBC units, *n* (%)	32 5 (3.2)
Patients receiving ≥10 RBC units, *n* (%)	282 (28.5)
Storage duration of freshest RBC unit, mean/SD; median (Q1, Q3)	19.7/8.2; 19 (14, 25)
Storage duration of oldest RBC unit, mean/SD; median (Q1, Q3)	25.2/9.0; 25 (18, 34)
Patients receiving	
Platelet transfusion, *n* (%)	5870 (52.4)
Plasma transfusion, *n* (%)	5262 (47.0)
Cryoprecipitate transfusion, *n* (%)	1378 (12.3)

Abbreviation: RBC, red blood cell.

[a]Including diagnose type of most responsible diagnosis, secondary diagnosis and post-admit comorbidity. Sepsis codes using ICD-10 codes listed by Joelley et al.[8]

Association between in-hospital mortality and RBC storage duration

The hazard ratios (HRs) and 95% CIs for Models 1–4 are shown in Figure 2. There were no statistically significant associations noted between red cell storage duration and in-hospital mortality observed in any of the models.

In Model 5, the use of patient categories who received fresh or old and a combination of fresh and old RBCs was compared against patients who received exclusively mid-aged blood. Model 5a compares RBCs stored at 1–7 days, 8–35 days and 36–42 days; Model 5b compares RBCs stored at 1–10 days, 11–33 days and 34–42 days; and Model 5c compares RBCs stored at 1–14 days, 15–28 days and 28–42 days. This analysis did not yield statistically significant results. Compared with the reference group of mid-age storage red cells, the use of fresher (HR 1.040, 95% CI 0.588–1.841, p-value = 0.893) and older (HR 1.038, 95% CI 0.769–1.1.402, p-value = 0.0801) aged RBCs did yield numerically higher HRs for mortality, but statistical significance was not reached. Figure 3 shows the HRs of the three models where the cut-off markers represented mutual exclusive exposure categories. As the inclusivity criteria for age that define the fresh and old groups were extended, the total number of patients in the mid-aged group decreased, yielding similar numbers in all three groups (see Appendix).

DISCUSSION

This is a retrospective exploratory cohort study of more than 11,200 adult patients in Hamilton, who had received at least one allogeneic

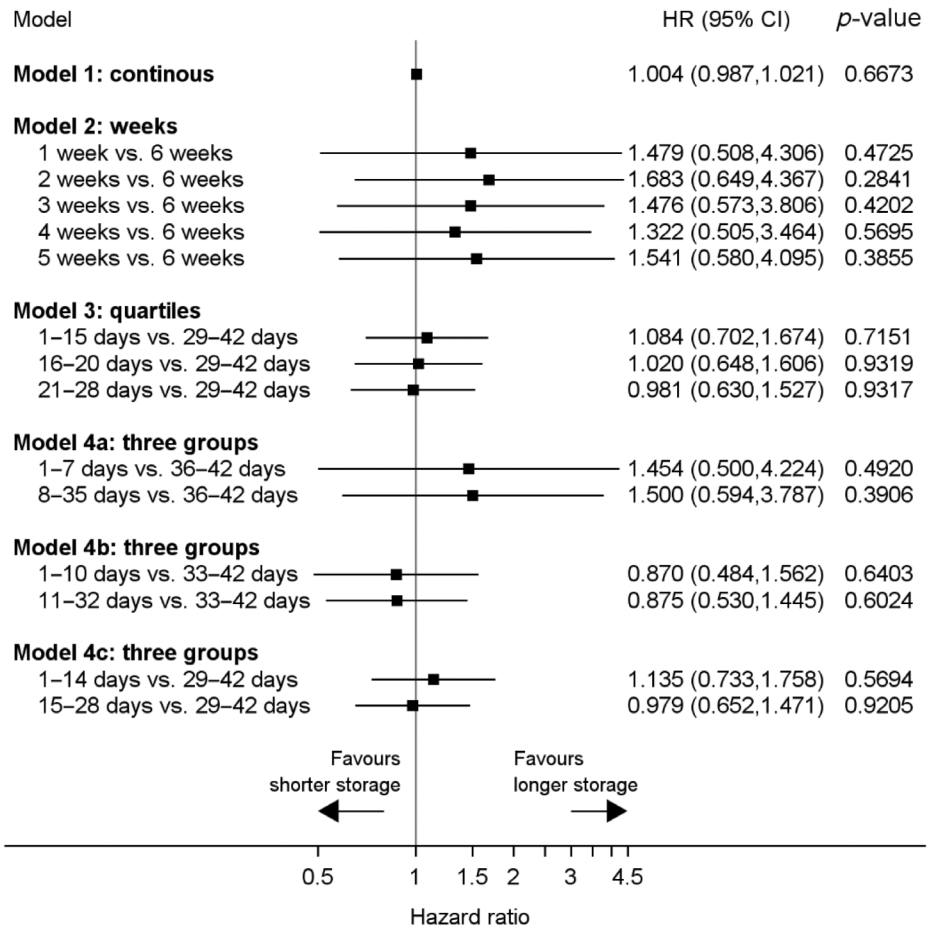

FIGURE 2 Hazard ratios (HRs) and 95% confidence intervals (CIs) for the comparisons in Models 1–4.

RBC transfusion during their hospitalization for cardiac surgery requiring bypass over a 14-year period (March 2004 to December 2017). In this study, there was no evidence of statistically significant harm or benefit with the use of fresher blood compared with mid-aged or older blood seen across a variety of regression models. When comparing exposure to fresh, middle-aged or older RBCs exclusively, the results were not statistically significant.

Many clinical settings require the use of RBCs for transfusion, including invasive surgical procedures, bleeding due to trauma, obstetrical haemorrhage and in bleeding patients with critically low haemoglobin levels. This results in approximately 112.5 million RBC units collected annually, according to the World Health Organization [9]. The potential impact of RBC storage duration on recipient morbidity and mortality is a question to which clinicians have long sought an answer. The usual concerns pertain to the biological impacts of storage lesions within a product over time [10]. An international pragmatic RCT of 20,858 in-patient adults, the INFORM trial, showed no difference between standard issue versus short-term storage RBCs as did the results of several other RCTs [2–4, 11]—effectively putting an end to the debate of the superiority of short-term RBCs [3]. Other large studies have explored the clinical impact of RBC storage age in various patient populations, including neonates, children, intensive care patients and cardiac surgery patients [3, 4, 11–20]. These studies have also shown no significant statistical difference in morbidity/mortality of fresher versus older blood. However, studies on blood storage have unexpectedly raised the question of potential harm of short-term stored blood [7, 21]. Questions around the potential for harm with blood stored for 36–42 days remain, as this storage duration was not adequately represented in the studies published to date [22–25]. Furthermore, definitions of fresh and old have varied significantly between published studies complicating this question, particularly as red cells are dynamic immunologic products transfused into an equally dynamic recipient; in our study, we evaluated various cut-offs of fresh, mid-age and old products to help detect meaningful patterns in a real-life setting.

Cardiac surgery and the use of extracorporeal circulation often require blood product support in the form of RBC transfusion. The impact of exposure of blood to contact surfaces, increased mechanical shear stress and changes in temperature triggers a cascade of sequences that have been linked to increased hospital stay, post-operative infections, myocardial, hepatic and renal dysfunction, and increased mortality [26]. The hypothesized mechanism that leads to these poor outcomes is the development of an inflammatory state that leads to increased production of reactive oxygen species and further production of pro-inflammatory cytokines [6]. The impact of the duration of red cells storage on this hyperinflammatory state seen in cardiac surgery is unclear. Previous studies have reported that transfusion of RBCs that

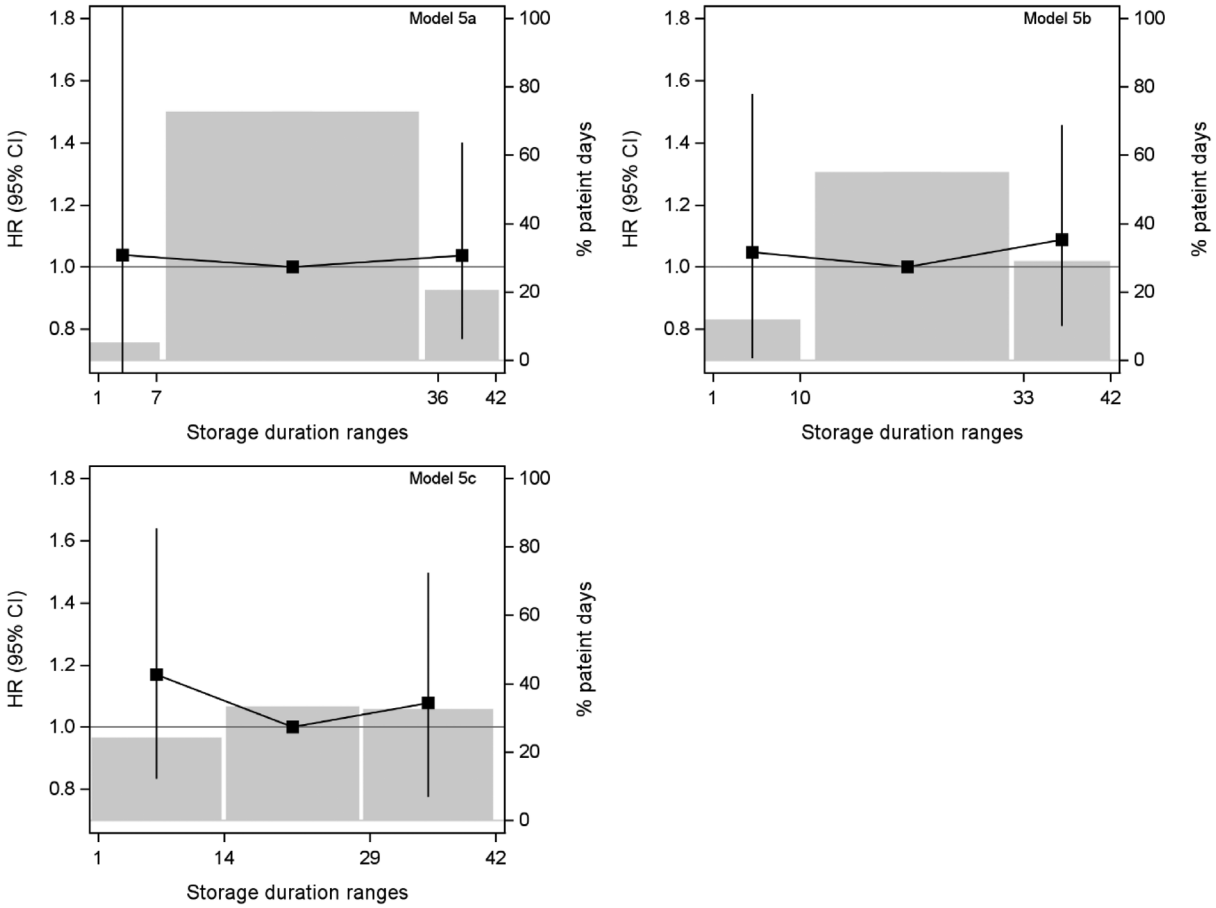

FIGURE 3 Hazard ratios (HRs) comparing three exclusive exposure groups: Model 5a: fresh red blood cells (RBCs) stored 1–7 days, mid-range 8–35 days and old 36–42 days; Model 5b: fresh RBCs stored 1–10 days, mid-range 11–32 days and old 33–42 days; Model 5c: fresh RBCs stored 1–14 days, mid-range 15–28 days and old 29–42 days. The heights of the three grey bars represent in each graph the number (%) of patient days spent in each exposure group. The widths represent storage duration ranges of the exposure groups. The small black squares and the vertical black lines represent HR and 95% confidence interval (CI) of two exposure groups compared with the reference group (mid-aged group).

have been stored for more than 2 weeks are associated with an increased risk of post-operative complications after cardiac surgery [5]. In this study, we found no statistically significant associations between short-duration red cell storage and mortality risks among cardiac surgery patients.

In our analysis (Model 5) where exclusively mid-age red cells were used as the reference, the use of fresher and older aged RBCs did yield numerically higher HRs for mortality, but statistical significance was not reached. A number of biological mediators may account for this observation. For red cell stored for prolonged durations, storage lesions (reviewed extensively in previous publications) may lead to impairment of oxygen transport and an increase in haemolysis and acidosis [27]. Other potential contributors include extracellular vesicles (EVs) [28, 29], which can (1) increase the procoagulant effect [30], (2) up-regulate the production of pro-inflammatory cytokines and (3) lead to increased rates of immune-mediated transfusion reactions, such as transfusion-related acute lung injury (TRALI) [31, 32]. Red cell-derived EVs tend to increase with storage, and is further influenced by numerous factors, including the storage conditions, the additive solutions utilized, the method of leukoreduction and the donors themselves [33]. For red cells stored for short durations, EVs produced from white cells and platelets can be identified in blood products stored for shorter durations, with the amounts related directly to the processing method [34]. Like red cell-derived EVs, their impact on the immune system has been shown to be potentially stimulatory and suppressive [31, 35–37]. The age of the RBC unit has a direct impact on the measurable quantity of cell-free DNA (cfDNA), which can activate the coagulation cascade and promote thrombosis [38]. Whole-blood-filtered, fresh RBCs have been shown to have more cfDNA than red cell filtered, old RBCs [39]. Fresh RBCs may also contain platelets as well as cfDNA [38]. In addition, despite universal leukoreduction, fresh RBCs contain residual leukocytes, which may get activated post-transfusion and stimulate the recruitment of additional pro-inflammatory cytokines.

The strengths of this paper include the length of study (14 years) and subsequently large patient population providing a robust data set. Patient groups were well defined with clear inclusion and exclusion criteria: a nearly equal number of male and female patients; the use of complex analytical approaches that controlled for covariates and confounders; and inclusion of models that used a variety of product age cut-offs were other strengths of this study.

The design of the study has some limitations including: the limited number of transfused RBC units in the fresh groups (e.g., 1–7 days) when compared proportionally with other older groups; not accounting for the effects introduced by the underlying reason for bleeding; the potential implication of different medical decisions; the severity of illness for an individual patient; the risk of using anticoagulants in this cohort, including the potential of trauma-induced emergency cardiac surgeries and its effect on mortality; the number of other blood products transfused; the impact of varying numbers of RBCs transfused at once; and the unequal proportionally transfused ABO blood groups. Haemoglobin was also used as one of the time-dependent covariates, which can be affected by bleeding or transfusion. Due to the nature of the data set, only in-hospital mortality was captured. We also did not assess outcomes such as nosocomial infection, which may be associated with transfusion of red cells stored for shorter durations [2]. ICD-10 codes for infection and sepsis are coded only at the end of the hospital admission, whether or not outcomes might have occurred prior to or following blood transfusion. As such we felt that infection could not be reliably assessed in our study due to an inability to establish temporality.

In conclusion, red cell storage duration was not significantly associated with in-hospital mortality among adult cardiac surgery patients in this retrospective cohort study.

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S.N., N.M.H. and R.B. conceptualized and designed, Y.L. interpreted the data and performed the statistical analysis, S.N., A.G. and K.B. created the first draft of the manuscript and N.H. supervised the research. All authors reviewed and edited the final manuscript.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

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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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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Evaluation of a rail logistics transmission system for the transportation of blood components within a medical centre

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Abstract

Background and Objectives: Rail logistics transmission systems (RLTSs) are commonly used for the transportation of blood samples, pathological specimens and other medical materials in many hospitals, as they are rapid, secure, cost-effective and intelligent. However, few studies have evaluated blood component transportation from blood banks to the patient care areas of hospitals using RLTS. In this study, we evaluate the RLTS used for the transportation of blood components within a medical centre.

Materials and Methods: The dispatch of blood components, including packed red blood cells (pRBCs), fresh frozen plasma (FFP), cryoprecipitate and platelet units, from a blood bank to critical care areas or general wards was done using RLTS. Parameters such as the delivery time, temperature, physical integrity and blood component quality were evaluated via analytical testing using specimens obtained before and after transportation by RLTS.

Results: The turnaround time and temperature of all tested blood units via RLTS transportation were able to meet the clinical demands of blood component delivery (median time: 323 s [118–668 s]; temperature variation: 4.5–8.9°C for pRBCs and FFP and 21.5–23.5°C for cryoprecipitate and platelet units). Furthermore, parameters of pRBC quality, including the haemolysis index and potassium and lactate dehydrogenase levels in plasma, were not significantly different before and after transportation through RLTS. Similarly, RLTS transportation affected neither the basic coagulation test results in FFP and cryoprecipitate specimens nor platelet aggregation and activation markers in apheresis platelet specimens.

Conclusion: Hospital-wide delivery of blood components via RLTS seems to be safe, reliable and cost-effective and does not have any negative impact on blood quality. Therefore, the establishment of standard criteria, protocols and guidelines based on further studies is needed.

Keywords

blood components, blood transfusion, guidelines, quality assessment, rail logistics transmission system, transportation

Highlights

- This study is the first to report a feasibility study of blood component distribution via a rail logistics transmission system (RLTS) within our medical centre, which primarily focused on delivery time, temperature, stability and reliability for system operation.
- The effect of mechanical forces and temperature on blood quality before and after RLTS transportation was assessed, including haemolysis, coagulation indices and platelet function.
- This study is an attempt to formulate a workflow for transporting blood components and then determine preliminary criteria for quality control.

INTRODUCTION

Blood transfusion is an important means to save lives, and its therapeutic efficacy depends on the timeliness of blood component transfusion. The transportation of all types of blood components from blood banks to the patient care areas of hospitals has long depended primarily on the use of traditional hospital porters, which is laborious, time consuming and inefficient.

Automated transportation solutions in the hospital setting seem to present an alternative for the transportation of blood components and are known to be a rapid, simple, secure, reliable and intelligent method for transporting medical material such as blood samples, pathological specimens and imaging findings in medical centres. Pneumatic tube systems (PTS) are one of the most widely used systems for transporting various types of medical materials, including blood samples [1–3], biopsy samples [4], urine samples [5], cerebrospinal fluid samples [6] and even blood components [2, 7–11]. However, based on the available literature, PTS have adverse effects on test results obtained from diagnostic samples [12, 13]. Because of the combined effects of rapid acceleration, deceleration, radial gravity forces and vibration, blood samples show increase in the levels of markers, including lactate dehydrogenase (LDH), potassium ($K+$), haemolysis index (HI), basic coagulation test results (activated partial thromboplastin time [APTT], prothrombin time [PT]) and platelet (PLT) activation markers (CD62P, CD63) after transportation via PTS [13–17]. As a result, PTSs were found to cause a variety of impairments that compromise blood quality and pose a potential risk to transfusion safety concerning the delivery of blood components to patients.

Unlike the PTS, the rail logistics transmission system (RLTS) operates smoothly with reduced vibrations and minimal acceleration forces, thereby having only a limited influence on the stability of cellular components. To date, RLTS is employed in various medical centres in China and has been found to be especially promising as a candidate product for hospital-wide blood component distribution. So far, little research has focused on the use of RLTS for the transportation of blood components in medical centres. In particular, the effect of vibration and acceleration forces on blood component quality remains to be defined.

The aim of our study was to evaluate the delivery time, temperature, physical integrity and quality of blood components distributed by RLTS to assess the feasibility of transporting blood components via RLTS. Then, a protocol and management guidelines were developed to advance this work for practical use.

MATERIALS AND METHODS

System description

As a Class A tertiary general hospital, our hospital occupies a land area of 303,333 m^2, has 2580 beds that are actually used and consists of 48 departments and 53 wards. There are seven buildings comprising clinical departments, medical technology departments and supporting departments, the largest of which is 18 floors above the ground and 2 floors below ground. The RLTS (Telelift Gmbh Ltd, Germany) used in our medical centre comprises 92 launch and collection stations connected by a network of UniCars (Figure S1A). Furthermore, the UniCar trolleys have a payload of 10–15 kg and a speed of 0.6 m/s during transportation involving horizontal running and 0.4 m/s during vertical climbing. A balancing device is used to prevent slipping or dumping of cargo due to collision of UniCars with each other or during vertical climbing (Figure S1B,C). The entire RLTS is controlled by a central computer system to allot each transmission task and monitor the whole process (Figure S1D).

UniCar modifications

The results of preliminary experiments showed that UniCars manufactured by Telelift Gmbh Ltd. do not possess the capacity to provide heat-preservation conditions for blood unit storage during transport (Figures S1C and 1c). A specially designed niche made of ethylene-vinylacetate copolymer (EVA) materials was processed and sculpted to suit the UniCar structure and construction, which enabled both thermal insulation and vibration absorption. Furthermore, special ice packs could be placed in the interlayer of the niche in a non-contact manner to provide the red blood cell (RBC) components with a low-temperature environment for a long time during long-distance transport. The structure and internal layout of the niche are shown in Figure 1a,b.

Transaction time

Transaction time is defined as the time between the launch of the car from the blood bank to receipt by the clinical stations that have demand for blood components. The overall transaction time comprises

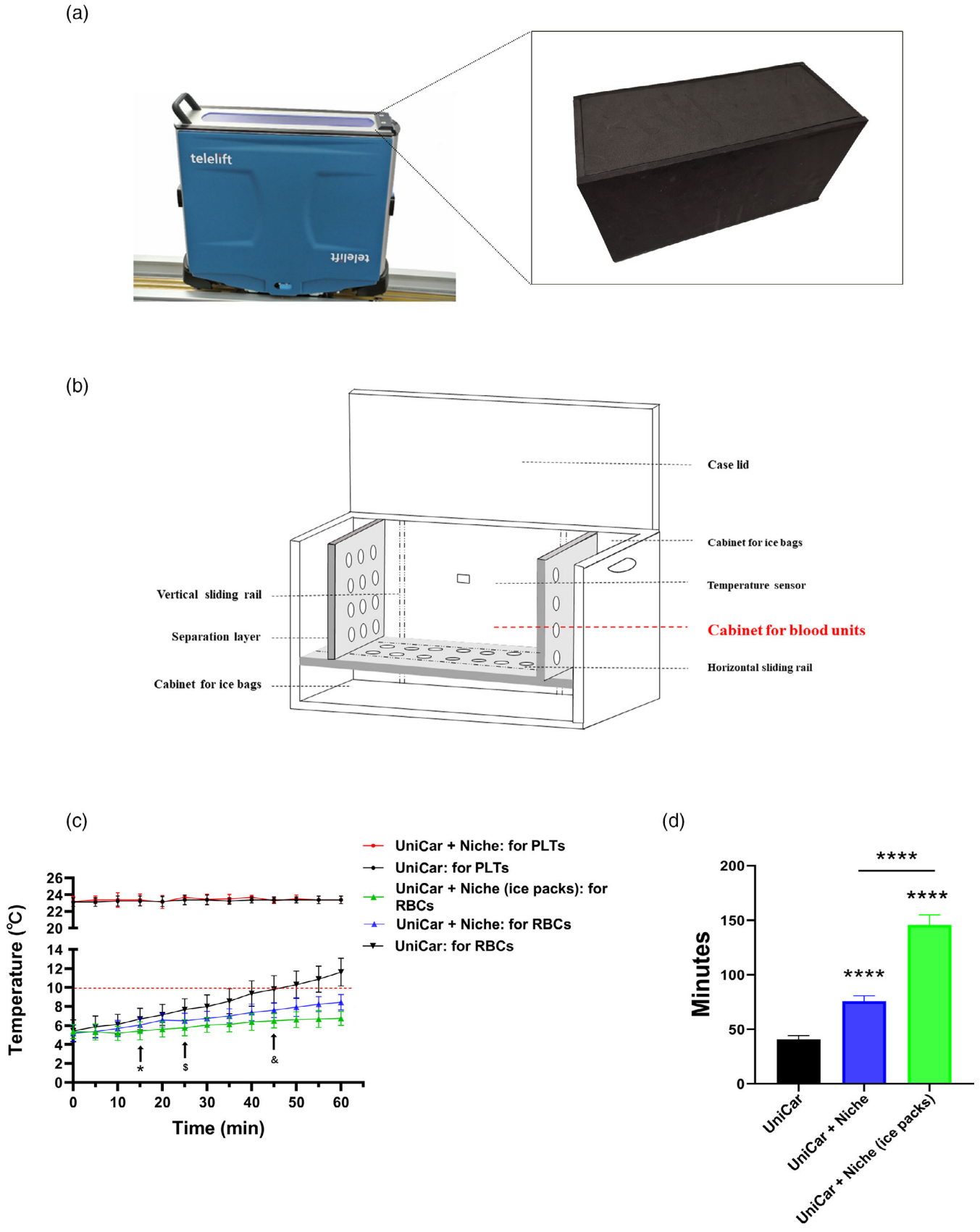

FIGURE 1 Legend on next page.

the time required for blood component loading, transmission and receipt, including delays due to busy and crowded stations.

Transaction temperature

The temperature of blood units was limited to within the range 1–10°C for packed RBCs (pRBCs) and fresh frozen plasma (FFP) and 20–24°C for cryoprecipitate and PLTs during transportation to the destination. An equipment monitoring system (EMS) comprising continuous temperature monitoring devices (Thermochron iButton, DS1925-F5#, Wdsen Electronic) was used to measure and record the temperature deviations of all blood components throughout the whole process. In brief, a series of sensors were placed in the middle of two-bag blood units, forming a 'sandwich'. The temperatures of the blood storage environment during transport were recorded automatically every minute.

Blood components

Sample preparation

The pRBCs, FFP, cryoprecipitate and apheresis PLTs from the Shanghai Blood Center were used as test samples in this study. The FFP and cryoprecipitate units used in this study were stored in a freezer at −30°C for 74.4 ± 11.3 and 99.3 ± 12.0 days, respectively. Then, all samples tested were thawed at 37°C using a special water-bath box for 15 min and kept in the blood refrigerator of the hospital at 2–6°C until use. The apheresis PLTs were stored at 20–24°C with horizontal vibration for an average of 3.3 ± 0.4 days.

Transport via RLTS

The blood units for testing were packed into sealed ziplock plastic bags, and subsequently placed into a dedicated niche as described above (Figure 1a,b). The transaction temperature was measured and recorded continuously by several temperature monitoring devices placed inside the niche to ensure that all blood units are in the acceptable temperature range.

Furthermore, UniCar trolleys loaded with the niches were transported from the blood bank to the destination site at a constant speed, as described above. A 10-mL aliquot each was removed from the blood unit before and after RLTS transportation under sterile conditions.

Physical integrity

A visual assessment was made before and after RLTS transportation to evaluate the physical integrity and leakage/breakage of the blood component bags. A range of indexes involving the results of the visual inspection of the blood components were evaluated.

Parameters used to evaluate the blood components

A range of parameters of blood component quality were evaluated via analytical testing on the specimens before and after RLTS transport. Haemoglobin (Hb), haematocrit (HCT), PLT count and mean PLT volume (MPV) were measured by an automated haematology analyser (XN-9000, Sysmex, Kobe, Japan). To assess haemolysis of the RBCs, a 10-mL aliquot each was removed from the pRBCs unit before and after RLTS transportation. Subsequently, the supernatant was extracted through centrifugation at 3000*g* for 10 min. Later, free Hb in the supernatant of pRBCs was tested using a clinical chemistry analyser (Architect c16000, Abbott, IL). The percentage of haemolysis was calculated based on the following formula: percent haemolysis (%) = [(100 − HCT) × plasma Hb concentration (in g/dL)]/total Hb concentration in the unit (in g/dL). In addition, the levels of K^+ and LDH in the supernatant were measured by a Beckman Coulter AU5800 analyser (Beckman Coulter, Brea, CA). To assess the influence of delivery via RLTS on FFP and cryoprecipitate, PT, prothrombin time-international normalized ratio (PT-INR), APTT, thrombin time (TT), Factor VIII (FVIII) and plasma fibrinogen (FIB) concentration and FVIII activity were assessed by a Sysmex CS 5100 coagulation analyser (Siemens Healthcare Diagnostics, Erlangen, Germany). For PLT units, pH was assessed by a routine blood gas equipment (GEM Premier 3500, Werfen, Lexington, MA).

Regarding PLT isolation and preparation, washed PLTs were obtained by centrifugation of the PLT sample at 280*g* for 5 min and then gently resuspended in Tyrode buffer (134 mM NaCl, 2.9 mM KCl, 340 μM $Na_2HPO_4{\cdot}12H_2O$, 12 mM $NaHCO_3$, 20 mM HEPES, 1 mM $MgCl_2$ and 5 mM glucose, pH 7.35, 37°C) to a suitable concentration for the following assay. Furthermore, the levels of P-selectin (CD62P), CD63 and PAC-1 on the surface of the cell membrane were measured by flow cytometry (Accuri C6, BD Biosciences, CA).

FIGURE 1 UniCar modifications and validation of thermal insulation performance. (a) Exterior profile of designed ethylene-vinylacetate copolymer (EVA) container. (b) Interior structure and lay-outs of the designed EVA container. Thermal insulation performance of the EVA container is evaluated in (c) and (d). (c) The temperature change curve of blood products over time. '*' indicates a significant difference at p 0.05 between the UniCar group and the UniCar + Niche (ice packs) group after 15 min; '$' indicates a significant difference at p <0.05 between the UniCar group and UniCar + Niche group after 25 min; '&' indicates a significant difference at p <0.05 between UniCar + Niche group and UniCar + Niche (ice packs) group after 25 min. (d) Average time required for the temperature of blood products to reach 10°C. Significant differences are denoted with asterisks: ****p < 0.0001; statistical analyses were performed using repeated-measures one-way analysis of variance followed by Dunnett's multiple comparisons test. The number of repeated tests for each group was 10. Two blood units were transported via rail logistics transmission system per dispatch, with a total of 50 blood units (26 packed red blood cells [RBCs] + 24 platelet [PLT]).

Briefly, the PLTs obtained by centrifugation described above were then resuspended in Tyrode's buffer at a concentration of 5×10^8 PLTs/mL. For PLT activation, a final concentration of 0.05 U thrombin as an agonist was incubated with sample aliquots for 10 min at 37°C. Then, the washed PLTs were stained with phycoerythrin (PE)-conjugated CD63 (BioLegend, San Diego, CA) monoclonal antibodies and fluorescein isothiocyanate (FITC)-conjugated CD62P (BioLegend) or APC-conjugated PAC-1 monoclonal antibodies (BioLegend) and protected from light at room temperature for 15 min. The percentages of positive events and the results were quantified by flow cytometry as previously described. PLT aggregation function response was assessed via multiple-electrode aggregometry (MEA) (Multiplate, Roche, Mannheim, Germany) and light transmission aggregometry (LTA) (Model 700, Chrono-Log, Havertown, PA).

Statistical analysis

Statistical analysis was performed with GraphPad Prism 9.0 (GraphPad Software Inc). Continuous variables are presented as the mean and SD or median and interquartile range, according to their distribution assessed by the Shapiro–Wilk test. Two groups were compared using the Student's *t*-test (normally distributed variables) or the Mann–Whitney test (non-normally distributed variables). For comparisons among multiple groups, one- or two-way analysis of variance (ANOVA) was used for data analysis. Results with $p < 0.05$ were considered statistically significant.

RESULTS

RLTS preparations and configurations

The RLTS connects each department of the hospital with transport tracks through launch and collection stations and carries out the transfer of materials between each department through carriage cars (UniCars). In this study, a range of complex transport scenarios were used to simulate the transport of blood components, including short and long distances and horizontal and vertical running (Figure S1A).

Transaction time

The time taken for blood component delivery depends on the distance and spatial layout of the path between the transfusion department and destination, trolley busyness and unexpected system failure. In this study, in non-emergency situations, the median transit time was 323 s (118–668 s). Furthermore, under the circumstances of emergent transfusion requirements, the median transaction time could be shortened to 179 s (169–182 s) from the blood bank to the patient care areas of hospitals, such as the emergency department, intensive care units and operating theatres.

Transaction temperature

Container modifications

In detail, as shown in Figure 1a,b, the structure and layout of this EVA container had the capacity to provide an independent space for a single blood unit (pRBCs or FFP) and ice packs to maintain cooling. Furthermore, several independent and adjustable spaces were fully formed with distinct segmentation, which was desirable to prevent the movement and dumping of blood units during UniCar operation. With this design, the extent of damage to the blood quality, such as haemolysis and PLT activation, was likely to be alleviated due to the prevention of repeated friction and collision between the blood units and container. A series of sensors were placed inside the EVA container to measure and record the internal temperature in real time. Once the temperature exceeds the normal range, an alarm warning and alarm system are activated and any information regarding abnormal conditions is sent to the system managers.

Temperature validation

To assess the feasibility of EVA containers used to load blood components, a performance test on the thermal insulation performance of the containers loaded with blood components was conducted.

For the transport of pRBCs and FFP, the results showed that the temperature of the units tested started to increase rapidly in the UniCar group, unlike the temperature in the UniCar + Niche group or in the UniCar + Niche (ice packs) group. The slowly increasing temperature of the units tested over 60 min was in the UniCar loaded with a niche. Moreover, the ice packs further seemed to enhance the thermal insulation of this special niche, with a mean increase in temperature of 1.4 ± 0.7°C from the beginning of blood unit transport up to 60 min (Figure 1c).

To further test the effect of thermal insulation, the time required to reach 10°C (the upper limit of temperature per regulatory requirements) was recorded. Not surprisingly, the time required for this temperature change was 78.8 ± 4.8 min, which could be further prolonged to up to 145.7 ± 9.2 min in the presence of ice packs. However, the time taken was 44.5 ± 5.1 min in the absence of EVA containers (Figure 1d).

In addition, for cryoprecipitate and PLT units transport, the transaction temperature was stable in the range of 20–24°C during their delivery (Figure 1c).

Quality validation of blood components

To accurately simulate blood component transportation via RLTS used in extreme conditions, a range of complex and rigorous scenarios including long horizontal distances (>500 m) and vertical heights (>60 m) between different buildings were considered. However, the time span of delivery was limited to 20 ± 0.7 min to guarantee good comparability among the different tested groups.

Physical integrity

Based on visual inspection, all blood units consistently maintained their original positions without inverting or shaking violently during RLTS transportation. Furthermore, neither leakage/breakage nor any discernable abnormal changes (such as clots, small aggregates, cloudiness, abnormal colour, protein precipitate or air bubbles) were found in the blood unit bags.

Packed RBCs

A total of 20 pRBC units were clustered into two groups: the 'fresh RBCs' group (stored for <21 days), and the 'old RBCs' group (stored for >21 days), which were randomly chosen for transportation via RLTS. The average age of fresh RBCs was 16.5 ± 0.9 days and that of old RBCs was 28 ± 1.5 days. Two randomly selected pRBC units were tested for each shipment. And, a total of 10 units in each group were transported through five dispatch experiments.

In the fresh RBC group, the levels of HCT, Hb, free Hb, HI and LDH and K+ levels were not significantly different before and after transport (Figure 2). But, the HI was higher in the old RBC group before RLTS transport than in the fresh RBC group (0.130 ± 0.014 vs. 0.063 ± 0.010), regardless of whether they were transported by the RLTS (Figure 2d). However, there was no significant difference between the pre- and post-transport values when transport was performed via RLTS, regardless of the pRBC storage period. Similarly, in addition to the increase in plasma free Hb levels being greater in the old RBC group, the storage of pRBCs caused a significant elevation of K+ and LDH levels (26.6 ± 2.8 vs. 21.5 ± 1.5 and 1517.4 ± 326.1 vs. 847.5 ± 50.4, respectively), but the level of these markers did not continue to increase significantly after RLTS transport (Figure 2e,f).

FFP and cryoprecipitate

Regarding the delivery of FFP, 2 units were transported via RLTS per dispatch, and 10 units were tested (a total of five dispatches). There were no significant differences in the values of routine haematology and coagulation parameters, including PT, PT-INR, APTT, TT and FIB, between the specimens tested before and after RLTS transport (Figure 3a–e).

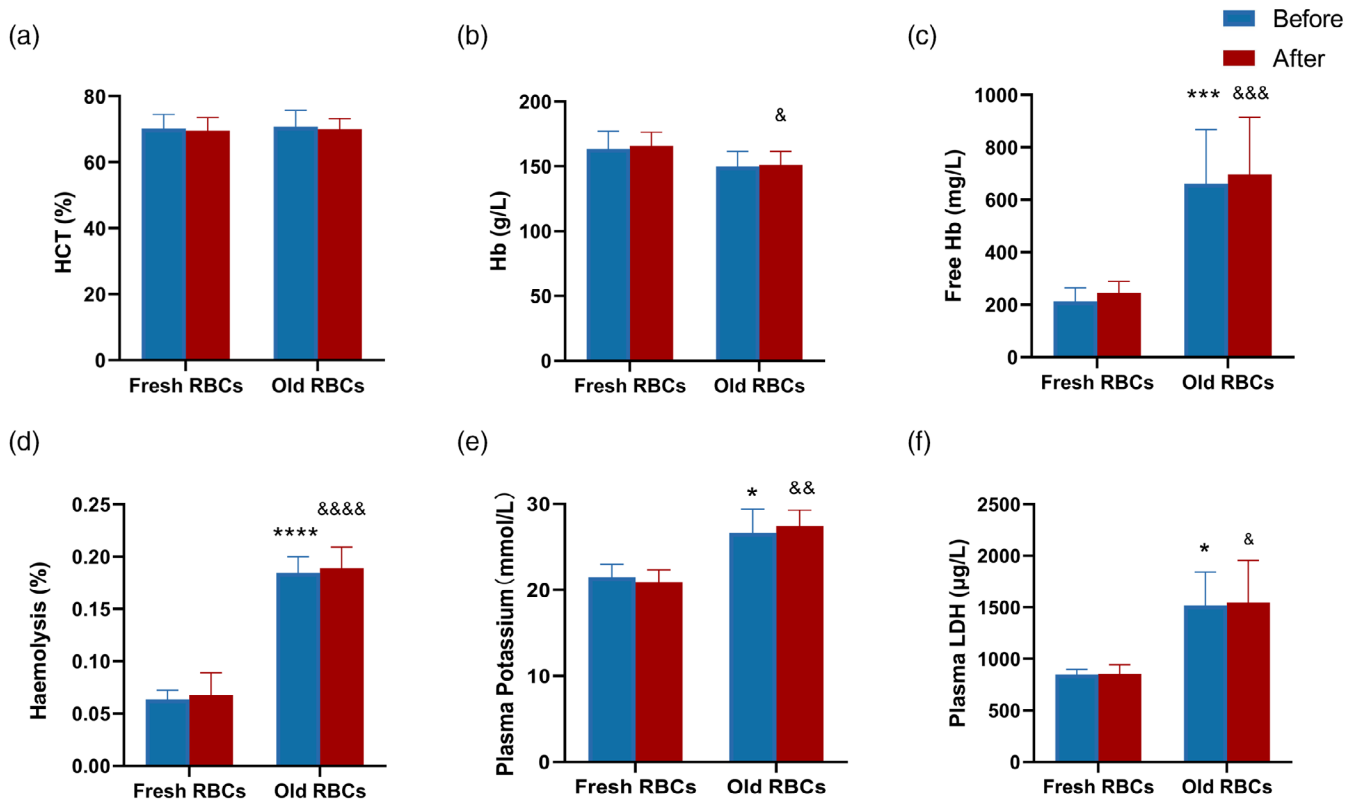

FIGURE 2 Comparison of packed red blood cells (pRBCs) parameters before and after rail logistics transmission system (RLTS) transportation. A series of test parameters of pRBCs before and after RLTS delivery for 'old pRBCs' or 'fresh pRBCs' were examined and calculated, including (a) haematocrit (HCT), (b) haemoglobin (Hb), (c) free Hb, (d) haemolysis ratio, (e) plasma potassium (K^+) and (f) lactic dehydrogenase (LDH). Data are mean ± SEM, $n = 10$ (10 test samples were sourced from the aliquot and removed from the blood unit before and after RLTS transportation). Statistical analyses were performed using repeated-measures one-way analysis of variance followed by Dunnett's multiple comparisons test. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ compared with 'fresh RBCs before RLTS delivery' group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, $^{\&\&\&\&}p < 0.0001$ compared with 'fresh RBCs after RLTS delivery' group.

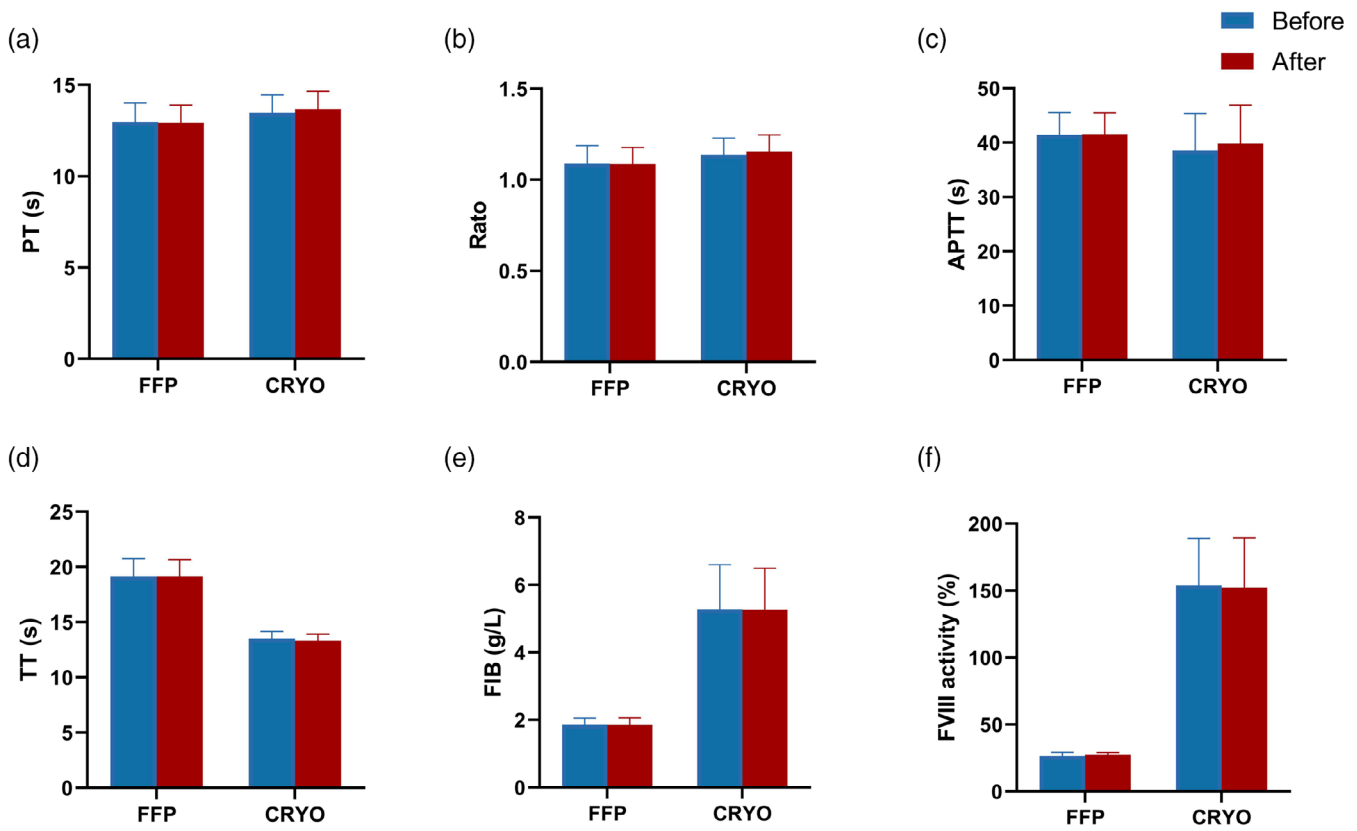

FIGURE 3 Comparison of fresh frozen plasma (FFP) parameters and pre-pooled cryoprecipitate parameters before and after rail logistics transmission system (RLTS) transportation. A series of test parameters of FFP and cryoprecipitate before and after RLTS delivery were examined and calculated, including (a) prothrombin time (PT), (b) prothrombin time-international normalized ratio, (c) activated partial thromboplastin time (APTT), (d) thrombin time (TT), (e) fibrinogen (FIB) and (f) Factor VIII activity (FVIII). Data are presented as mean ± SEM, $n = 10$ (10 test samples were sourced from each aliquot which was removed from the blood unit before and after RLTS transportation).

In addition to the routine coagulation parameters described above, the FVIII activity assay was considered for evaluating the haemostatic properties of FFP and cryoprecipitate in this study. Not surprisingly, no significant impact of RLTS transport was seen on FVIII activity (Figure 3f).

PLT concentrates

Five dispatches of PLT units via RLTS were performed, totalling 10 units of PLT evaluated (2 units per dispatch). A quantitative assessment of metabolism, activation and PLT function in vitro after RLTS transport was performed. The results are shown in Table 1. There was no statistically significant difference in the pH (p =0.093), PLT (p =0.626) count or MPV (p =0.473) between the two groups. The PLT aggregation function response to agonists was assessed using MEA and LTA in this study. However, the data showed no statistically significant differences in percentage maximum aggregation for LTA (adenosine diphosphate [ADP]: $p = 0.425$; U46619: $p = 0.485$) and area under the curve (AUC) values for MEA (ADP: $p = 0.851$; U46619: $p = 0.423$) between the pre- and post-RLTS samples (Table 1). Then, the expression changes in the activation markers CD62P, CD63 and PAC-1 did not differ in samples between the two groups, either during the resting state or after PLT activation stimulated by thrombin (0.05 U) (Table 1).

DISCUSSION

In the case of blood component delivery, replacing traditional hospital porters with automated transportation solutions will become an inevitable trend to accommodate the growing demands for blood and those of an expanding medical centre, characterized by long distribution distance and the presence of more buildings and clinical departments. With the widespread application of RLTS in recent years, since 2018, approximately 80% of the materials in our institution are transported automatically with RLTS systems. To ensure system safety and the maintenance of quality, system validation of RLTS for the transportation of blood components is key. A sequence of different transport scenarios, including horizontal running, vertical climbing and flipping and long distances, was considered and arranged in this study with the aim of sufficiently mimicking the real environment of blood component delivery by RLTS.

Reducing the delivery times of blood components can shorten the wait for a rapid replenishment of blood components and

TABLE 1 Comparison of platelet concentrate parameters before and after rail logistics transmission system (RLTS) transportation.

	Before RLTS[a]		After RLTS[a]		
Parameter	**Mean (SD)**	**Median (IQR)**	**Mean (SD)**	**Median (IQR)**	***p*-Value**
Platelet count ($\times 10^9$/L)	940 (86.1)	946 (73.0)	920 (87.6)	908 (96.0)	0.626[a]
MPV variation (fL)	9.10 (0.15)	9.09 (0.24)	9.07 (0.19)	9.10 (0.21)	0.473[a]
PDW	16.20 (0.39)	16.25 (0.57)	16.21 (0.35)	16.25 (0.52)	0.685[a]
pH variation	7.4 (0.1)	7.4 (0.1)	7.4 (0.1)	7.4 (0.2)	0.093[a]
Activation markers					
%CD62P+ (resting)	5.78 (1.70)	6.0 (3.20)	6.06 (1.71)	6.2 (2.7)	0.732[a]
%CD62P+ (activated)	93.31 (3.12)	92.6 (3.70)	93.20 (2.83)	93.1 (3.01)	0.938[a]
%CD63+ (resting)	5.87 (2.42)	6.9 (4.60)	6.15 (1.86)	6.3 (1.71)	0.787[b]
%CD63+ (activated)	90.95 (5.52)	93.7 (7.30)	91.64 (4.83)	94.0 (7.82)	0.781[b]
%PAC-1+ (resting)	22.57 (8.80)	23.50 (12.97)	22.46 (8.32)	23.10 (10.57)	0.918[b]
%PAC-1+ (activated)	88.32 (3.95)	89.0 (6.10)	89.85 (2.78)	90.1 (1.40)	0.355[a]
MEA					
ADP (AUC)	10.9 (3.6)	9.1 (4.2)	9.7 (3.9)	8.7 (4.8)	0.851[a]
AA (AUC)	9.1 (3.5)	9.6 (4.3)	9.4 (4.3)	9.1 (5.3)	0.423[a]
LTA					
ADP-induced MAR (%)	29.52 (2.29)	30.13 (3.00)	31.25 (2.68)	32.08 (3.11)	0.425[a]
U46619-induced MAR (%)	40.34 (3.11)	40.75 (4.23)	39.51 (3.14)	41.50 (4.22)	0.485[a]

Note: $N = 10$. For multiple-electrode aggregometry (MEA) or light transmission aggregometry (LTA), the following agonists were tested: adenosine diphosphate (ADP) (10 μM), U46619 (0.5 μM), arachidonic acid (AA) (0.5 mM). Platelet activation stimulated by thrombin (0.05 U) for flow cytometry analysis.

Abbreviations: AUC, area under the curve; IQR, interquartile range; MAR, maximum aggregation rate; MPV, mean platelet volume; PAC-1, platelet activation complex-1; PDW, platelet distribution width.

[a]Paired Student's *t*-test.

[b]Wilcoxon signed-rank test.

correction of coagulation dysfunction and haemostatic disorders. In this study, a range of additional measures were taken, including the use of priority dispatch, preferential pass and arriving stations, to minimize the delivery time by setting the UniCar logistics system depending on the specific clinical demand. In addition to transaction time, another concern is the temperature of the blood units during transport by the RLTS. Accordingly, maintaining the acceptable temperature range needed to meet the storage demands of different blood components during transport is an important prerequisite to ensure blood quality. Therefore, a series of improvements were applied to UniCars in this study, including the use of thermal insulation devices and ice packs. In addition, many sensors were provided on the interior surface of the niches, which were used in real-time monitoring and recording of the temperature of the blood components. In addition to maintain the temperature, the materials that the niche is made of can have specific strengths, including the ability to absorb vibrations and prevent leakage, and great plasticity to enable adaptation to different containers. Overall, these improvement initiatives provide a guarantee for physical integrity, quality and biosecurity during blood component transportation.

Prior to the formal clinical application of blood component transportation by RLTS, caution should still be exercised. Although there were numerous strengths, we still focused on whether RLTS would have a negative effect on blood quality. Previously, PTS are widely used to transport various clinical specimens (blood, urine, cerebrospinal fluid, etc.) to diagnostic laboratories, including blood components. Thus, several factors that have adverse impacts on blood quality, for example, rapid accelerations, vibrations and formation of air bubbles during the course of PTS, had to be considered in this study.

Past investigations suggest that rapid accelerations (multiples of gravitational force) in PTS are significantly positively correlated with the degree of haemolysis [18]. Mullins et al. [19] demonstrated that a longer duration of forceful acceleration tended to increase the HI and plasma LDH in blood samples after they were transported by PTS routes. Thus, in 2021, the 'Updated AABB Guide to Pneumatic Tube Delivery System Validation' focused on blood component quality by addressing 'vibration that can cause haemolysis, which is detrimental to integrity'. Promisingly, our results in the present study showed that RLTS transport, with features of low velocity and smooth operation, seems to result in no bubbles in blood bags and has limited effect on the haematological parameters of pRBCs (Figure 2 and Table S1), such as Hb levels, HCT, HI and LDH and K^+ levels in plasma. As is well known, pRBC samples stored in blood bank conditions undergo a progressive series of physical and biochemical alterations known as RBC storage lesions [20, 21]. There is no doubt that a variety of factors, for example, vibrations, pressure and rapid accelerations,

collectively exacerbate the aggravation of the structure, cell viability and microcirculatory flow of aged RBCs [22]. Our results showed that aged RBCs transported by RLTS do not exhibit a significant increase in the HI or LDH and K^+ levels in plasma (Figure 2).

Earlier studies have suggested that PTS used for the delivery of patient samples were likely to affect the results of routine haematology and coagulation tests. Le Quellec et al. [16] found a statistically significant shortening of the APTT and an increase in D-dimer levels after blood sample transport by the PTS. In addition, Slavík et al. [18] showed that, despite no obvious effect on basic coagulation test results, PTS transportation with a speed of 6 m/s, to some extent, resulted in a statistically significant difference in the use of the thrombin generation test (TGT) as a sensitive monitoring method. However, our results showed that for the delivery of FFP or cryoprecipitate, there was no significant difference in the value of routine haematology and coagulation parameters, including PT, PT-INR, APTT, TT and FIB, between the specimens tested before and after RLTS transport. In addition, several labile coagulation factors, such as FVIII and FV, have half-lives that are sensitive to PTS transportation conditions [23]. In contrast, our results showed no significant impact of RLTS transport on FVIII activity results.

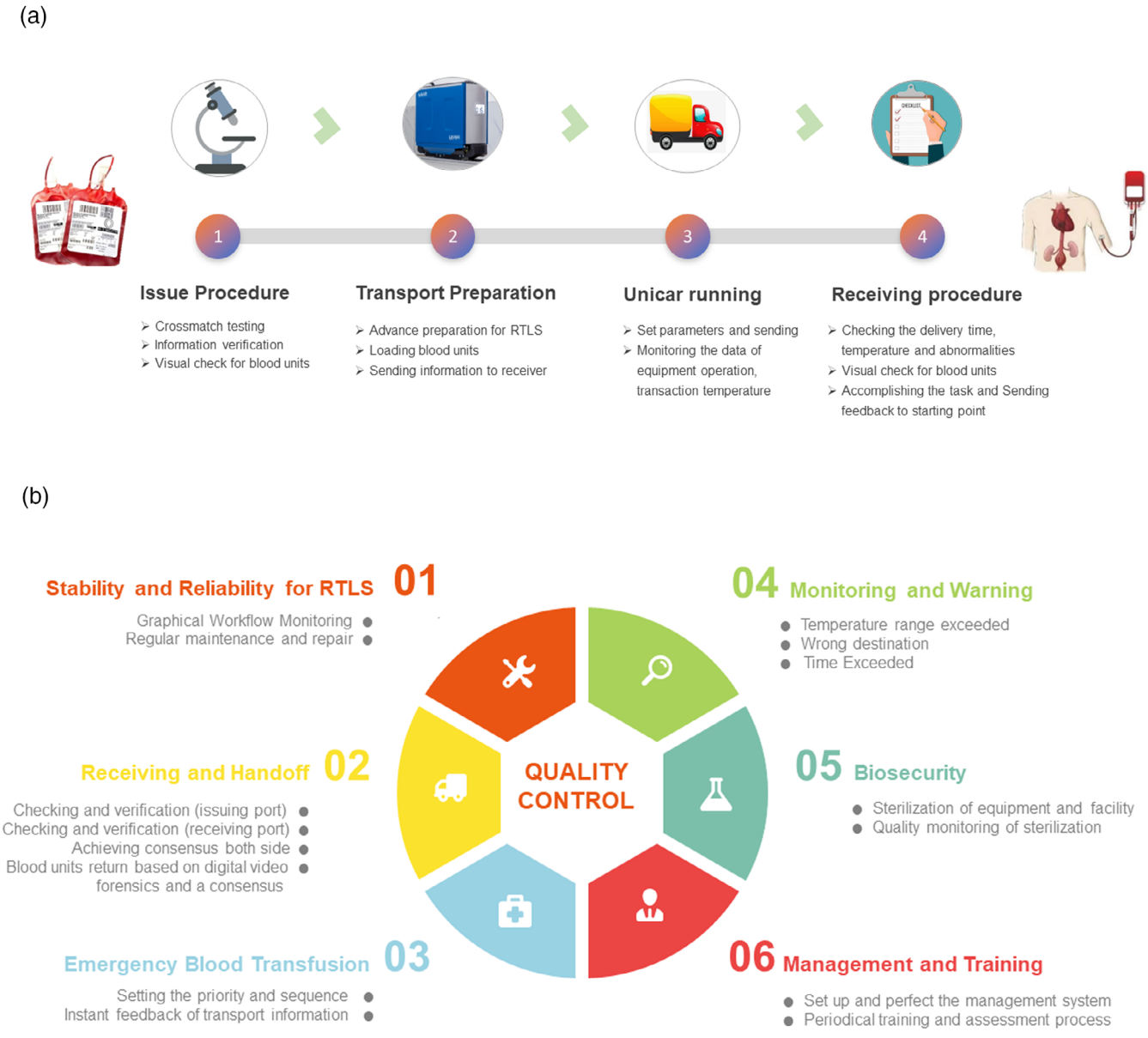

FIGURE 4 Schematic of workflow and quality control procedures for considering rail logistics transmission system (RLTS) for blood component distribution. (a) Flow diagram shows the node times for blood component transportation via the RLTS, which consist of several parts: issue procedure, transport preparation, UniCar running and receiving procedure. (b) The quality control approach for blood component transportation via RLTS consists of six steps: (1) stability and reliability of RLTS; (2) receiving and handing over; (3) emergency blood transfusion; (4) monitoring and warning; (5) biosecurity; (6) management and training.

As the smallest tangible blood cells in peripheral blood, PLTs in vitro show a set of morphological changes and functional responses when exposed to environmental conditions such as shear stress, temperature and pressure [24]. Similarly, PTS used to transport samples had a negative influence on the test results of PLT function assays, including LTA [25, 26], MEA [15, 27, 28] and thrombelastogram [29]. However, our flow cytometry data showed that there was no statistically significant difference in activation marker expression within the PLT population resulting from RLTS transport and non-RLTS transport. Additionally, RLTS transport had no impact on the results of PLT aggregation assessment via LTA or MEA.

As a preliminary validation, we carried out a feasibility study of blood component distribution by RLTS that primarily focused on time consumption, temperature maintenance and blood quality. The parameters of tested samples from all blood units after delivery via RLTS met the requirements specified by the criteria that were used (Table S1). As early as 2005, to ensure product safety and the maintenance of quality, the American Association of Blood Banks (AABB) released guidelines for validating PTS for blood components to provide direction when pneumatic tube technology can be considered for blood component distribution. Thus, based on the knowledge gained through this study, we attempted to formulate a workflow for transporting blood components and then formulated preliminary criteria for quality control that addressed the stability and reliability of RLTS, receiving and handing over of samples, emergency blood transfusion, monitoring and warning, biosecurity and management and training (Figure 4).

There are several limitations associated with this study. As a preliminary validation, the first limitation is the limited number of tested samples. The experimental sample size will be further increased in our following implementation study. In addition to the blood products tested, whole blood, washed or irradiated RBCs will be considered in follow-up studies. Additionally, only a small number of blood products of a single type were transport by RLTS during each test. For massively bleeding patients, massive transfusion protocols (MTPs) will be promptly activated in response to emergency blood request. RLTS transportation of all blood component types in a single travel, including RBC, plasma, cryoprecipitate and PLT units, will require further study. Because of the occurrence of public health emergencies and the need for prompt emergency treatment, efficacy, safety and feasibility of blood units transported via RLTS remain to be assessed. Altogether, there is still much to assess before RLTS can be routinely used in medical centres.

In conclusion, our study preliminarily verified the feasibility of RLTS for blood component distribution. Although PTS are regarded as a promising candidate, some deteriorative impacts on blood quality transport by PTS must be revisited. In contrast to many PTS, RLTS transport does not have a significant influence on the quality of different blood components, according to our results. To our knowledge, no published study has yet validated RLTS transport as a method for blood component distribution. Thus, the results of this study are expected to provide assistance and suggestions for developing guidelines to validate and use RLTS to transport blood components.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

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

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Design and practice of an emergency blood allocation system based on radiofrequency identification technology

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Abstract

Background and Objectives: Currently, blood allocation is solely done by scanning barcode labels for each bag of blood, with low efficiency. However, the rapid allocation of emergency blood is required owing to the rapid increase in blood consumption during unconventional emergencies. This study aimed to design and apply radiofrequency identification (RFID) technology for the rapid allocation of blood in batches with advantages in time, efficiency and accuracy.

Materials and Methods: A blood emergency allocation system based on RFID technology was designed using a multi-label anti-collision algorithm and tested with automatic information check, a comparative study of scanning speed and accuracy, data analysis and other methods.

Results: The optimal packing quantities of suspended red blood cells and fresh frozen plasma were 40 and 50 bags per box, respectively. The application of rapid batch allocation of blood using RFID technology was performed, and the data sent and received by RFID scanning and barcode scanning were compared.

Conclusion: The designed RFID blood emergency allocation system could effectively achieve the rapid and batch allocation of emergency blood and has the advantages of stability, efficiency and accuracy in blood emergency allocation and management.

Keywords

design, emergency blood allocation, practice, radiofrequency identification technology

Highlights

- Currently, blood allocation is solely done by scanning barcode labels, which in situations of emergency with high demand can be unacceptably slow.
- A blood emergency allocation system based on radiofrequency identification (RFID) technology was designed.
- The RFID blood emergency allocation system could effectively achieve rapid and batch allocation of blood.

INTRODUCTION

Radiofrequency identification (RFID) is a non-contact, automatic identification technology. It uses radiofrequency to carry out non-contact two-way communication to exchange data for identification purposes. It can simultaneously identify multiple target objects and is fast and convenient to operate [1]. During World War II, RFID technology was first used by the British Royal Air Force to identify domestic aircraft [2]. Blood collection and supply management began in Germany in 2003, when RFID technology was used to track blood products [3]. In 2009, the Blood Center of

Wisconsin in the United States adopted RFID to replace barcodes for the management of blood product libraries. After a comprehensive evaluation, they found that RFID could help overcome many common challenges in the identification and tracking of blood products, such as process inefficiency [4]. The International Society for Blood Transfusion Working Party on Information Technology established a Task Force on RFID in 2006 to study the status quo of RFID development and drafted guidelines for the medical application of RFID in blood transfusions [2].

The continuous development of RFID technology in the field of blood management has effectively improved blood quality and management. The Suzhou Blood Center introduced RFID technology to achieve automatic identification, batch handover and accurate positioning of blood in blood establishments, which led to the development of the Jiangsu provincial local standard 'DB32/T 4413-2022 Specifications for the radiofrequency identification (RFID) technology application of qualified blood in blood establishment' [5]. It greatly improved the levels of intelligent management of blood inventory. At present, the application of RFID technology in blood management is mostly for security monitoring from blood collection to distribution in blood stations; however, the technology has not been applied to blood allocation management in different institutions and regions. Blood allocation is still limited to the method of scanning barcode labels for each bag of blood, which has low efficiency. However, during some emergencies, blood consumption increases sharply over a short period. Hospitals or blood stations must allocate blood urgently, and methods have been proposed for the rapid allocation of emergency blood. On 27 October 2021, 'WS/T 789-2021 Label and identification codes for blood products' [6] was issued by the National Health Commission of China to unify domestic blood coding standards and facilitate cross-regional allocation of blood.

Presently, no relevant application design or practice exists for using RFID technology to improve the efficiency and ability of rapid blood batch allocation. Therefore, we propose an RFID-based blood emergency allocation system to allow blood information sharing and rapid allocation, which has advantages in terms of time, efficiency and accuracy and provides a new mode for allowing emergency blood supply and guarantee.

MATERIALS AND METHODS

Materials

Our study looks at the research and design of an emergency blood allocation system based on RFID technology for rapid blood allocation. This includes research on multi-label anti-collision algorithm, optimal number of blood transport boxes, automatic information checks and other difficult problems.

Methods

Rapid writing of RFID chip blood information

The radiofrequency printer was equipped with a radio frequency writing function. In the process of printing finished blood labels, information such as the blood donation code and product code is written into the electronic product code (EPC) area of the chip in accordance with the 'WS/T 789-2021 Standard for Label and Identification Code of Blood Products'. When the read–write device scans the chip, the information in the chip is obtained by analysing the EPC, and complete blood information is searched for and matched in the information system for verification.

Use of ultra-high frequency RFID tags

Ultra-high frequency (UHF) (820–960 MHz) RFID tags have the largest reading range and speed with strong environmental adaptability and anti-interference ability and are not affected by ultra-low temperature and deformation, and thus are very suitable for blood management. Studies have shown that the use of UHF RFID tags does not affect the preservation quality of red blood cells, platelets or frozen plasma [7–9]. To this end, we followed the ISO18000-6C and ISBT128 standards and used a UHF RFID blood bag tag [2, 10, 11].

RFID system multi-label anti-collision algorithm

We propose a multi-tag anti-collision algorithm for an UHF RFID identification system. Based on the time-slot ALOHA algorithm in an RFID system, this algorithm makes full use of the information collected by the read–write device in the previous frame, retains the information of the frame before tagging as prior information, estimates the number of tags based on the H function and dynamically adjusts the frame length. This algorithm is more accurate in estimating the number of labels, improving the throughput of the system and accelerating the recognition speed of the labels (ALOHA algorithm is a kind of random access algorithm; when the tag wants to send data information, it can randomly send information at any time, and then the communication between the tag and the read–write device begins). In addition, the algorithm solves the problem of the ALOHA algorithm in which the number of tags increases too much or the required time slots grow slowly for a large number of tags. Based on the hash function as the allocation principle, the time slot allocation of labels is performed to reduce the number of labels in a collision. Based on the relationship between the average value and proportion of collision time slots and proportion of idle time slots in the collision time slots, the error rate generated by the estimation method was small [10, 12]. The frame length time slot number can be set as 1.6 times the label, and label recognition rate can be controlled at 500 μs. The average recognition time for each tag is approximately 600 μs. Therefore, the prediction algorithm is based on continuous, free time slots; that is, the hash function algorithm is simple, uniform and has a constructed library that can accelerate the skipping of invalid time slots. Under large-scale tags, it can maintain a high throughput and is stable at 63%. This multi-label anti-collision algorithm has significant advantages in improving the algorithm throughput, transmission cost and delay [13].

Automatic information check

Rapid blood allocation was performed using the RFID blood allocation system. Based on emergency blood allocation requirements, the information system generates blood allocation tasks, the blood sender system generates blood outbound information and the blood receiver system generates blood inbound information. The read–write device of the blood sending side quickly scans the outgoing blood in batches and automatically checks the outgoing information in the RFID blood allocation system. After the check is found correct, blood is collected and transported. After the blood reaches the receiving side, the read–write device quickly scans the blood in batches and confirms that the blood information is in one-to-one correspondence with the stored information in the allocation system to automatically check the blood information. This process is illustrated in Figure 1.

In the traditional blood allocation process, scanning the blood barcode bag by bag and manually checking the label information of the blood bag with receipt information are necessary. The process (shown in detail in Figure 2) is more time consuming, laborious and prone to errors. The RFID blood rapid allocation system can achieve rapid batch scanning of blood allocation out and into the warehouse; it no longer relies on manual blood bag scanning and information checks, and thereby not only reduces the allocation workload and allocation time but also greatly improves the efficiency and accuracy of blood allocation.

Comparative study on the scanning speed and accuracy of RFID and barcode

The suspended red blood cells and fresh frozen plasma were placed in batches of 20, 40 and 60 bags/20, 40 and 50 bags, respectively, in the blood transport box. The RFID read–write device was placed 10 cm above the blood, and the required time and readings were recorded

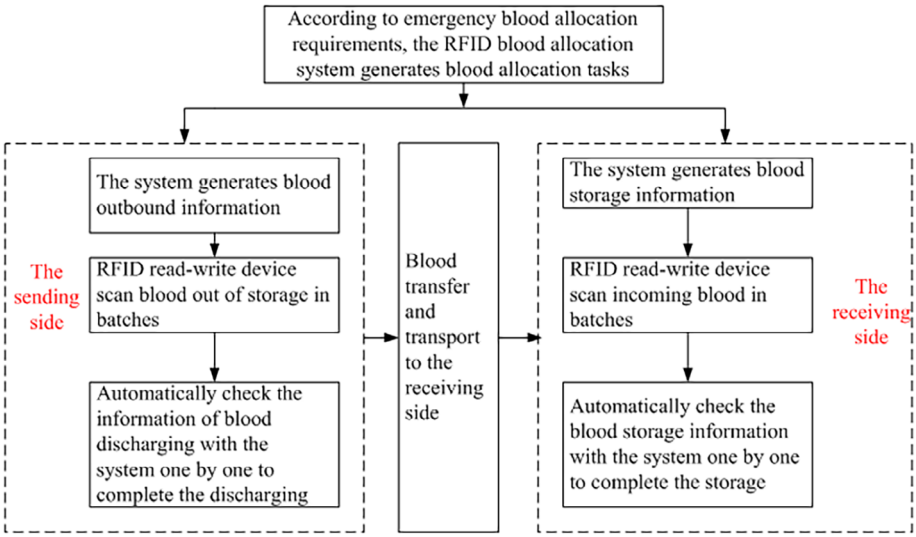

FIGURE 1 Allocation and information checking process of radiofrequency identification (RFID) blood emergency allocation system.

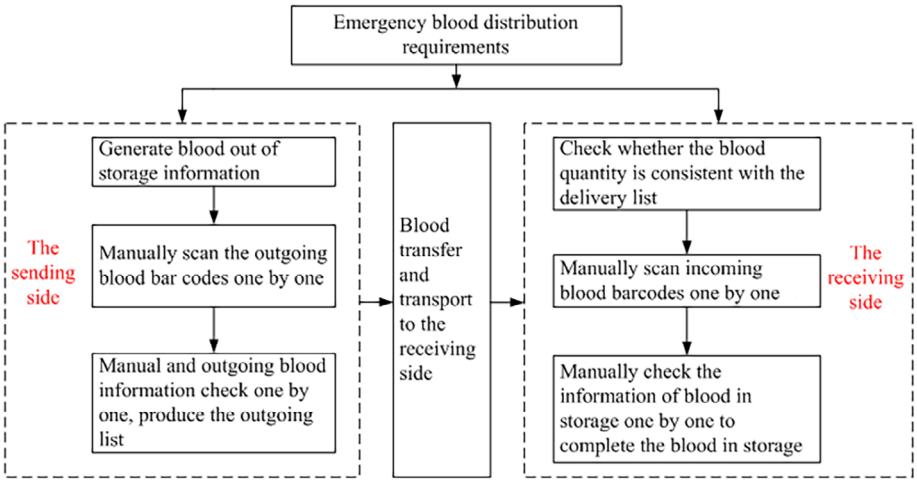

FIGURE 2 Barcode blood allocation and information checking process.

(the amount of blood component prepared by separating from 200 mL whole blood is 1 unit. In this paper, one bag of suspended red blood cells is 1 unit, and one bag of fresh frozen plasma is 1 unit) [5]. Each group was tested 10 times to determine the optimal packing quantity. RFID and bar code were used to scan at a distance of 10 cm above the box containing 40 bags of red blood cells or 50 bags of fresh frozen plasma, and the scanning accuracy and time were compared 10 times.

Influence of transportation on the speed and accuracy of RFID scanning

We compared the accuracy and time of RFID scanning before and after transportation, which was about 26 km.

Statistical analysis

SPSS 26 software was used for statistical analysis. The measurement data were expressed as mean ± SD ($x \pm s$), and the t-test was used for comparison between groups. $p \leq 0.05$ was considered statistically significant.

RESULTS

Determining the optimal packing quantity

A multi-label anti-collision algorithm was used to determine the optimal amount of blood per box for the RFID system. The suspended red blood cells were placed in 20, 40 and 60 bags per box based on the blood placement rules and scanned in sequence from top to bottom, left to right. The scanning accuracy of 20 bags per box was 100%, 40 bags per box was 100% and 60 bags per box was 98.33%. Therefore, the optimal packing quantity of the suspended erythrocytes was determined to be 40 bags per box.

Fresh frozen plasma was placed in 20, 40 and 50 bags per box based on the blood placement rules and scanned in sequence from top to bottom, left to right. The scanning accuracy of 20 bags per box was 100%, 40 bags per box was 100% and 50 bags per box was 100%, as shown in Table 1. Therefore, the optimal packing quantity of the fresh frozen plasma was determined to be 50 bags per box.

TABLE 1 Packing unit number and scanning accuracy (radiofrequency identification).

Variety	Units/box	Scanning accuracy
Red blood cells	20	100%
	40	100%
	60	98.33%
Fresh frozen plasma	20	100%
	40	100%
	50	100%

Note: Referring to the box size, the length is 565*cm × 335*cm × 275*cm. One unit of suspended red blood cells is one bag, and 1 unit of fresh frozen plasma is one bag.

Application of RFID technology for rapid batch blood allocation

To ensure efficient blood supply, emergency blood allocation is often required in Suzhou City and its districts. The Wu Jiang Branch Station has adopted the same RFID technology as the Suzhou Blood Center for blood stock management and blood delivery. The Suzhou Blood Centre and Wu Jiang Branch Station are approximately 26 km apart. Blood transportation is in accordance with 'WS/T 400-2012 Blood Transportation Requirements' [14]. (Blood components for transporting red blood cells should be maintained at 2–10°C. Transport of frozen plasma should be done under frozen conditions). Five boxes (200 bags) containing suspended red blood cells and six boxes (300 bags) containing freshly frozen plasma were transported. On the ChangTai Expressway, the average speed was 43.33 km/h, taking an average of 36 min. Before and after blood delivery, RFID scanning/barcode scanning was conducted at the Suzhou Blood Centre and Wu Jiang Branch Station, respectively, for sending and receiving data, and the data were compared [15], as shown in Tables 2–4.

DISCUSSION

Blood is an important medical resource necessary to support medical emergencies. In emergency planning and mass casualty events, blood consumption increases in a short period, and hospitals may not have a large quantity of stored of blood. Local blood collection and supply institutions are often unable to meet emergency blood supply demands for accident rescue, and they also need to transfer blood from other locations. Therefore, rapid trans-regional blood allocation is an important part of the blood emergency guarantee. A randomized controlled trial involving massive transfusion (MT) at 12 Level-I trauma centres showed that, independent of the product ratios, every 'minute' delay from the time of MT protocol activation to the time of initial cooler arrival increased the odds of mortality by 5% [16]. A recent SHOT report described 133 cases of blood transfusion delays that could have resulted in death, including logistical issues [17]. During the explosion accident at Kunshan Factory in 2014, 1.61 million millilitres of frozen plasma and 847 units of red blood cells were transferred to Suzhou Blood Centre from blood banks in the outer regions during 2–10 August, and the highest number of frozen plasma units transferred in a day was 12,700 (about 1.27 million millilitres) [18]. Rapid distribution of blood is essential for treating patients in hospitals. Routine blood collection and supply work uses barcodes for bag-by-bag blood storage and distribution, which involves a large workload and has poor efficiency and cannot meet the requirements of rapid batch blood storage and distribution.

TABLE 2 Comparison of scanning data of suspended red blood cells and fresh frozen plasma.

Variety	Units/box	Number of scans	Barcode scanning(s)	RFID scanning(s)
Red blood cells	40	10	182.30 ± 29.22	3.70 ± 0.675
Fresh frozen plasma	50	10	338.00 ± 26.73	2.00 ± 0.00

Note: Compared with barcode group, $p < 0.05$. One unit of suspended red blood cells is one bag, and 1 unit of fresh frozen plasma is one bag.
Abbreviation: RFID, radiofrequency identification.

TABLE 3 Comparison of scanning data of suspended red blood cells and fresh frozen plasma after rapid storage after transportation (approximately 26 km: Wu Jiang branch station).

Variety	Units/box	Number of scans	Barcode scanning(s)	RFID scanning(s)
Red blood cells	40	10	181.90 ± 13.09	3.70 ± 0.95
Fresh frozen plasma	50	10	340.80 ± 21.31	2.00 ± 0.00

Note: Compared with barcode group, $p < 0.05$. One unit of suspended red blood cells is one bag, and 1 unit of fresh frozen plasma is one bag.
Abbreviation: RFID, radiofrequency identification.

TABLE 4 Comparison of outgoing and incoming scanning before and after transportation of radiofrequency identification (RFID) group.

Variety	Units/box	Number of scans	Barcode scanning(s)	RFID scanning(s)
Red blood cells	40	10	100%	100%
Fresh frozen plasma	50	10	100%	100%

Note: One unit of suspended red blood cells is one bag, and 1 unit of fresh frozen plasma is one bag. 100% means all red blood cells packed in 40 units/box were detected and all fresh frozen plasma packed into 50 units/box were detected.

Therefore, rapid batch blood storage and distribution is a key and challenging problem in emergency blood supply for blood collection and supply institutions.

RFID technology is superior to the traditional barcode recognition in several aspects. RFID does not require visualization, allows multiple labels to be read simultaneously, stores more information on the chip, includes status monitoring sensors (such as for time and temperature) and supports automatic identification and data acquisition [4]. Therefore, RFID may be used for blood transfusion service [19]. Transfusion medicine is in the early stages of adopting RFID technology to support blood collection, processing, labelling, inventory and distribution of blood products [2]. We designed and applied RFID technology to a blood emergency allocation system. Research has shown that the blood emergency allocation system using RFID technology plays a significant role in the speeding up of emergency blood transfers. The application of RFID technology to the rapid allocation of blood indeed reflects that RFID tags store blood information efficiently, conveniently and easily, and can be quickly and accurately docked with the information system. Research has shown that RFID tags of different frequencies have different performances in actual operations. In April 2010, ISBT published guidelines for the use of RFID technology in transfusion medicine, recommending ISO/IEC18000-3 Mode 1 high frequency (HF) 13.56 MHz RFID tags, taking into account international standardization and harmonization issues [2]. However, in terms of read and write times, distance, number of targets, environmental adaptability and anti-interference ability, UHF (820–960 MHz) RFID tags are better than low-frequency and HF RFID labels, more suitable for applications in the management of blood and also the direction for future development [7]. This study shows that UHF RFID tags perform well in the rapid allocation of emergency blood in the case of transportation and various environmental interferences, and their accuracy and recognition speed are very good.

The UHF RFID tag itself has some limitations and restrictive factors, such as follows: the RFID system communication broadband cannot be expanded infinitely; the characteristics of message transmission are limited; labels do not have a carrier monitoring function; and labels cannot communicate with each other. In addition, in the practical application of RFID, electronic tags are in a mobile dynamic working environment, and new tags may enter or exceed the scope of reading and writing at any time during communication. Multiple tags communicate with the read–write device through competition. Therefore, a collision occurs, and the anti-collision algorithm completes the smooth communication of the labels. To this end, we designed the multi-tag anti-collision algorithm in the UHF RFID identification system, and proposed an anti-collision algorithm based on hash function, thereby overcoming the collision caused by the communication between multiple tags and read–write devices in the dynamic working environment of UHF RFID tags. This study shows that this algorithm can indeed complete the smooth communication of tags and can well be applied to the environment of rapid allocation of emergency blood.

Finally, the determination of the optimal packing quantity was carried out. Scanning accuracy is affected by the penetration of the RFID read–write device and the number of blood bags inside the blood box [5]. In the experiment, 40 bags of suspended red blood cells and 50 bags of fresh frozen plasma were loaded into cardboard boxes and placed in an open environment. The read–write device was placed within 10 cm above the blood sample,

which guaranteed 100% accuracy during scanning. After transportation, the label did not appear damaged or fall off, the time of scanning record in and out of storage did not change compared with before departure and the chip performance remained stable. The RFID blood emergency allocation system can be used for rapid exit and batch entry and automatic information comparison, which not only saves time and effort but also has higher accuracy compared with the traditional exit and manual one-by-one checking, thus effectively realizing the rapid batch allocation of emergency blood.

Life is precious, and in an emergency every minute counts. Therefore, shortening the time spent in the whole chain of blood transfusion is necessary. As proved through this study, the RFID blood emergency allocation system can safely, stably, accurately and efficiently meet the requirements of rapid blood allocation inside and outside a warehouse, which has unique advantages. Of course, there are some limitations in this study: the sample size was limited, and the blood components transported were limited to red blood cells and plasma. In future research, the size and other blood components will be further expanded. With the continuous development and improvement in RFID technology, an RFID blood emergency allocation system will realize a wider application range, higher application efficiency and better application effects.

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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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Polymorphisms in the promoter regions of *RHD* and *RHCE* genes in the Chinese Han population

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Abstract

Background and Objectives: The Rh blood group system is the most polymorphic human blood group system. Previous studies have investigated variants in the *RHD* and *RHCE* promoter. The relevance of these variants to the Chinese Han population is further clarified in this study.

Materials and Methods: In total, 317 donors (223 Rh D-positive [D+], including 20 Del and 94 Rh D-negative [D−]) were randomly selected. The promoter regions and exon 1 of *RHD* and *RHCE* were amplified through polymerase chain reaction (PCR) whose products were directly sequenced using forward and reverse primers.

Results: Expected PCR products of the *RHD* promoter and exon 1 were amplified in 223 D+ individuals, including 20 Del individuals, and were absent in 81 of 94 D− individuals. Expected PCR products of *RHCE* were observed in all donors. Two single nucleotide variants (SNVs) were observed in the *RHD* promoter region. Moreover, 11 SNVs were observed in the promoter and exon 1 of *RHCE*. rs4649082, rs2375313, rs2281179, rs2072933, rs2072932, rs2072931 and rs586178 with strong linkage disequilibria were significantly different between the D+ and D− groups. [A;C] was the most common haplotype in the *RHD* promoter (NC_000001.11:g.[−1033A>G;−831C>T]). [G;T;T;A;T;A;C;G;A;C;G] was the most predominant haplotype in both total and D− groups. In D+ individuals, [A;C;T;G;C;G;C;G;C;C;C] was the most frequent haplotype in the *RHCE* promoter (NC_000001.11:g.[−1080A>G;−958C>T;−390T>C;−378G>A;−369C>T;−296G>A;−144C>G;−132G>A;−122C>A;28C>T;48C>G]).

Conclusion: We speculate that the SNVs/haplotypes found in this article cannot significantly affect gene expression. The present study findings should help elucidate the molecular basis of the polymorphic expression of *RHD* and *RHCE* promoter regions.

Keywords

gene, polymorphism, promoter, *RHCE*, *RHD*

Lin-Nan Shao and Zi-Wei Zheng contributed equally to this work.

Highlights

- This is the first study investigating the polymorphisms in the *RHD* and *RHCE* promoter regions by using the sequencing method in Chinese Han population.
- rs4649082, rs2375313, rs2281179, rs2072933, rs2072932, rs2072931 and rs586178 with strong linkage disequilibria were significantly different between the D+ and D− groups.
- The present study findings should help elucidate the molecular basis of the polymorphic expression of *RHD* and *RHCE* promoter regions.

INTRODUCTION

The Rh blood group system is the most polymorphic human blood group system with RhD and RhCE polypeptides encoded by the genes *RHD* and *RHCE* [1, 2]. These two genes are approximately 30 kb apart, have opposite orientations and exhibit 93.8% homology over all introns and coding exons [3]. Depending on the CcEe phenotype, RhD and RhCE proteins differ by 32–35 amino acids [4]. Individuals are classified as Rh D-positive (D+) and Rh D-negative (D–) according to the presence or absence of the D antigen on the red blood cell surface. The incidence of the D+ phenotype is ~85% in Caucasians, ~95% in sub-Saharan Africans and >99.5% in eastern Asians [5]. Furthermore, the Rh system plays a pivotal role in the field of blood transfusion and severe haemolytic disease of fetus/newborn because of its high polymorphism and strong immunogenicity [6, 7]. Numerous *RHD* and *RHCE* variants derived from hybrid genes or nucleotide polymorphisms exist [8, 9]. These variants are categorized as partial and/or weak antigens [10] and present challenges to blood transfusion service. Knowledge about *RH* variants in blood donors and patients is necessary to make blood transfusion safer [11, 12].

A promoter is a sequence located upstream of the 5′-flanking region of a gene. It is a significant player in the highly efficient expression and regulation of target genes at the transcriptional level. Promoter variants affect gene expression and regulation. GATA motifs, which are binding sites for the erythroid-specific transcription factor GATA-1, have been shown to play a role in gene expression [13]. In the Duffy blood group system, a mutation in the GATA-1 binding site of the Duffy gene's promoter region results in the Duffy-null phenotype that prevents gene transcription in erythroid cells [14]. Most studies have investigated the exon and intron regions of *RHD* and *RHCE* but relatively few studies exist on their promoters. Fennell et al. found that variants in the GATA-1 binding site at 5′ UTR-115C of *RHD* and 5′ UTR-83T of *RHCE* showed a significant reduction in *RH* gene expression [13]. Denomme et al. [15] investigated the 1246 bp 5′-flanking regions of the *RHD* and *RHCE* promoter, and found that the proximal *cis*-regulatory region of the *RHD*/*RHCE* promoter is 105 bp with binding motifs for Sp1, GATA-1 and E2F transcription factors and a 55-bp core devoid of known binding motifs. The aim of this study was to characterize the polymorphisms of the ~1.3 kb 5′-flanking regions of the *RHD* and *RHCE* promoters in the Chinese Han population to evaluate their impact on gene expression and to deliver more systematic population-specific data.

MATERIALS AND METHODS

Patients and samples

The study participants were restricted to healthy, unrelated Chinese Han blood donors. In total, 317 donors were randomly selected. All participants provided informed consent for sequence analysis. All procedures were performed in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national). The Declaration of Helsinki approval for this study was granted by the local ethics committee.

Serological tests

We performed D phenotyping by using standard monoclonal antisera (Shanghai Hemo-Pharmaceutical & Biological Co., Ltd.) and read the tests for agglutination in tubes. Moreover, samples were typed as D− for the D-elute (Del) phenotype through an adsorption–elution test in tubes. If the result was positive, the sample was determined to belong to the Del phenotype; otherwise, it was determined to be a truly D− phenotype.

DNA extraction

Genomic DNA was extracted from proteinase K-treated peripheral whole blood samples by using a commercially available DNA isolation kit on a MagCore automated nucleic acid extractor (RBCBioscience, Taipei, Taiwan, China) according to the manufacturer's instructions.

Genotyping

The promoter regions and exon 1 of *RHD* and *RHCE* genes were amplified through polymerase chain reaction (PCR). *RHD*- and *RHCE*-specific forward primers used were 5′-TCCACTTTCCACCTCCCTGC-3′ and 5′-TCCACCTTCCACTTCCCTGT-3′, respectively. The reverse

TABLE 1 Genotype/allele frequencies in the promoter and exon 1 of *RHD* and *RHCE*.

Gene	SNV	Genotype or allele	Total		Rh D+		Rh D−		p^a
			N (%)	p^b	*N* (%)	p^b	*N* (%)	p^b	
RHD[c]	rs191568964	−1033 AA	232 (98.3)	<0.05	219 (98.2)	<0.001	13 (100.0)	NA	0.888
		−1033 AG	3 (1.3)		3 (1.3)		0		
		−1033 GG	1 (0.4)		1 (0.4)		0		
		−1033 A	467 (98.9)		441 (98.9)		26 (100.0)		1.000
		−1033 G	5 (1.1)		5 (1.1)		0		
RHD[c]	rs557082901	−831 CC	229 (97.0)	<0.05	216 (96.9)	<0.001	13 (100.0)	NA	0.810
		−831 CT	6 (2.5)		6 (2.7)		0		
		−831 TT	1 (0.4)		1 (0.4)		0		
		−831 C	464 (98.3)		438 (98.2)		26 (100.0)		1.000
		−831 T	8 (1.7)		8 (1.8)		0		
RHCE	rs4649082	−1080 AA	105 (33.1)	<0.05	98 (43.9)	0.743	7 (7.4)	0.126	<0.05
		−1080 AG	125 (39.4)		98 (43.9)		27 (27.8)		
		−1080 GG	87 (27.4)		27 (12.1)		60 (63.8)		
		−1080 A	335 (52.8)		294 (65.9)		41 (21.8)		<0.05
		−1080 G	299 (47.1)		152 (34.1)		147 (78.2)		
RHCE	rs2375313	−958 CC	105 (33.1)	<0.05	98 (43.9)	0.743	7 (7.4)	0.126	<0.05
		−958 CT	125 (39.4)		98 (43.9)		27 (27.8)		
		−958 TT	87 (27.4)		27 (12.1)		60 (63.8)		
		−958 C	335 (52.8)		294 (65.9)		41 (21.8)		<0.05
		−958 T	299 (47.1)		152 (34.1)		147 (78.2)		
RHCE	rs591570	−390 TT	316 (99.6)	0.978	222 (99.6)	0.973	94 (100.0)	NA	1.000
		−390 CT	1 (0.3)		1 (0.4)		0		
		−390 CC	0		0		0		
		−390 T	633 (99.8)		445 (99.8)		188 (100.0)		1.000
		−390 C	1 (0.1)		1 (0.2)		0		
RHCE	rs2281179	−378 GG	105 (33.1)	<0.05	98 (43.9)	0.743	7 (7.4)	0.126	<0.05
		−378 AG	125 (39.4)		98 (43.9)		27 (27.8)		
		−378 AA	87 (27.4)		27 (12.1)		60 (63.8)		
		−378 G	335 (52.8)		294 (65.9)		41 (21.8)		<0.05
		−378 A	299 (47.1)		152 (34.1)		147 (78.2)		
RHCE	rs2072933	−369 CC	105 (33.1)	<0.05	98 (43.9)	0.743	7 (7.4)	0.126	<0.05
		−369 CT	125 (39.4)		98 (43.9)		27 (27.8)		
		−369 TT	87 (27.4)		27 (12.1)		60 (63.8)		
		−369 C	335 (52.8)		294 (65.9)		41 (21.8)		<0.05
		−369 T	299 (47.1)		152 (34.1)		147 (78.2)		
RHCE	rs2072932	−296 GG	105 (33.1)	<0.05	98 (43.9)	0.743	7 (7.4)	0.126	<0.05
		−296 AG	125 (39.4)		98 (43.9)		27 (27.8)		
		−296 AA	87 (27.4)		27 (12.1)		60 (63.8)		
		−296 G	335 (52.8)		294 (65.9)		41 (21.8)		<0.05
		−296 A	299 (47.1)		152 (34.1)		147 (78.2)		
RHCE	rs201048836	−144 CC	275 (86.7)	<0.05	193 (86.5)	0.082	82 (87.2)	<0.05	0.457
		−144 CG	36 (11.3)		27 (12.1)		9 (9.6)		
		−144 GG	6 (1.8)		3 (1.3)		3 (3.2)		
		−144 C	586 (92.4)		413 (92.6)		173 (92.0)		0.870
		−144 G	48 (7.5)		33 (7.4)		15 (8.0)		

TABLE 1 (Continued)

Gene	SNV	Genotype or allele	Total		Rh D+		Rh D−		p^a
			N (%)	p^b	*N* (%)	p^b	*N* (%)	p^b	
RHCE	rs771559205	−132 GG	312 (98.4)	0.887	218 (97.8)	0.866	94 (100.0)	NA	0.327
		−132 AG	5 (1.5)		5 (2.2)		0		
		−132 AA	0		0		0		
		−132 G	629 (99.2)		441 (98.9)		188 (100.0)		0.329
		−132 A	5 (0.7)		5 (1.1)		0		
RHCE	rs2072931	−122 CC	106 (33.4)	<0.05	99 (44.4)	0.816	7 (7.4)	0.126	<0.05
		−122 AC	125 (39.4)		98 (43.9)		27 (28.7)		
		−122 AA	86 (27.1)		26 (11.7)		60 (63.8)		
		−122 C	337 (53.1)		296 (66.4)		41 (21.8)		<0.05
		−122 A	297 (46.8)		150 (33.6)		147 (78.2)		
RHCE	rs749601047	+28 CC	316 (99.6)	0.978	222 (99.6)	0.973	94 (100.0)	NA	1.000
		+28 CT	1 (0.3)		1 (0.4)		0		
		+28 TT	0		0		0		
		+28 C	633 (99.8)		445 (99.8)		188 (100.0)		1.000
		+28 T	1 (0.1)		1 (0.2)		0		
RHCE	rs586178	+48 CC	105 (33.1)	<0.05	98 (43.9)	0.602	7 (7.4)	0.126	<0.05
		+48 CG	124 (39.1)		97 (43.5)		27 (28.7)		
		+48 GG	88 (27.7)		28 (12.6)		60 (63.8)		
		+48 C	334 (52.6)		293 (65.7)		41 (21.8)		<0.05
		+48G	300 (47.3)		153 (34.3)		147 (78.2)		

[a]Between Rh D+ and Rh D− groups.
[b]*p*-values are of Hardy–Weinberg equilibrium.
[c]Expected PCR products of the *RHD* promoter and exon 1 were amplified in 223 D+, including 20 Del individuals, and were absent in 81 of 94 Rh D− individuals.

primers for *RHD* and *RHCE* were the same, that is, 5′-CTTGATAG-GATGCCACGAGCC-3′. The final reaction volume of 25 μL PCR mixtures included 1 μL of purified genomic DNA (ca. 50–200 ng), 1 μL of forward and reverse primers (20 μM), 12.5 μL of AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA), 2 μL of 360 GC Enhancer and 7.5 μL of distilled water. The PCR procedure was as follows: 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 63°C for 30 s and 72°C for 2 min, and a final extension step at 72°C for 7 min. The amplification products were 1286 and 1290 bp for *RHD* and *RHCE*, respectively. These products were analysed through electrophoresis on ethidium bromide-stained 1% agarose gel. Samples with negative results needed to be reconfirmed with an internal control (*GAPDH* gene). The PCR products were directly sequenced using the forward and reverse primers in an ABI 3730 XL DNA sequencer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's manual. The sequencing results were analysed using DNAstar software.

Statistical analysis

Allelic, genotypic and haplotypic data were analysed using the χ^2 test or Fisher's exact test. The relative risk estimate was evaluated based on the odds ratio at the 95% confidence interval. The Hardy–Weinberg equilibrium (HWE) test for genotypic data was conducted using the χ^2 test. When several dependent or independent statistical tests were performed simultaneously on a single dataset, Bonferroni-corrected *p*-value (*pc*) adjustment was made to the *p* values. Statistical analyses were performed using SPSS version 21.0. Haplotype constructions were generated from the observed genotypes by using SHEsis Online Version [16, 17]. Standardized linkage disequilibrium values (r^2) were measured using Haploview 4.2 (Broad Institute, Cambridge, MA, USA).

RESULTS

Gene polymorphisms

Expected PCR products of the *RHD* promoter and exon 1 were amplified in 223 D+, including 20 Del individuals, and were absent in 81 of 94 D− individuals. Expected PCR products of *RHCE* were observed in all 317 donors. The genotype distributions and the genotype/allele frequencies of the single nucleotide variants (SNVs) in the promoter region and exon 1 of *RHD* and *RHCE* (ignoring hemizygotes) are presented in Table 1. In the *RHD* promoter region, two SNVs,

TABLE 2 Haplotype frequencies in the promoter and exon 1 of *RHD* and *RHCE* genes.

Gene	Haplotypes	*N* (%) Rh D+	*N* (%) Rh D−	*N* (%) Del	OR (95% CI)	*p*[a]	*pc*
RHD	[A;C]	433 (97.1)	26.00 (100.0)	40.00 (100.0)	0.971 (0.955–0.987)	1.000	
	[A;T]	8.00 (1.8)	0	0	0.982 (0.970–0.994)	1.000	
	[G;C]	5.00 (1.1)	0	0	0.989 (0.979–0.999)	1.000	
RHCE	[G;T;T;A;T;A;C;G;A;C;G]	149.03 (33.4)	147.00 (78.2)	15.00 (37.5)	0.140 (0.094–0.208)	<0.001	<0.001
	[A;C;T;G;C;G;C;G;C;C;C]	261.85 (58.7)	26.00 (13.8)	24.00 (60.0)	8.872 (5.629–13.984)	<0.001	<0.001
	[A;C;T;G;C;G;G;G;C;C;C]	29.12 (6.5)	15.00 (8.0)	1.00 (2.5)	0.802 (0.420–1.533)	0.498	
	[G;T;T;A;T;A;G;A;C;C;G]	2.00 (0.4)	0	0	0.996 (0.989–1.002)	1.000	
	[A;C;T;G;C;G;C;A;C;C;C]	1.13 (0.3)	0	0	0.998 (0.993–1.002)	1.000	
	[A;C;C;G;C;G;G;A;C;C;G]	1.00 (0.2)	0	0	0.998 (0.993–1.002)	1.000	
	[A;C;T;G;C;G;G;A;C;C;C]	0.87 (0.2)	0	0	0.998 (0.993–1.002)	1.000	
	[A;C;T;G;C;G;C;G;C;T;C]	0.03 (0.0)	0	0	1.000 (1.000–1.000)	1.000	
	[G;T;T;A;T;A;C;G;A;T;G]	0.97 (0.2)	0	0	0.998 (0.993–1.002)	1.000	

Note: Haplotypes in *RHD* and *RHCE* genes represent NC_000001.11:g.[−1033A>G;−831C>T] and NC_000001.11:g.[−1080A>G;−958C>T;−390T>C;−378G>A;−369C>T;−296G>A;−144C>G;−132G>A;−122C>A;28C>T;48C>G] (e.g., following SNVs for rs191568964, rs557082901 and rs4649082, rs2375313, rs591570, rs2281179, rs2072933, rs2072932, rs201048836, rs771559205, rs2072931, rs749601047, rs586178, respectively).

Abbreviations: CI, confidence interval; *pc*, Bonferroni-corrected *p*-value; OR, odds ratio.

[a]Between Rh D+ and Rh D− groups.

namely rs191568964:A>G and rs557082901:C>T, were observed in four and seven donors, respectively. Moreover, 11 SNVs were observed in the promoter and exon 1 of *RHCE*. The variant rs749601047 in *RHCE*, that is, c.28C>T (p.Arg10Trp), was found in a 28-year-old female with the phenotype CcEe. To our knowledge, it is found in Asia for the first time. Figure S1 shows the sequencing results. Some SNVs, namely rs191568964, rs557082901, rs4649082:A>G, rs2375313:C>T, rs2281179:G>A, rs2072933:C>T, rs2072932:G>A, rs201048836:C>G, rs2072931:C>A and rs586178:C>G, were not in HWE. Nevertheless, rs4649082, rs2375313, rs2281179, rs2072933, rs2072932, rs2072931 and rs586178 were in accordance with HWE after all the donors were divided into D+ and D− groups. In addition, the frequencies of these seven SNVs were significantly different between the D+ and D− groups (p <0.001).

Distribution of haplotypes

Table 2 presents the haplotypes of *RHD* and *RHCE* (ignoring hemizygotes). Three haplotypes (NC_000001.11:g.[−1033A>G;−831C>T]) were found in the *RHD* promoter. [A;C] was found to be the most common haplotype in all three groups. Ten haplotypes (NC_000001.11:g.[−1080A>G;−958C>T;−390T>C;−378G>A;−369C>T;−296G>A;−144C>G;−132G>A;−122C>A;28C>T;48C>G]) were found in the *RHCE* promoter. [G;T;T;A;T;A;C;G;A;C;G] was the most dominant haplotype in D− groups (78.2%); however, [A;C;T;G;C;G;C;G;C;C;C] was the most frequent haplotype in both D+ and Del individuals (58.7% and 60.0%, respectively). The distributions of these two haplotypes were significantly different between the D+ and D− groups (pc <0.001).

Linkage disequilibrium

In the *RHD* promoter, linkage disequilibrium (LD) with a pairwise r^2 of 0 was observed between rs191568964 and rs557082901 for the D+, D− and total groups. The LD results for the promoter and exon 1 regions of *RHCE* are presented in Figure 1. Strong LD with a pairwise r^2 of at least 0.98 was observed among rs4649082, rs2375313, rs2281179, rs2072933, rs2072932, rs2072931 and rs586178.

DISCUSSION

RHD and *RHCE* molecular polymorphisms have been observed in exons and introns among various populations [18–22] but knowledge about promoter region polymorphisms is limited. Promoters can be positively or negatively regulated by various transcription factors and other transcription-specific protein effectors. Studying the promoter region is important for analysing the transcription factor binding motifs and gene expression mechanisms, providing basic data for future research. Here we investigated promoter polymorphisms in *RHD* and *RHCE* in the Chinese Han population.

Among the 94 D− individuals, 81 individuals lacked exon 1 of *RHD*, while promoter deletion was also observed. This result is in agreement with previous studies reporting that more than 70% of the D− phenotypes in the Chinese Han population are explained by the entire *RHD* deletion mechanism [23, 24], but confirmatory genotyping would need to be performed. The remaining 13 individuals exhibited D− phenotypes, but exon 1 and promoter regions were not missing. This indicated the presence of other D− mechanisms in these individuals.

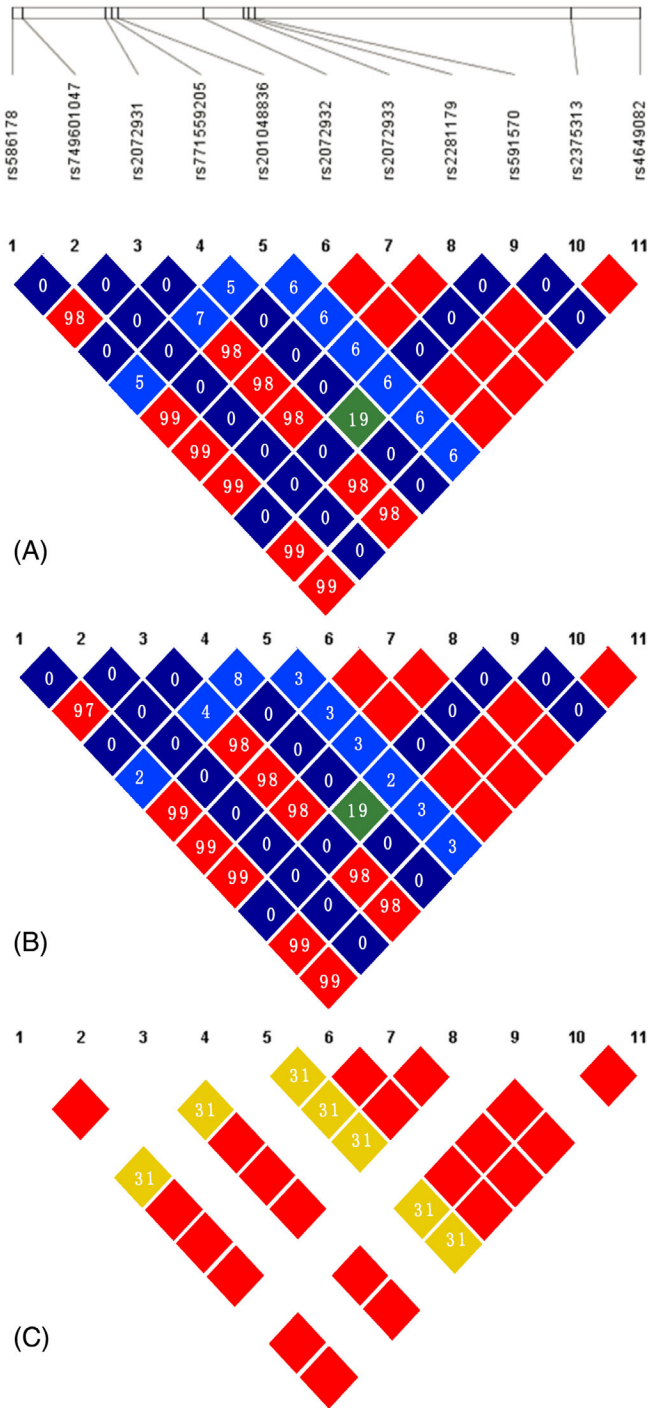

FIGURE 1 Linkage disequilibrium (LD) analysis of 11 SNVs in *RHCE* promoter and exon 1 regions. The figure was generated using Haploview 4.2 software. The numbers in the squares (0–99) refer to the pairwise LD measured as r^2. The red squares without numbers represent complete LD. Eleven SNVs include rs586178, rs749601047, rs2072931, rs771559205, rs201048836, rs2072932, rs2072933, rs2281179, rs591570, rs2375313 and rs4649082. (a) LD analysis of the *RHCE* promoter and exon 1 regions of the total group. (b) LD analysis of the *RHCE* promoter and exon 1 regions of the D+ group. (c) LD analysis of the *RHCE* promoter and exon 1 regions of the D− group.

All D+ individuals including 20 Del individuals possessed *RHD* promoter regions. Our data indicated no essential difference in the *RHD* promoter region between the Del and D+ individuals. We found nine and two SNVs in the *RHCE* and *RHD* promoter regions, respectively. Moreover, the wild-type genotypic frequencies of the two SNVs were >97%. Although *RHD* is formed by a duplication of *RHCE*, These two genes are 93.8% identical to each other [10, 25]. According to our results, the *RHD* promoter seems to be more conservative than the *RHCE* promoter.

Of note, in the *RHD* promoter region, [A;C] was the most common haplotype found in the D+ and D− groups. It means that this haplotype has no association with D antigen expression. In the *RHCE* promoter region, the allelic distributions of rs4649082, rs2375313, rs2281179, rs2072933, rs2072932 and rs2072931 were inverted and significantly different between the D+ and D− groups. Moreover, these six SNVs were in accordance with HWE in both D+ and D− groups but exhibited bias in the total group. This is because the proportion of D− individuals we selected (29.65%) far exceeded the actual number in the Chinese Han population (ca. 0.3%). Haplotype analysis can clarify how alleles are organized along the chromosome and can potentially capture *cis* interactions between two or more causal variants [26]. In the haplotype analysis, the aforementioned six SNVs exhibited very strong LD with $r^2 > 0.98$. An interesting study by Denomme et al. [15] found that there was no statistical difference in promoter activity between *RHD* and *RHCE* from −1163 to promoter region 3′ and that there was no significant positive or negative effect on the promoter activity from −1163 to −138. Moreover, it is noteworthy that the proximal *cis*-regulatory region of the *RHD/RHCE* promoter is 105 bp. The SNVs/haplotypes we found in the promoters of the Chinese Han population are not in this key region, so we speculate that these SNVs/haplotypes cannot significantly affect gene expression. In the Chinese population, C antigen is predominant in D+ individuals and c antigen is most common in D− individuals [22, 27–29]. Moreover, despite the presence of false-positive results, the polymorphism of rs586178 is closely related to Rh C/c [30, 31]. The haplotypes [A;C;T;G;C;G;C;C;C] and [G;T;T;A;T;A;C;G;A;C;G] (containing rs586178 and with strong LD) have significant differences in D+ and D− groups, which may be due to these two haplotypes being actually related to Rh C or c.

On sequencing the *RHCE* promoter region of 30 Japanese individuals, Tanaka et al. [32] found only three nucleoside substitutions at positions −292, −283 and −210 (corresponding to rs2281179, rs2072933 and rs2072932, respectively), which were associated with the C/c phenotype. By contrast, our data revealed that in the Chinese Han population, one more SNV, rs2072931, located closer to *RHCE* exon 1 had strong LD with these three SNVs. In addition, our study has the advantages of having more participants, a longer sequencing length of the promoter region and discovering more SNVs. The limitation of this study is the small sample size of the donors, especially for D− and Del individuals. Therefore, further studies enrolling a larger sample size are warranted.

Compared with more comprehensive studies on the exon and intron regions of *RHD* and *RHCE*, relatively few studies have investigated their promoter regions. To the best of our knowledge, this is the first study investigating the polymorphisms in the *RHD* and *RHCE* promoter regions by using the sequencing method in Chinese Han population. We found that the *RHD* promoter was more conservative than the *RHCE* promoter. Moreover, in the *RHCE* promoter region, the allelic distributions of rs4649082, rs2375313, rs2281179, rs2072933, rs2072932 and rs2072931, which exhibited very strong or even complete LD, were significantly different between the D+ and D− groups. We speculate that these SNVs/haplotypes found in this study cannot significantly affect gene expression. Taken together, the present study findings should be beneficial in elucidating the molecular basis of the polymorphic expression of Rh antigens.

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Z.S.H. and L.X.H. conceptualized the study; S.L.N. and Z.Z.W. investigated the data; Z.S.T. and S.W.Q. analysed the data; X.Y.X. provided resources; S.L.N. wrote the draft.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict 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

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Blood donor genotyping for prediction of blood group antigens: Results from 5 years' experience (2017–2022)

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Abstract

Background and Objectives: For 5 years, routine genotyping has been performed for selected blood groups of blood donors in the Copenhagen Capital Region, Denmark. The result is summarized in the following.

Materials and Methods: Genotyping was carried out by an external service provider using the competitive allele specific PCR (KASP) technology. The genotypes were returned to the blood bank and translated into phenotypes by a proprietary IT application.

Results: In total, 65 alleles from 16 blood group systems (ABO, MNS, Rh, Lutheran, Kell, Duffy, Kidd, Diego, Yt, Dombrock, Colton, Landsteiner-Wiener, Cromer, Knops, Vel, secretor status) and the HPA1, HPA5 and HPA15 antigens were interrogated. After translation, phenotypes were imported into the laboratory information management system of the blood bank. The results from 31,538 genotyped blood donors were used to calculate the allele frequencies for a Danish blood donor population. *ABO* genotyping was done for sample ID purposes. Determination of the 1061delC single nucleotide polymorphism (SNP) (NM_020469.2), most frequently characteristic of *ABO*A2*, was validated against a series of 1287 samples with *Dolichos biflorus* lectin determination of the A1 phenotype.

Conclusion: We report allele frequencies and phenotype frequencies for 16 blood groups from a total of 31,538 genotyped blood donors. Blood products were supplied from a total of 64,312 active blood donors, and of these active blood donors 25,396 (39.5%) were genotyped. These donors represent a valuable resource for patient treatment. This genotyping has enabled the provision of rare genotyped donor blood for patients with alloantibodies and rare reagent cells for serology.

Keywords

blood donor, genotyping, transfusion

Highlights

- Determination of blood groups from blood donors is performed by classical serotyping or by genotyping.
- We describe here a collection of 31,538 genotyped blood donors for 15 blood groups.
- The genotyping system has proved very satisfactory, cost-efficient and reliable, with a reproducibility of over 99%.

INTRODUCTION

Blood transfusion is an established life-saving treatment. Currently, 45 different blood groups are recognized by the International Society of Blood Transfusion (ISBT) [1]. In routine settings, usually, only groups ABO and RhD are considered. When a recipient has irregular blood group antibodies, it is essential to select donor blood that is antigen-negative for the corresponding antibodies in cases where antigen incompatibility is known to potentially cause adverse effects. If prevention of the development of alloantibodies is desired, choosing the appropriate antigen-negative blood products is important. This is particularly crucial if the patient is expected to receive multiple blood transfusions.

Determination of blood groups from blood donors can be achieved in several ways. The two predominant methods are classical serotyping, which requires that antibody reagents are obtainable, or by genotyping, which requires that the molecular genetic basis of the blood group is known. Several genotyping systems have been described, and some are commercially available [2–8].

In healthcare systems, there is an incentive to continuously optimize the use of resources.

We, therefore, sought more cost-efficient ways of acquiring blood donor typing information than possible with traditional serotyping using current instrumentation. This prompted us to design a cost-effective modular genotyping system where additional blood alleles could be quickly entered into the current selection if so desired.

We designed a proprietary screening system of blood donor genotyping for selected blood groups replacing the previous serotyping except for ABO and RhD. The system was validated and described earlier [9]. Genotyping was performed by an external service provider and thus did not require investments in instrumentation or workforce.

It is rare in the health sciences that technological progress replace old methods and at the same time give even more information at lower costs. But the reported blood donor genotyping system is one example that does both.

Before issuing blood for transfusion to a patient, a confirmatory serotyping is performed in cases of known alloantibodies in the recipient. Thus, an erroneous genotype can be detected, and a transfusion reaction in the recipient is avoided. For dispensing antigen-negative blood for the prevention of immunization, however, one has to solely rely upon the genotyping result.

This paper aims to assess the results of a multi-year blood donor genotyping effort and reports the huge resource these data represent.

MATERIALS AND METHODS

The data in the present study were generated from 35,305 blood donor samples collected from 31,538 individuals at the Department of Clinical Immunology, Copenhagen University Hospital, Copenhagen, Denmark, from 2017 to 2022.

The blood donor genotyping system was described earlier [9]. Briefly, blood was obtained from voluntary blood donors according to routine procedures; within 2 weeks of storage at 4°C, 200 µL blood from each donor was pipetted in a single well of a resealable deep-well microtitre plate compatible with robotic handling by the genotyping robots. Dedicated software and a robot (NEO Immucor analyser) were used for pipetting. The software is designed to take 100 µL from both the top and bottom parts of a tube to ensure that the haematocrit (the proportion of red blood cells to the total blood volume) reflects the authentic value. Re-suspending the blood in all tubes is thus not needed. Microtitre plates are produced as needed, and optimal use of resources is ensured by waiting until sufficient number of samples is available to fill a full plate. The microtitre plates are heat-sealed and stored at −20°C until shipped on dry ice to the contract research organization (CRO) for genotyping in batches of eight plates (Figure 1). The genotyping is performed in batches of eight deep-well microtitre plates, and in one plate in each batch six different known donor blood samples are pipetted as a batch control of the clinically most important genotypes. These control donor samples are kept frozen in aliquots of 2 mL. Also, two wells in each microtitre plate are left empty for technical controls. One batch of eight microtitre plates thus has 746 wells available for samples.

A sample is taken for genotyping from a blood donor the third time the donor donates blood, indicating the intention of the donor to return for future donations. Blood donors are not intentionally genotyped more than once unless more than four ‘no calls’ are returned, in which case the donor is flagged for one extra genotype attempt at the next donation.

As the genotyping data get accumulated, the genotypes are entered into a compiled spreadsheet that enables direct searching among the genotypes. This spreadsheet is maintained to enable direct searches among genotypes, and the data quality is protected in several ways. Our laboratory management information system (LIMS) allows the storage of genotypes but does not give access to direct searches based on genotypes. All data presented in this paper were extracted from this compiled dataset and from existing phenotyping data in the LIMS.

Upon receipt of the genotypes from the CRO, they are translated into virtual phenotypes by a proprietary, validated translation table of the canonical blood groups using Labware. Subsequently, the predicted phenotypes are imported into LIMS to become available for search for donors with a given desired phenotype. A number of quality control (QC) steps are included to continuously monitor the reliability of the genotyping data and address safety concerns [9].

The ABO*A1 phenotyping was done by *Dolichos biflorus* according to standard procedures. In this report, we give the rs numbers of the variants interrogated and the cumulative number of the different alleles typed, as well as some internal discrepancies and ‘no calls’ of the different assays. We do not in this report attempt to resolve any inconsistencies by sequencing or other methods.

To evaluate reproducibility, we sifted through the data and found 7213 samples encompassing donors who had been tested more than once. Some of these repeat samples were due to our >4 ‘no calls’ rule, which required a sample to be repeated once if >4 ‘no calls’ were found, but most were due to inadvertently repeating a genotype.

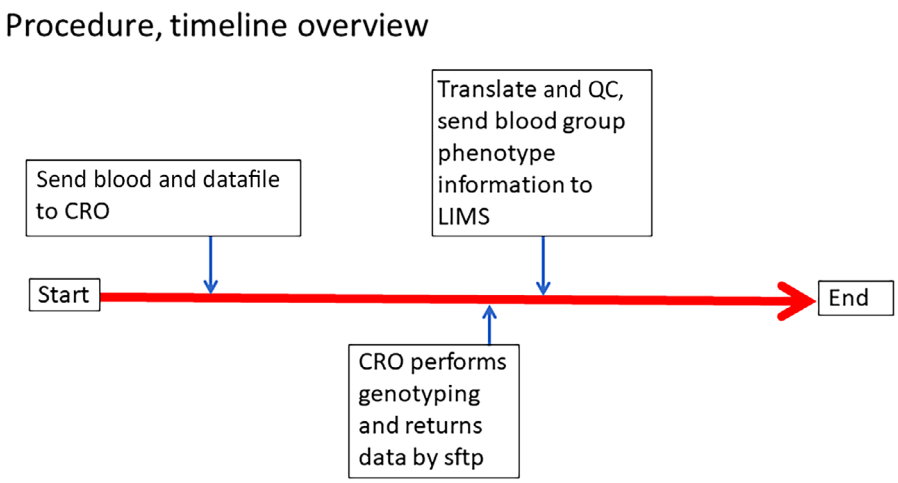

FIGURE 1 Schematic depiction of organization of one cycle of the genotyping. About 1 month from blood draw to return of genotyping results. About 7000 donors were genotyped annually. CRO, contract research organization; LIMS, laboratory management information system.

The repeat samples, however, represent an opportunity to evaluate the data for reproducibility. Repeat genotypes from the same donor with four or less 'no calls' in all repeat genotypes were compared in a spreadsheet. The reproducibility calculations were done as follows: in case of a genotype result of 'no call' or other non-base calls, or discrepant base calls of two samples from the same donor, both sample determinations were labelled as wrong and only a genotype result with bases called consistently was labelled as correct, even though the true genotype of the sample was not known. The number of times a consistent, presumed-correct base call was found was divided by all determinations of that primer set, and a percentage is calculated.

Ethics statement

In this paper we exclusively address anonymized data collected as part of routine genotyping of blood donors.

RESULTS

A total of 35,305 blood donor samples were genotyped, with an average of about 7000 blood donor samples genotyped every year. For some donors, one or more primer sets failed to call the genotypes, but only a total of 101 samples (0.285%) had >4 'no calls' including otherwise unusable results. Samples with >4 'no calls' result in an incomplete phenotype and could also indicate that the DNA was of insufficient amount or quality. For these samples, the genotyping results were not transferred to the LIMS, and the donor was genotyped again at the next blood donation.

From the 31,538 blood donors genotyped, there were 25,396 active blood donors at the time of this survey of historically acquired data. 'Active' was defined as having given blood at least once within the last 3 years from the date of this investigation.

The blood group genes, the rs numbers interrogated, the calculated allele frequencies and the predicted phenotypes are shown in Table 1.

Apart from blood groups where one allele was very rare and the homozygote was found less than five times, Hardy–Weinberg equilibrium was found for all blood group alleles except for Fy^a/Fy^b and $Fy^{ab}/Fynull$, *M/N* and *LU*A/LU*B*. Despite the large number of genotypes, for some blood groups the rarer allele was found only in 2–53 instances, except for the Lu blood group. The number of 'no calls' for the Lu primer set was not larger than for the other primer sets, indicating that the Lu competitive allele specific PCR (KASP) assay worked as well as the other primer sets. No additional attempt was made to resolve the 'no calls' for the other blood group systems which ranged between 79 and 526 with an average of 169 (0.48%) 'no calls' from about 35,000 determinations of each of the 51 KASP assays, totalling 1,785,000 individual genotypes.

The calculated and predicted phenotype frequencies were in good accordance with the phenotype frequency from the literature [10]. The donor population is skewed towards more group O donors, reflecting deliberate recruiting efforts.

A few alleles were not detected in the homozygous form but detected only in the heterozygous form. Thus, all the primer sets are expected to function technically.

The *ABO* determinations were used for sample ID control purposes and were not imported to the LIMS, except for the *ABO*A2* determination from those *ABO*A2* groups based on the c.1061delC. This information is used to select platelets from homozygous *ABO*A2* donors or *ABO*O/ABO*A2* heterozygous donors, which are for practical purposes used as group O donor platelets as no A antigen is expressed on these platelets [11]. The c.1061delC is also found in some Aweak alleles including some ABO*A3 types and in ABO*O.01.22 O22. Of the 1287 donors genotyped as *ABO*A2* either *ABO*A2/ABO*O.01*, *ABO*A2/ABO*O.02*, *ABO*A2/ABO*A2* or *ABO*A2/ABO*B* for the c.1061delC, 1282 were phenotyped as A1 negative and 5 (0.389%) were designated as A1 positive, giving very good concordance between the serological phenotyping of A1 and genotyping of *ABO*A2*. Genotyping has been shown to find more *ABO*A2* types than lectin typing [12].

TABLE 1 Frequency of blood group genotypes of Danish blood donors in the Danish Capital Region.

Blood group and PLT genes	Gene	Allele (ref. allele in bold)	rs number	SNP[a]	Frequency of minor allele	Antigen	% Occurence[b]	% Published occurence[c]	Antigen	% Occurence[b]	% Published occurence[c]
1	ABO	***ABO*A1.01***/*ABO*O.01*	rs8176719	261delG	See ABO in Tables 2 and 3						
		ABO*A1.01/*ABO*B or ABO*O.02*	rs7853989	526C>G							
		ABO*A1.01/*ABO*B*	rs8176741	657C>T							
		ABO*A1.01/*ABO*A2*	rs56392308	1061delC							
2	GYPA	***GYPA*01***/*GYPA*02*	rs7682260	59C>T	0.467	M	77	78	N	71	72
	GYPB	*GYPB*03*/***GYPB*04***	rs7683365	143C>T	0.300	S	51	55	s	91	89
4	RH	***RHD*01***/*RHD*01N*	RHD specific	RHD exon 5	0.452	D	79	85	D−	21	15
		RHD*01/*RHD*01N*		RHD exon 7	0.451	D	80		D−	20	
		*RHCE*C +/−*	RHCE intron 2	i2-3031 In109[d]	0.598	C	64	68	C−	36	32
		RHCE*01 *(RHCE*c)*	rs676785	307C	0.423	c[e]	82	80	c−	18	20
		*RHCE*E/***RHCE*e***	rs609320	676G>C	0.154	E	28	29	e	98	98
		RHCE*01/*RHCE*02.08*	rs138268848	122A>G	0.016	Cw	2.9	2	Cw−	97	98
5	BCAM	*LU*01/****LU*02***	rs28399653	230G>A	0.044	Lu[a]	8.0	8	Lu[f]	99.9	100
6	KEL	*KEL*01.01/****KEL*02***	rs8176058	578C>T	0.039	K	7.3	9	k	99.8	100
		KEL*02/*KEL*01M.01*	rs8176058	578C>G	0.002	na			Kweak	0.0032	Rare
		*KEL*02.03/****KEL*02.04***	rs8176059	841C>T	0.013	Kp[a]	2.2	2	Kp[f]	100	100
		*KEL*02.06/****KEL*0.07***	rs8176038	1790T>C	0.000	Js[a]	0.054	<0.01	Js[f]	100	100
8	ACKR1	***FY*01***/*FY*02*	rs12075	125G>A	0.432	Fy[a]	68	66	Fy[f]	81	83
See also Duffy in Table 5		***FY*wt***/*FY*01N.01/FY*02N.01*	rs2814778	-67T>C	0.006	See also Duffy in Table 5					
		FY*wt/*FY*01/02W*	rs34599082	265C>T	0.012						
9	SLC14A1	***JK*01***/*JK*02*	rs1058396	838G>A	0.480	Jk[a]	77	77	Jk[f]	73	74
10	SLC4A1	*DI*01/****DI*02***	rs2285644	2561C>T	0.002	Di[a]	0.070	0	Di[f]	100	100
		*DI*02.03/****DI*02***	rs75731670	1972G>A	0.000	Wr[a]	0.048	<0.01	Wr[f]	100	100
11	ACHE	***YT*01***/*YT*02*	rs1799805	1057C>A	0.041	Yt[a]	99.8	>99.8	Yt[f]	7.8	8
14	ART4	*DO*01/****DO*02***	rs11276	793G>A	0.372	Do[a]	62	67	Do[f]	85	82
15	AQP1	***CO*01.01***/*CO*02*	rs28362692	134C>T	0.047	Co[a]	99.8	99.5	Co[f]	8.8	10
16	ICAM4	***LW*05***/*LW*07*	rs77493670	299A>G	0.007	Lw[a]	100	100	LW[f]	1.0	<1
18	FUT2	***FUT2*01***/*FUT2*01N.02*	rs601338	461G>A	0.443	See also secretor in Table 2					
21	CD55	***CROM*01***/*CROM*-01*	rs60822373	679G>C	0.002	Cra+	100	100	Cra−	0.0064	na
22	CR1	***KN*01***/*KN*02*	rs41274768	4681G>A	0.038	Kn[a]	99.9	95	Kn[f]	7.2	3.5
34	SMIM1	***VEL*01*** */VEL*01N.01*	rs566629828	64_80delAGCCTAGGGGCTGTGTC	0.023	Vel+	100		Vel−	0.041	
PLT	ITGB3	***ITGB3*001***/*ITGB3*002*	rs5918	176T>C	0.168	HPA-1a	97	98	HPA-1b	30	28
PLT	ITGA2	***IGTB2*001***/*IGTB*002*	rs1801106	1600G>A	0.079	HPA-5a	99	100	HPA-5b	15	21
PLT	CD109	***CD109*001***/*CD109*002*	rs10455097	2108C>A	0.496	HPA-15a	74	77	HPA-15b	76	65

Note: An overview of genotyping results with genes and variants interrogated, 'no calls' and calculated frequencies are shown. Hardy–Weinberg test result is calculated only if more than five genotypes are found for all homo- and heterozygous samples. Frequencies are from [10, 16]. Table 1 includes the number of allele counts and some additional information can be found under supporting information as Table S1.

Abbreviations: na, not available; PLT, platelet antigens.

[a]Bases given as defined by ISBT (16).

[b]Most antigens are directly predicted from a single SNP, and some are deduced from the result of several SNP determinations.

[c]Frequency in Caucasians from FACTSBook [10] and Curtis et al. [16].

[d]Defined from the intron in GenBank: U66340.1.

[e]Deduced from *RHCE*c* negative samples.

[f]*p*-Value above 0.05 is considered Hardy–Weinberg equilibrium and cannot be calculated if less than five of any category.

TABLE 2 ABO genotypes, 34,745 total called results of all ABO SNPs.

Number				%	
ABO genotype		Non-secretor	Secretor	Non-secretor	Secretor
O.01/O.01	13,058	2606	10,452	20	80
A1/O.01	7168	1424	5744	20	80
A2/O.01	2712	541	2171	20	80
B/O.01	3078	593	2485	19	81
O.02/O.01	686	135	551	20	80
A1/A1	1196	236	960	20	80
A1/A2	824	171	653	21	79
A1/B	1010	193	817	19	81
A1/O.02	186	37	149	20	80
A2/A2	138	31	107	22	78
A2/B	381	84	297	22	78
A2/O.02	65	14	51	22	78
B/B	196	40	156	20	80
B/O.02	84	17	67	20	80
O.02/O.02	13	3	10	23	77
Total	30,795	6125	24,670	20	80

Note: ABO and secretor frequencies show that secretor status is independent of ABO blood group.

TABLE 3 ABO groups with called genotype but fall outside the conventional ABO algorithm.

	Assay name					
Number of samples	ABO_O1 rs72238104[a]	ABO_B_O2 rs7853989	ABO_B rs8176741	ABO_A2 rs56392308[b]	Comment	Serotype
11	–/–	C/C	C/C	C/–[c]	Unknown	O
1	–/–[c]	C/C	C/C	C/–	Unknown	A
8	–/–	C/G	C/C	C/C	*O.01/O.03* or *O.48* or *O.49* or *O.50*	O
42	–/–	C/C	C/T	C/C	*O.10/O.01*	O
1	–/–[c]	C/G	C/T	C/C	Unknown	B
3	–/–	C/G	C/T	C/C	*O.01/O.24*	O
1	G/–	C/G[c]	C/C	C/–	Unknown	A
2	G/–	C/C	C/T[c]	C/C	Unknown	B
10[d]	G/–	C/C	C/T[c]	C/C	Unknown	A
1	G/–	C/C	T/T[c]	C/C	Unknown	A
3[e]	G/–	C/G	T/T[c]	C/C	Unknown	B
1	G/–[c]	G/G	T/T	C/C	Unknown	B
2	G/–	C/C	C/T[c]	C/–	Unknown	A1–
2	G/–	C/C	C/T[c]	C/–	Unknown	A
1	G/–	C/G	T/C	C/–[c]	Unknown	B
1	G/G	C/C[c]	T/C	C/–	Unknown	AB

Note: ABO variants. ABO genotyping is used for ID control purposes except for ABO*A2 results where homozygous A2 or heterozygous A2O predicted donors are used as universal platelet donors.

[a]– Synonymous with c.261delG.

[b]– Synonymous with c.1061delC.

[c]Results that may be erroneous or resulting in an unknown ABO genotype.

[d]This result was confirmed for one donor.

[e]This result was confirmed for three donors.

TABLE 4 Comparison of C and c determinations (there were no phenotypes available for all donors).

	Assay name				
No. of samples	*RHD* RHD_exon 5	*RHD* RHD_exon7	*RHC* RHCE_C_intron2	*RHc* rs676785	Phenotype
Presumed erroneous *RHC* genotype determinations					
1	MINUS/PLUS[a]	?	MINUS/PLUS[a]	C/C	D−C−e−c+E+
1	MINUS/MINUS	MINUS/MINUS	MINUS/PLUS[a]	C/C	D−
10	MINUS/MINUS	MINUS/MINUS	MINUS/PLUS[a]	C/C	D−C−e−c+E+
Presumed erroneous *RHc* genotype determinations					
1	MINUS/MINUS	MINUS/MINUS	MINUS/MINUS	T/C[a]	D−C−E−c+e+
3	MINUS/MINUS	MINUS/MINUS	MINUS/MINUS	T/C[a]	D−

Note: Inconsistencies in RhCc determination. The conflicting genotype results of homozygous c and heterozygous C in the same person will not be transferred to the laboratory management information system. In all cases in the table but two, phenotype data were available, and phenotypes confirmed the c homozygous genotype. Due to homology between *RHD* and *RHCE* genes, the assays for RHD and RHC are performed in a MINUS/PLUS format.
[a]Results that may be erroneous.

TABLE 5 Duffy variants.

	Assay name					
Sample	Duffy_Fya/a rs2814778	Duffy rs12075	Duffy_Fyx rs34599082	Explanation	Expected phenotype	Serotype
1	T/C	G/G	C/C	*FY*01N.01* [14]	FY(a+b−)	na
2	T/C	G/G	C/C			na
4	T/T	G/G	C/T	*FY*01W* [15]	Fy(a+b−)	na
5	T/T	G/G	C/T			na
7	T/C	A/A	C/T	*FY*02N.01/FY*02W*	Fy(a−b−)	Fy(a−b−)
8	T/C	A/A	C/T			Fy(a−b−)

Note: Rare combinations of variants found in the Duffy genotyping are shown.
Abbreviation: na, not available.

Secretor status (FUT2) was independent of the ABO blood group (Table 2).

For the ABO blood group, determinations are based on several primer sets, and a number of instances were noted where all primer sets were called but where the translation table did not allow prediction of a phenotype (Table 3) by a simple conventional translation. As ABO serotype determinations existed for all blood donors, it was possible to compare the genotyping results from these instances. These discrepancies indicated that it would be possible to modify the translation to include at least some of the ABO determinations where all primer sets are called but translation to a phenotype is not possible with our current translation table. But as previously noted, the ABO translations are for donor ID purposes only, except for the *ABO*A2* predictions.

The *RHD* determinations were used to screen for the DEL type. Therefore, discrepancies between phenotype and genotype were investigated with an extended *RHD* genotyping.

Some inconsistencies were noted for RHCE. Of 6338 samples found homozygous for RHc, 6197 samples were found RHC negative, 13 samples were RHC positive and 128 were 'no call' for *RHC*. In the 13 samples where we found a positive RHC genotype, together with a homozygous RHc positive genotype, a phenotype was performed on 11 of these samples which turned out to be C-c+ (Table 4). These C-negative samples were D negative as expected.

The genotyping for the Fy antigen was based on three primer sets interrogating rs12075 (Fya/Fyb), rs2814778 (Fy^{ab}/Fynull) and rs34599082 (Fy^{ab}/Fy^{ab}weak). Some rare combinations are shown in Table 5. It appears that Duffy determinations are not in Hardy–Weinberg equilibrium: there are too many of the two homozygotes and/or too few of the heterozygotes.

For assessment of the reproducibility, the repeat genotyping results on independently drawn blood samples were examined. The 7213 repeat samples from all the 35,305 blood samples genotyped were found as follows: 3175 donors were genotyped twice, 234 donors were genotyped in triplicate, 27 donors in tetraplicate, 7 donors in pentaplicate and 3 donors in hexaplicate. Altogether, 3767 genotypes were unnecessarily repeated. The reproducibility was assessed as excellent and based on the batch controls and on the duplicate genotype determinations only. If a duplicate genotype was discrepant, both genotypes for this allele were scored as wrong when,

in fact, most likely, one would be correct. The average reproducibility estimated in this conservative way of all results over all primer sets investigated was 98.76%; assuming only one of the two duplicates is erroneously determined, the correct score would reach 99.38%. When samples with >4 'no calls' (a total of 70 samples) were removed from the calculation, the overall reproducibility was 99.15% on average for all primer sets. The lowest percentage was for the MN determinations, only 94.83%, and the highest percentage was for Duffy, 99.65%.

The reproducibility results are summarized in Table S2, which also includes an assessment of reproducibility based on the 51 genotype batch controls.

We addressed the question of reliability of base determination from bases called in samples with >4 'no calls' by estimating the frequency of discrepant results from bases actually called in the duplicate sample in the >4 'no call' samples between the two duplicate samples and compared with the ≤4 'no call' samples irrespective of primer set. For the samples with>4 'no call', there were 0.892 discrepant base calls per sample (calculation based on 28 data points) compared with the duplicate sample from the same donor, and for the ≤4 'no call' samples, there were 0.061 discrepant base calls per sample (calculation based on 3147 data points) compared with the duplicate sample from the same donor.

DISCUSSION

We report allele frequencies and the frequency of the homozygous and the heterozygous genotypes of 16 blood groups as determined from our genotyping of 31,538 individual donors (i.e., from 35,305 samples minus those samples tested more than once). The data are from the largest genotyped Danish donor cohort so far, based on dedicated blood group genotyping.

We currently use the genotype results for finding blood donors suitable for blood donation to patients with alloantibodies or predicted antigen-negative donors to antigen-negative recipients to prevent alloimmunization. Also, *ABO* genotype determinations are made for ID control purposes except for the *ABO*A2* genotype used for selecting donor blood.

Overall, this blood donor genotyping system has proven very useful and enabled the procurement of rare blood [13]. The reproducibility is high, the workload minimal and the throughput virtually unlimited; only the capacity of the CRO sets the limit and fresh blood from rare donors is often available. Only a LIMS, a pipetting robot and a heat sealer are the necessary investments locally; most blood banks would already have this equipment.

As the *ABO*O.01* deletion is upstream of the *ABO*B* and *ABO*A.02* specific sequences, the phenotype will be O unless another one base insertion is present downstream of the c.261delG, reconstituting the reading frame. Two exceptions, however, were found (Table 3); perhaps a genotyping error in the *ABO*O.01* determination is the basis. The samples were not sequenced.

The *RHD* genotyping is used in conjunction with other analyses to detect the DEL types.

We have developed our blood donor genotyping system as a very economical blood donor genotyping system encompassing the most common blood groups of clinical relevance including three platelet groups as well as some selected blood groups of rarer clinical relevance.

Occasionally, a result was not called, and out of the 35,305 blood donor samples, only 101 samples had four or more 'no calls'. As a precautionary measure, from samples with >4 'no calls', no other results were translated into phenotypes, as it was surmised that the DNA quality of that sample was poor. Thus, the other results from that sample which were called were assumed to be less reliable. By this single step, incorrect genotyping results were reduced by 56% [9]. Robustness of the genotyping was indicated by the relatively few >4 'no calls' as well as the low number of 'no calls' for each genotype assay (Table S1).

Currently, we use six selected donors with known genotypes that are included separately in each batch of eight plates to serve as controls. Instead of this approach, it is possible to clone all polymerase chain reaction products for all alleles in a few plasmids and use the linearized plasmids as positive controls; alternatively, we can have all the relevant sequences with all allotypes synthesized and cloned into a plasmid that can be linearized and used as a positive control for all reactions. This would necessitate only one well per batch as genotype control and leave five more wells for blood donor samples and remain a positive control of all allotype reactions.

The observed discrepancy between the RHC genotype and C phenotype (Table 4) can either be due to a genotyping error or to the occurrence of the RHC-specific insertion in other RH alleles. Possibly the C intron assay, albeit better than an assay for the C SNP rs586178, could still be improved.

The rare combinations found with the GATA box and the c.265C>T variant on the *FY*A* allele have been previously described [14, 15]. Also, two donors had a combination of the *FY*02N.01* and *FY*02W* alleles (Table 5). Twenty-four donors were found as homozygous *FY*02N.01* and thus possibly with benign ethnic neutropenia; this was not investigated further.

We have no good explanation for the seeming lack of Hardy–Weinberg equilibrium for Fy^a/Fy^b.

Explanations for the other deviations from Hardy–Weinberg equilibrium (MN and Lu) are also somewhat enigmatic, but perhaps a slight suboptimal function of the primers or even the emigration of many years is now reflected in these findings. The reproducibility was judged from both repeat genotyping of donors and the genotyping of the controls included in every batch as batch controls, and the reproducibility was deemed as good, although we know of no formal QC criteria to evaluate the results against. The assessment rendered further evidence for the internal rule of not entering results from samples with >4 'no calls' into the LIMS, as these samples had a slightly lower reproducibility percentage. Because of the way the data were deemed acceptable, the assessment is rather stringent. Also, the finding of more discrepant base calls in the >4 'no call' sample results than among the ≤4 'no call' samples lends support to our internal rule of not entering >4 'no call' sample results into the LIMS but retesting them once.

The many replicates were surprising but are most likely largely due to a delay of entering genotyping information into the LIMS. An awaited IT solution should circumvent this excess testing.

The costs for the complete genotyping of one sample yielding the prediction of 51 antigens is about half of that of phenotyping with determination of 11 antigens—and some phenotypes are impossible to perform due to lack of reagents—making genotyping the only option. Turnaround time for genotyping varied slightly and was about 1 month from shipment of samples to digital receipt of the genotypes by secure ftp. The genotyping was performed with primer sequences that were designed in proprietary software.

In some instances, a CE-marked commercial genotyping kit was used for genotyping patients in whom serotyping could not be used due to recent massive blood transfusion.

In conclusion, the genotyping system has proven very satisfactory, cost-efficient and reliable with a reproducibility of over 99% using the described calculation, and the genotyped donors represent a valuable resource that on several occasions has enabled the timely issuing of rare antigen-negative blood to patients with alloantibodies.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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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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SHORT REPORT

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A novel allele of *FUT2* gene containing a deletion of nine bases (c.461_469delGGACCTTCT) in a Chinese Han blood donor

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Abstract

Background and Objectives: The *FUT2* gene is responsible for the synthesis of the H antigen in body secretions. It is highly polymorphic and population specific. We investigated the *FUT2* gene polymorphism in Chinese blood donors and found a novel deletion mutation in one non-secretor individual. This study aimed to identify mutation(s) responsible for a non-secretor phenotype.

Materials and Methods: The Lewis blood group of a Chinese Han blood donor was typed using the standard serological technique and the *FUT2* gene of the sample was analysed by Sanger sequencing. Clone sequencing was performed for determining the haplotype of the *FUT2* gene. Bioinformatics tools were used for predicting the effect of the deletion on the *FUT2* gene.

Results: A novel nine-base deletion (c.461_469delGGACCTTCT) in the *FUT2* gene was identified in a Chinese Han blood donor. Two haplotypes $Se^{390,418}$ and $se^{204,249,461\_469del,772,993}$ were determined by clone sequencing. According to the prediction of bioinformatics tools, the mutation at c.461_469delGGACCTTCT might not influence the activity of the Se enzyme.

Conclusion: We identified a new *FUT2* mutation, the deletion of nine bases (c.461_469delGGACCTTCT), in a Chinese Han blood donor. This deletion was reported for the first time.

Keywords

deletion, *FUT2*, SNP

Highlights

- The *FUT2* gene is responsible for the synthesis of the H antigen in body secretions.
- It is highly polymorphic and population specific.
- We report here a new *FUT2* mutation, the deletion of nine bases (c.461_469delGGACCTTCT), in a Chinese Han blood donor.

INTRODUCTION

H blood group system consists of a single antigen, the H antigen, which serves as a crucial precursor for the A and B antigens. The expression of the H antigen is controlled by two genes: *FUT1* and *FUT2*. The *FUT1*

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gene governs the H antigen on red blood cells (RBCs), while the *FUT2* gene regulates the H antigen in body secretions, excluding the cerebrospinal fluid. The *FUT2* gene encodes the secretor-type α(1,2)-fucosyltransferase (Se enzyme) that adds a fucose to the type I oligosaccharide chain, producing the H antigen [1]. The *FUT2* gene, located on chromosome 19, consists of two exons and encodes a 343-amino acid protein, the Se enzyme. Individuals who carry at least one active *FUT2* allele (Se) are secretors and possess H antigens in their body secretions. Non-secretors carry homozygotes for the inactive *FUT2* allele (se) and lack the H antigen [2]. Additionally, weak secretors express lower levels of H antigens compared with secretors. Weak secretors can be either homozygotes (Se^w/Se^w) for the weak-secretor allele or heterozygotes (Se^w/se). Moreover, the *FUT2* gene also influences the Lewis phenotypes, while the *FUT3* gene controls the expression of the Lewis antigens. Individuals with homozygotes for non-functional *FUT3* alleles exhibit the Lewis-negative phenotype (Le(a−b−)) regardless of their secretor status. Conversely, individuals with at least one functional *FUT3* allele can have three different Lewis-positive phenotypes depending on the secretor status. Secretors display the Le(a−b+) phenotype, non-secretors have the Le(a+b−) phenotype and weak secretors possess the Le(a+b+) phenotype [1].

Since 1995, the *FUT2* gene has been cloned and separated [2], and numerous *FUT2* alleles resulting from single nucleotide polymorphisms (SNPs), deletion-based mutations and non-allelic homologous recombination have been identified [3]. *FUT2*01N.02* (c.461G>A) is most common in Caucasian and African with a frequency of approximately 20%. Conversely, the *FUT2*01W.02.01* (c.418A>T) is specific to East and Southeast Asian populations including Chinese, Japanese and Vietnamese with a frequency of around 50%. The synonymous variant c.390C>T is found in various populations, containing Chinese. Considering the genetic diversity and ethnicity-specificity of the *FUT2* gene, we analysed its polymorphism in Chinese blood donors. An allele with a new deletion (c.461_469delGGACCTTCT) was discovered in a non-secretor donor. Our objective was to characterize this allele and investigate its association with the non-secretor phenotype.

MATERIALS AND METHODS

Sample collection and Lewis typing

The peripheral blood sample was collected with informed consent from a Han nationality blood donor in Dalian who is blood group O and RhD positive. The ethical procedures were taken and approved by the Ethics Committee of Dalian Blood Center. For evaluating the secretor status, Lewis typing was performed using a standard tube agglutination test with monoclonal anti-Le^a and anti-Le^b antibodies (Rapid Labs Ltd, Colchester, UK).

Polymerase chain reaction (PCR) and DNA sequencing

Genomic DNA was extracted from ethylenediaminetetraacetic acid (EDTA) anti-coagulated blood using an Automated Nucleic Acid Extractor (RBC Bioscience Corporation, Taiwan) following the manufacturer's instruction. The entire coding region of *FUT2* was amplified in a 50 μL reaction of 25 μL AmpliTaq Gold® 360 Master Mix (Thermo Fisher Scientific, Lithuania), 1 μL 360 EC Enhancer, 1 μL forward primer, 1 μL reverse primer (final concentration 0.4 μM each) and 150 ng DNA. The temperature profile was 94°C for 1 min, followed by 35 cycles of denaturing at 92°C for 30 s, annealing at 55°C for 50 s, extension at 72°C for 40 s and a final extension at 72°C for 1 min. Subsequently, the PCR amplicons were sequenced by BGI Tech Solutions (Beijing, China). The primer sequences used (Table 1) were obtained from a previous literature [4]. For determining the *FUT2* gene haplotypes, the PCR amplicon containing the novel mutation was cloned into a plasmid and sequenced by General Biol (Chuzhou, China).

TABLE 1 Primer sequences used in this study.

Primer	Sequence (5′–3′)
FUT2_Amp_F	TGCCAAGTATTTACACACCTGAAG
FUT2_Amp_R	GATTTCTGTTACTTGCAGCCCA
FUT2_F1_R	AGTCGTTCAGGTGGTAGTTCTGC
FUT2_F2_F	TGTACGCCCTGGCCAAGA
FUT2_F3_F	CCATGTCATGCCAAAAGTGTG

Note: FUT2_Amp_F and FUT2_Amp_R were used for PCR amplification. FUT2_F1_R, FUT2_F2_F and FUT2_F3_F were used for Sanger sequencing.

In silico analysis

We utilized Mutation Taster (http://mutationtaster.org/) and MutPred-Indel (http://mutpred.mutdb.org/) to predict the possible impact of the new deletion on Se enzyme function and the pathogenicity of this deletion, respectively. Swiss Model (http://www.swissmodel.expasy.org/) was then used for predicting 3D structures for both wild-type Se enzyme and variant caused by the c.461_469delGGACCTTCT mutation. Subsequently, Swiss-Pdb Viewer 4.0.1 was performed for marking specific amino acid sites on the generated 3D protein structures. In addition, the long coding region of the *FUT2* gene (1032 bp:343 amino acid) was used for numbering the nucleotide positions.

RESULTS

Lewis typing

Based on the serological test results, the Lewis blood group of the subject was determined as Le(a+b−). Specifically, the subject's RBC exhibited 2+ strength of the agglutination with monoclonal anti-Le^a and no agglutination with monoclonal anti-Le^b. Therefore, it can be speculated that the subject is a non-secretor.

FUT2 genotyping and haplotype analysis

In this study, we identified a deletion site and six SNPs at the *FUT2* locus in the donor. Of them, the c.461_469delGGACCTTCT is a nine-base deletion, while c.204A>G (rs492602), c.249C>T (rs681343), c.390C>T (rs281377) and c.993A>G (rs485186) are synonymous mutations and c.418A>T (rs1047781) and c.772G>A (rs602662) are missense mutations. All of these variants were found to be heterozygous. The deletion site c.461_469delGGACCTTCT led to a change in four amino acids at positions 154–157, which were encoded by the coding region sequence of *FUT2* at positions 460–471. Due to the deletion of nine bases at positions 461–469, the remaining three bases recombined to form a new codon, which encoded the amino acid tyrosine (Tyr) at position 154 in the variant Se enzyme sequence (Figure 1). This new amino acid in the variant sequence was identical to the amino acid present at position 157 in the wild-type sequence. Consequently, compared with the wild type, the variant lacked three amino acids, namely, p.154delW, p.155delT and p.156delF. The amino acids following position 157 remained unchanged, but their positions have shifted forward by three positions. As a result, the length of the amino acid sequence has changed from 343 amino acids to 340 amino acids.

Then, we determined two haplotypes, which were identified as allele $Se^{390,418}$ and allele $se^{204,249,461\_469del,772,993}$ (Figure 2). The allele carrying the novel deletion c.461_469delGGACCTTCT represents a new variant of the *FUT2* gene observed in a Chinese blood donor.

In silico analysis

The mutation at c.461_469delGGACCTTCT was predicted as a 'polymorphism' by Mutation Taster, suggesting that it might not influence the activity of the Se enzyme, and the deletion with a MutPred-Indel score of 0.55889, indicating that the deletion has some potential to be pathogenic, but it is not strongly predicted to be so. Furthermore, the 3D structures obtained through the Swiss Model demonstrated that the amino acid sequence of the Se enzyme with

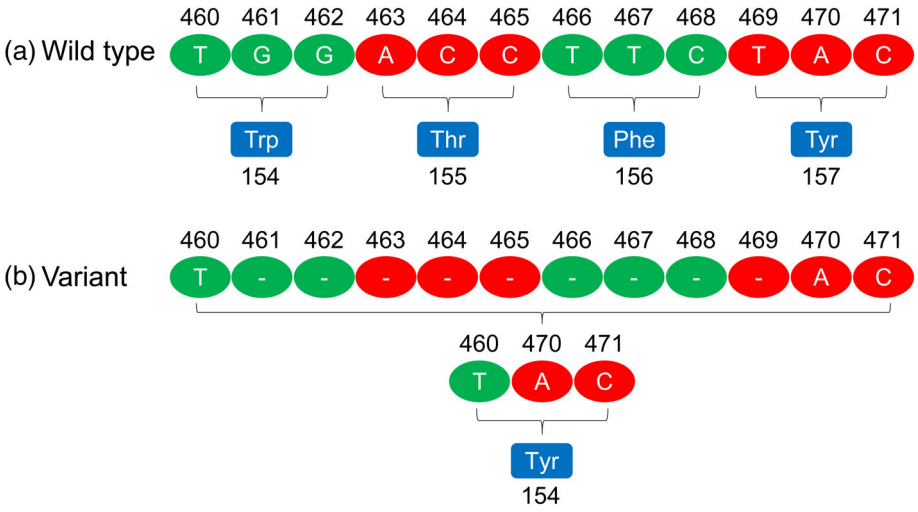

FIGURE 1 The c.461-469delGGACCTTCT deletion in the *FUT2* gene caused a change in the amino acid sequence of the Se enzyme. (a) In the wild-type *FUT2* gene, the bases at positions 460–471 encode four amino acids. (b) In the variant *FUT2* gene carrying the c.461-469delGGACCTTCT deletion, the bases at positions 460–471 encode only one amino acid. The '-' indicates a base deletion. The amino acids in images included tryptophan (Trp), threonine (Thr), phenylalanine (Phe) and tyrosine (Tyr).

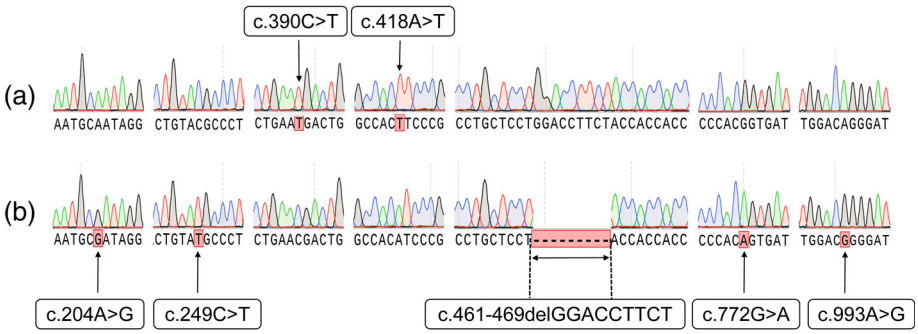

FIGURE 2 Two haplotypes obtained by Clone sequencing in the *FUT2* gene of a Chinese donor. (a) $Se^{390,418}$ haplotype and (b) $se^{204,249,461\text{-}469del,772,993}$ haplotype.

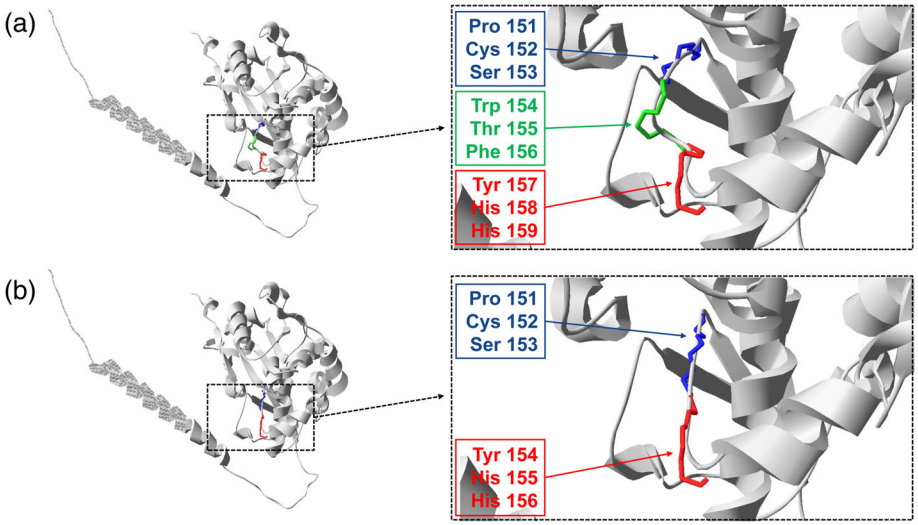

FIGURE 3 (a) 3D structure of the wild-type *FUT2* enzyme, with a focus on a selected region. The amino acids at positions 151–153, 154–156 and 157–159 were represented by the colours blue, green and red, respectively. (b) 3D structure of the variant *FUT2* enzyme resulting from the c.461_469del GGACCTTCT mutation, with a specific section enlarged. The amino acids at positions 151–153 and 154–156 were represented by the colours blue and red, respectively. The amino acids in images included proline (Pro), cysteine (Cys), serine (Ser), tryptophan (Trp), threonine (Thr), phenylalanine (Phe), tyrosine (Tyr) and histidine (His).

the c.461_469delGGACCTTCT mutation lost three amino acids at positions 154, 155 and 156 (Figure 3). Interestingly, it appeared that the amino acid at position 153 in the wild-type sequence was directly connected to the amino acid tyrosine (Tyr) at position 157. Coincidentally, in the variant Se enzyme with the c.461_469delGGACCTTCT mutation, the amino acid at position 154 was also tyrosine (Tyr).

DISCUSSION

In our study, we identified a deletion at c.461_469delGGACCTTCT and six SNPs at c.204A>G, c.249C>T, c.390C>T, c.418A>T, c.772G>A and c.993A>G by Sanger sequencing and two haplotypes $Se^{390,418}$ and $se^{204,249,461\_469del,772,993}$ by clone sequencing in the *FUT2* gene of a Chinese donor. The six SNPs have been reported previously [5]. However, the c.461_469delGGACCTTCT deletion site is a novel discovery.

Previous studies have indicated that the SNP c.390C>T does not influence the activity of the Se enzyme. However, the SNP c.418A>T has been found to reduce or even eliminate the expression of the Se enzyme, resulting in a weak-secretor or non-secretor phenotype. This can lead to the Lewis(a+b+) or Lewis(a+b−) phenotype, respectively [6]. Thus, the allele $Se^{390,418}$ can cause weak-secretor or non-secretor status. Moreover, the $Se^{390,418}$ allele is prevalent and restricted to East and Southeast Asian populations [7]. On the other hand, the deletion at c.461_469delGGACCTTCT was found to be linked with four SNPs at c.204A>G, c.249C>T, c.772G>A and c.993A>G, forming a new allele $se^{204,249,461\_469del,772,993}$ in our study. It is important to note that these four SNPs are most accompanied by the c.461G>A mutation. The $se^{204,249,461,772,993}$ allele has been previously reported in African, Peruvian and Chinese populations, and this allele can cause a non-secretor phenotype [5, 8, 9]. Based on the prediction from the Lewis phenotype, the donor may be a non-secretor lacking H antigen in body secretions. However, the mechanism for the non-secretor phenotype related to the new allele $se^{204,249,461\_469del,772,993}$ remains undetermined in our study. The synonymous SNPs c.204A>G, c.249C>T and c.993A>G do not lead to non-secretor phenotype [2]. For the c.772G>A, a study suggested that c.772G>A is responsible for some non-secretor cases [10], while another study demonstrated that the allele with c.772G>A encoded an efficient Se enzyme [11]. Consequently, the relationship between c.772G>A and the non-secretor phenotype remains uncertain. Thus, the allele $se^{204,249,461\_469del,772,993}$ may be relevant to the non-secretor status due to the c.461_469delGGACCTTCT. However, it is essential to note that the bioinformatics tools predicted that the c.461_469delGGACCTTCT may not affect the function of Se enzyme. Further investigations are necessary to determine the exact influence of this allele on secretor status. Additionally, strong linkage disequilibrium was observed between several SNPs in the intron and the coding region of *FUT2* [12], suggesting a potential influence of the non-coding region on the secretor status.

Excepting the SNPs, various types of deletions have been identified in the human *FUT2* gene, such as the *FUT2*0N.03* (Fusion gene 1 between *FUT2* and *Sec1*) is predominantly found in Japanese populations with a frequency of about 5%–9% [7]. Non-allelic homologous recombination events mediated by log terminal repeat have been observed in the *FUT2* gene of Peruvian [8]. These deletions both involve the loss of coding regions within the *FUT2* gene, causing the absence of H antigen expression in body secretions. Furthermore, several deletions, such as c.811delC and c.721_723delGTC in the *FUT2* gene, have been associated with non-secretor alleles [6, 9]. In our study, we identified a novel nine-base deletion at c.461_469delGGACCTTCT within the *FUT2* gene. In silico analysis

suggested that this deletion might not affect the function of the Se enzyme. Additionally, the bioinformatics tools are greatly valuable for predicting the effect of amino acid substitutions on enzyme function. However, the results are only prediction and cannot ensure 100% accuracy. Therefore, further experimental investigations are needed to verify the actual impact of this mutation on the Se enzyme function.

In summary, we discovered a novel allele carrying the c.461_469delGGACCTTCT deletion in the *FUT2* gene of a Chinese Han blood donor. This deletion was first reported, and this deletion site may not affect the Se enzyme function.

ACKNOWLEDGEMENTS

X.L. designed the study; S.Z. and L.W. performed serological testing, PCR amplification and in silico analysis; S.Z., L.W., W.S., Y.X. and L.S. analysed and interpreted the data; S.Z. and L.W. drafted the manuscript; all authors reviewed and edited the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict 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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SHORT REPORT

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Increasing the time-to-freezing for clinical apheresis plasma meets quality specifications

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Australian Government

Abstract

Background and Objectives: In Australia, the vast distances between blood collection centres and processing facilities make it challenging to align supply with demand. Increasing the time to freezing for clinical plasma beyond 6 h would alleviate supply issues. This study aimed to determine the quality of clinical apheresis plasma frozen within 12 h of collection.

Materials and Methods: Apheresis plasma ($n = 20$) collected at donor centres was immediately transported to a blood processing facility, stored at 26°C and sampled aseptically at 6, 8 and 12 h post collection. Frozen samples were thawed, and coagulation factors (F) II, V, VII, VIII and XIII, von Willebrand factor (vWF) and fibrinogen were measured using a coagulation analyser.

Results: FVIII concentrations declined in plasma frozen at 6, 8 and 12 h post collection (1.22 ± 0.27, 1.21 ± 0.25 and 1.16 ± 0.24 IU/mL, respectively) but not significantly ($p = 0.3338$). Importantly, all components met the FVIII specification (>0.7 IU/mL) for clinical plasma. Fibrinogen concentrations were stable from 6 to 12 h ($p = 0.3100$), as were vWF concentrations ($p = 0.1281$). Coagulation factors II, V, VII and XIII were not significantly different ($p > 0.05$ for all factors).

Conclusion: Clinical apheresis plasma can be frozen within 12 h of collection, allowing collections from donor centres further from processing centres and increasing supply.

Keywords

apheresis plasma, coagulation factor VIII, time to freeze

Highlights

- Coagulation factor VIII concentrations decreased in plasma frozen at 12 h post collection but were not significantly different from those in plasma frozen at 6 h.
- Fibrinogen and von Willebrand factor antigen concentrations were maintained in plasma frozen at 12 h post collection compared with plasma frozen 6 h post collection.
- Immunoglobulin G was stable in plasma frozen 6, 8 and 12 h post collection.

INTRODUCTION

Plasma demand continues to grow; in 2021–2022 the demand for plasma-derived products increased by 13% in Australia (internal communication). Clinical fresh frozen plasma (cFFP) is primarily transfused to correct deficiencies of clotting factors, for which a specific concentrate is not available, in patients with active bleeding or at risk of bleeding [1]; as a replacement fluid in

thrombotic thrombocytopenic purpura; and for clotting factor replacement in congenital or acquired bleeding diseases [2]. The exponential growth in fractionated plasma products such as intravenous immunoglobulin (IVIg) is also putting pressure on supply.

In Australia, there are vast distances between blood collection centres and blood processing facilities, making it challenging to align clinical plasma supply with demand, especially for AB plasma. Australia is mandated to follow the Council of Europe (CoE)'s *Guide to the preparation, use and quality assurance of blood components*. Historically, apheresis plasma was required to be frozen within 6 h of collection. However, in the 19th Edition of the CoE guidelines, the requirement for apheresis plasma has been updated to 'Freezing must commence either within 6 hours of collection or within a timeframe validated to result in a component meeting specification' [3]. As such, the time to freeze apheresis plasma could be extended if the quality is acceptable.

The stability of plasma frozen at various temperatures (−18°C, −25°C, −65°C), for different periods (1, 3, 7 years), and the stability of thawed plasma up to 5 days have been previously examined [4, 5]. There are also studies examining the time to separation and freezing of plasma derived from whole blood (<8 h, 18–24 h) [2, 6, 7], but limited studies have investigated the time to freezing for apheresis plasma. Given the limited data, the aim of this study was to compare the quality of clinical apheresis plasma frozen within 6, 8 and 12 h of collection.

MATERIALS AND METHODS

This project was approved by the Australian Red Cross Lifeblood Ethics Committee.

Apheresis plasma suitable for clinical use was collected according to standard procedures using the Aurora plasmapheresis platform (Fresenius Kabi, Bad Homburg, Germany), with end-saline infusion ($n = 20$; 10 group O and 10 non-group O). Plasma was packed and transported to a manufacturing facility within 2 h of collection. Upon arrival, plasma was incubated (Labec, Marrickville, NSW, Australia) at 26 ± 1°C (worst case scenario for blood shipping in Australia) for up to 12 h. Sample segments were prepared aseptically at 5.5, 7.5 and 11.5 h post collection and frozen in a rapid plasma freezer (RPF; Arrowsmith and Grant Refrigeration, Dandenong South, VIC, Australia) and stored below −25°C until tested. The remaining apheresis plasma collections were split into two to three components, depending on volume, and then frozen in an RPF and stored below −25°C.

Plasma segments were thawed in a 37°C water bath, and then visually inspected for turbidity, clots, fibrin strands, red cell or haemoglobin contamination or any other discolouration before being aliquoted for testing. Plasma was tested immediately for coagulation factors VIII and XIII, fibrinogen, von Willebrand factor antigen (vWF:Ag), activated partial thromboplastin time (APTT) and

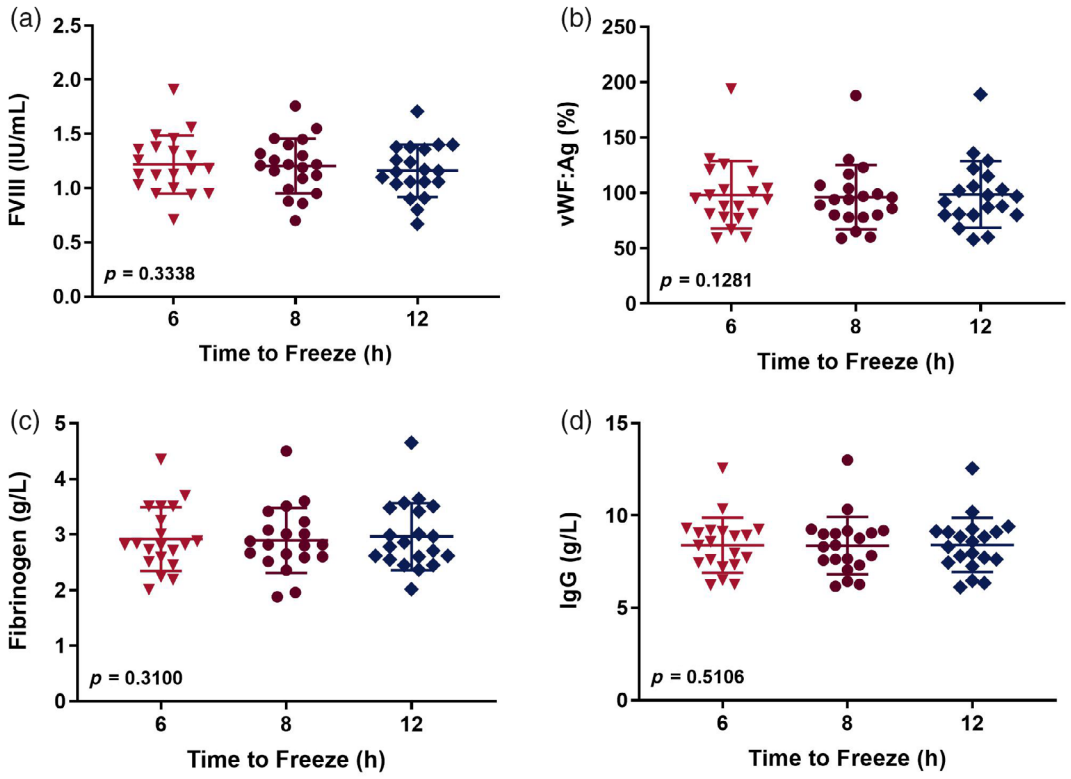

FIGURE 1 Coagulation factors in apheresis plasma that was frozen 6, 8 and 12 h post collection. (a) Coagulation factor VIII (FVIII), (b) von Willebrand factor antigen (vWF:Ag), and (c) fibrinogen were measured using a coagulation analyser. (d) Immunoglobulin G (IgG) was measured in plasma using a biochemistry analyser. Data points represent individual components; the horizontal lines represent the mean, and the error bars represent SD ($n = 20$). The horizontal dashed line is the minimum quality control specification for FVIII. *p*-Values were obtained using repeated-measures one-way analysis of variance.

antithrombin III (ATIII). Aliquots were frozen at −80°C for subsequent testing of prothrombin time (PT), coagulation factors II, V and VII, protein C, complement components C3a and C5a and immunoglobulin G (IgG).

Coagulation assays including PT, APTT, coagulation factors (FII, FV, FVII, FVIII, FXIII), protein C, fibrinogen, ATIII and vWF:Ag were performed using an automated coagulation analyser (STACompact; Diagnostica Stago Ltd., Asnieres, France). These factors were tested using one-stage clotting assays according to the manufacturer's instructions using STA reagents and standardized using reference plasma.

Complement components, C3a and C5a, were measured using a solid-phase sandwich enzyme-linked immunosorbent assay (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. IgG was measured using a biochemistry analyser (Architect c8000, Abbott Diagnostics, Abbott Park, IL) according to manufacturer's instructions.

Statistical analysis

Data were analysed using MS Excel and GraphPad Prism version 7.03. Mean and SDs were calculated. Comparisons of 6-, 8- and 12-h data were made using a repeated-measures one-way analysis of variance. For all analyses, p <0.05 was considered statistically significant.

RESULTS

The mean apheresis plasma volume was 819 ± 30 mL. The temperature during shipping ranged from 23.0 to 27.9°C. The mean temperature of the apheresis plasma during incubation was 25.9 ± 0.5°C. Although the aim was to freeze plasma after 6, 8 and 12 h, the actual times at which the plasma entered the RPF were 5:45 ± 0:25, 7:41 ± 0:28 and 12:14 ± 0:34 h:min post collection, and the freezing cycle took a further 25 min to complete.

TABLE 1 Apheresis plasma quality indicators over the extended time to freeze, 6, 8 and 12 h (mean ± SD, $n = 20$).

Parameter	6 h	8 h	12 h	p-Value[a]
ATIII (IU/mL)	1.05 ± 0.10	1.06 ± 0.10	1.05 ± 0.10	0.7819
APTT (s)	30.5 ± 2.2	30.4 ± 2.3	30.6 ± 2.1	0.1166
PT (s)	12.9 ± 0.9	12.9 ± 0.9	13.0 ± 0.9	0.0735
FII (IU/mL)	0.86 ± 0.12	0.86 ± 0.13	0.85 ± 0.12	0.4857
FV (IU/mL)	0.95 ± 0.25	0.94 ± 0.26	0.93 ± 0.23	0.6969
FVII (IU/mL)	0.96 ± 0.24	0.96 ± 0.26	0.94 ± 0.23	0.3108
FXIII (IU/mL)	1.42 ± 0.37	1.33 ± 0.34	1.38 ± 0.32	0.0676
Protein C (IU/mL)	0.98 ± 0.16	0.97 ± 0.16	0.96 ± 0.16	0.1877
C3a (μg/mL)	4.9 ± 1.0	4.8 ± 1.0	4.8 ± 1.0	0.5815
C5a (μg/mL)	57.5 ± 12.3	56.2 ± 11.4	56.4 ± 11.0	0.2123

Abbreviations: APTT, activated partial thromboplastin time; ATIII, antithrombin III; C, complement component; F, factor; PT, prothrombin time.
[a]p-Value determined using repeated-measures one-way analysis of variance.

The only quality control specification for apheresis plasma is FVIII (≥0.70 IU/mL). The mean FVIII concentration after a 12-h post-collection hold prior to freezing was 1.16 ± 0.24 IU/mL (Figure 1a). However, apheresis plasma is also manufactured into cryoprecipitate, for which vWF:Ag and fibrinogen are also quality control requirements (Figure 1b,c). There was no significant differences in vWF:Ag ($p = 0.1281$) or fibrinogen ($p = 0.3100$) concentrations after a 12-h hold post collection.

Additional coagulation parameters, namely PT, APTT, FII, FV, FVII, FXIII, ATIII and protein C, were maintained during the 12-h hold of apheresis plasma (Table 1). There was no significant difference in complement activation during the 12-h hold, as determined by C3a or C5a measurements (Table 1). IgG concentrations were also stable (Figure 1d).

DISCUSSION

Plasma demand is continually growing, and one way to expand clinical supply is to extend the time to freezing for clinical apheresis plasma. This enables clinical collections to be made at collection centres further from the processing facility and allows for unexpected transport delays. This study evaluated the quality of apheresis plasma after an extended hold at worst case scenario blood shipping temperatures (26°C) for 6, 8 and 12 h post collection, and found that the quality was not significantly affected by the increased time-to-freezing. The quality of the apheresis plasma, particularly for FVIII, vWF and fibrinogen, aligned with that reported in the literature [8].

FVIII is the most labile coagulation factor in plasma and therefore used as a quality indicator for plasma components [9, 10]. FVIII is known to degrade with time, especially when plasma is stored at room temperature [10]; however, there needs to be a balance between the feasibility of processing and quality. Although there were no significant differences in FVIII concentrations in apheresis plasma frozen 6, 8 and 12 h post collection, one component failed to meet the minimum specification (≥0.70 IU/mL) when frozen 12 h post collection. This component was from a group O positive donor. Blood groups contribute to the levels of some coagulation factors. In particular, group O donors have the lowest FVIII plasma levels [6, 9]. The CoE guidelines require the average FVIII concentration to be ≥0.70 IU/mL, and a minimum of 90% of the units tested must meet this value [3]. Therefore, the plasma in this study would meet this specification (mean FVIII in plasma frozen 12 h post collection was 1.16 ± 0.24 IU/mL with a pass rate of 95%).

vWF plays a major role in blood coagulation; its primary functions are binding to other proteins and in platelet adhesion to wound sites [11]. The vWF antigen concentrations were not significantly different in apheresis plasma frozen 12 h post collection ($p = 0.1281$). One plasma component had a much higher concentration (1.88–1.94 IU/mL), which was above the normal reference range (0.50–1.60 IU/mL). This component could be considered an outlier, but even if it were excluded from this study, it would not affect the outcome, and therefore the data were not excluded from the analyses.

Fibrinogen plays a crucial role in haemostasis, as it is the substrate for fibrin clot formation. The fibrinogen concentrations did not change

in apheresis plasma that was frozen 12 h post collection ($p = 0.3100$). One plasma component had a fibrinogen concentration above the normal range (2–4 g/L), which was outside two SDs of the mean and could also be considered an outlier.

Due to growing demand for IgG components, there is a strong interest in IgG levels in plasma for fractionation, and it has become a commonly used quality indicator for plasmapheresis collections [12]. There were no significant differences in IgG concentrations in apheresis plasma frozen 12 h post collection ($p = 0.5106$).

The main limitation of the study was that the samples at each time point were from a segment and not from a routine apheresis-split component. Although segments are used for routine quality control testing, there can be some discrepancies compared to the blood pack due to differing rates of freezing and thawing. As a segment would freeze faster, better preservation of coagulation factors would be expected. However, the segment would also thaw faster and may be exposed to higher temperatures, thus potentially degrading the coagulation factors. To use apheresis-split components at each time point, 3 times as many plasma components would be required, and because of the high demand in plasma, this was not feasible at the time of the study.

In conclusion, there were no significant differences in the quality of apheresis-derived plasma that was frozen 6, 8 and 12 h post collection while being maintained at a temperature of 26°C. The recommendation based on the data presented here is that the time-to-freezing apheresis clinical plasma could be extended from 6 to 12 h post collection. This allows collections from more donor centres further from blood processing centres, and for unexpected transport delays from closer donor centres, thereby increasing supply to meet the growing demand for plasma. The findings from this study have been confirmed in a manufacturing validation, and the data has been submitted to our regulatory authority for review.

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K.M.W., D.C.M. and S.I. designed the research study, K.M.W., R.G.W., E.M. and D.M. performed the research, and collected and analysed the data and K.M.W. wrote the first draft of the manuscript. All authors critically reviewed and edited the manuscript.

CONFLICT OF INTEREST STATEMENT

D.C.M. has received research funding from Macopharma and Cryogenics Holdings in the past 2 years. The other authors have no conflict of interests to declare.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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INTERNATIONAL FORUM

Vox Sanguinis ISBT International Society of Blood Transfusion

International Forum on Management of Blood Donors with Culture-Positive Platelet Donations: Summary

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INTRODUCTION

Bacterial contamination of platelets (Plts) stored at room temperature represents the highest risk for infection and fatality of any transfusable blood component. Contamination mitigation procedures include optimization of arm disinfection, sample diversion of initially collected blood, shorter dating or pathogen inactivation to avoid or prevent log-phase bacterial growth as well as detection of bacteria through immunoassay for bacterial antigens, assays for bacterial metabolic activity or bacterial culture [1]. Point-of-care assays are operationally challenging for transfusion services, which often prefer that the blood collection establishments (BCEs) employ enhanced culture strategies or provide pathogen-reduced Plt products.

The aim of this International Forum was to understand not only how Plt bacterial culture strategies are being employed by blood collectors internationally but, more specifically, how culture results impact donor management and ongoing donation eligibility. Using a proposed consensus definition of bottle and unit culture results following an automated sample incubator alarm [2], we desired to catalogue BCE procedures and donor work-ups as well as evaluation strategies for various scenarios, ranging from machine false-positive alarms to potentially bacteremic blood donors.

SUMMARY OF RESPONSES

Participants

An invitation to participate in this International Forum was sent to 19 BCEs either routinely employing Plt bacterial culture or with extensive Plt culture quality control programs. Of these, 14 (74%) respondents provided program demographics and summarized their culture practices. The Brazil—Hospital Centre data were not included in this summary as that centre only produces pathogen-reduced Plts, with 1% cultured at outdate for quality control purposes. Here we describe responses from the remaining 13 BCEs.

We provided a rubric for respondents categorizing four levels of suspicion for Plt unit contamination with organisms of varying pathogenicity and environmental, skin or bloodstream mechanisms for bag entry (Table 1). Participants described their evaluation strategies for each of five scenarios, selecting from within the same 12 options to investigate the equipment used in the collection, observe phlebotomy and culture inoculation techniques, address various aspects of donor health and make decisions about future donor eligibility. Respondents were provided the opportunity to provide additional details if there were additional considerations or actions important in investigation of the various scenarios. The five scenarios included definite or likely

TABLE 1 Rubrics for alarm investigation outcomes.

Alarm investigation definitions	Confirmed positive	Unconfirmed positive (inoculation contamination or unit self-sterilization)	Indeterminate	False positive (machine error)	False negative (confirmed septic reaction, visually abnormal or point-of-care test-positive and culture-positive)
Culture instrument alarm	Yes	Yes	Yes	Yes	No
Instrument bottle culture	Pos	Pos	Pos/ND	Neg	ND
Single or split Plt unit culture	Pos	Neg	Neg/ND	Neg/ND	Pos (unit-only or unit and patient)
Any additional Plt unit culture(s)	Pos/Neg/ND	Neg	ND	Neg/ND	Pos/Neg/ND

Note: *Organism definitions*: Low-pathogenicity skin organisms: *Cutibacteria*; Pathogenic skin organisms: *Staphylococcus epidermidis*/*Staphylococcus aureus*; Unusual environmental opportunistic pathogens: *Acinetobacter*/*Serratia* species; Common pathogens: dental (*Streptococcus viridans*), ENT/respiratory (*Streptococcus pneumoniae*) and GI/GU (coliforms) organisms; Common pathogens with specific disease associations: *Streptococcus bovis* group, nutritionally-deficient *Streptococci*. *Levels of suspicion for unit contamination*: Definite: any confirmed positive or false negative; Likely: unconfirmed positive/indeterminate with a common pathogen or bottle positive and unit associated with a transfusion reaction; Possible but unlikely: unconfirmed positive/indeterminate with any skin organism or indeterminate with an environmental pathogen; Unlikely: false positive or indeterminate with any skin or environmental organism and units uneventfully transfused.
Abbreviations: ENT, ear, nose and throat; GI, gastrointestinal; GU, genitourinary; ND, not done; Neg, negative; Plt, platelet; Pos, positive.

unit contamination with three different types of organisms (common pathogens, environmental opportunistic pathogens and skin bacteria), possible but unlikely unit contamination in settings often considered inoculation contamination, and machine false-positive alarms.

Participants were also asked to describe specific evaluation practices around process monitoring, transfusion service-reported contamination, multi-donor investigations of pools associated with culture alarms and the approach to donors associated with more than one alarm.

BCE demographics and culture strategies

A broad range of collectors participated, from small hospital-based programs to large national services providing hundreds of thousands of Plt doses annually (Table 2, reported alphabetically by country names). All but one collector culture all their apheresis collections or whole blood-derived (WBD) Plt pools prior to distribution. The Brazil—Regional Centre cultures 20% of units prior to distribution for quality control purposes. Each employs a slightly different strategy and offers different Plt products suspended in either 100% plasma or ~65% platelet additive solution (PAS)/~35% plasma.

Ten of the 13 centres use the BacT/ALERT (bioMérieux, Inc.; Marcy-l'Étoile, France) automated culture platform, while the remainder culture using a Bactec device (Becton, Dickinson & Co.; Franklin Lakes, NJ, USA). One BacT centre and all three Bactec centres perform only aerobic cultures, with the balance equally inoculating Plt samples into both aerobic and anaerobic bottles. There was a near-even split between sampling of the apheresis mother bag (6 of 13) versus sampling each split unit daughter bag (7 of 13). All but one centre inoculates 8–10 mL into each bottle, with one collector (Oman) culturing 3 mL in aerobic paediatric Bactec bottles. A 12–24-h sampling delay was associated with a 5-day outdate (excepting one U.S. centre employing a WBD Plt pooling set approved for only 5-day storage, inoculating at least 36 h after collection). Most of the remaining centres inoculate beginning 36–48 h after collection and have 7-day Plt dating, although two with 7-day dating noted sampling some time on the day after collection. Four centres (31%) send Plt units into the labelling and distribution process directly after sampling, while three (23%) wait 6 h for the sample to register in their product database and the balance (6, 46%) wait 12 h before releasing to afford additional time for early-alarming units not to be distributed. Five participants published their culture protocols and provided references [3–13].

Written procedures

All 13 (100%) centres have procedures governing overall management of bacterial culture alarms and policies specifying immediate actions after alarms sound. Differences between responding centres are summarized in Table 3 (sorted by annual Plt distributions). All but one centre (12, 92%) specifies timeframes for hospital transfusion service notification of involved co-components. The majority (11, 85%) state within their procedures when blood collector physician involvement is required to adjudicate result interpretations and make donor eligibility decisions, while only 8 (62%) procedurally require donor healthcare provider (HCP) assessments to assist in eligibility determination after selected alarm events. Of 11 centres producing WBD Plt pools, 6 (55%) specify how to resolve potential contamination to the individual donor level and have procedures governing the work-up of donors involved in positive pools.

Despite a broad array of bottle and product culture result scenarios, only 10 (77%) prescribe elements of some investigations, to include equipment, staff and donor assessments. Just over one-third (5, 38%) prescribed donor communications for specific confirmed pathogens, though only 1 of 13 centres (Australia) specify tests required to continue donating after sentinel pathogens are identified (e.g., endoscopy for *Streptococcus bovis*). More than half (7, 54%) can identify donors associated with prior alarm investigations.

TABLE 2 Participant demographics and summary of culture practices.

Country		Australia	Brazil	Canada-1	Canada-2	Denmark	New Zealand	Oman	Sweden	UK-England	UK-Scotland	USA-1	USA-2	USA-3
Service type (100% culture, except as noted)		National	Regional (20% QC)	National	Regional	National	National	Hospital-based	Regional	National	National	National	Community	Hospital-based
Annual Plt doses distributed		>120,000	<20,000	>120,000	20,000–120,000	20,000–120,000	<20,000	<20,000	<20,000	>120,000	20,000–120,000	>120,000	20,000–120,000	<20,000
Products offered (suspension medium and expiration)	Apheresis Plts	PAS 7d	Plasma 5d	Plasma 7d	Plasma 7d	PAS and Plasma 7d	PAS 7d	Plasma 5d	PAS 7d	Plasma 7d	PAS and Plasma 7d	Plasma 7d	Plasma 7d	Plasma 7d
	Buffy coat WBD Plt pools	PAS 7d	N/A	Plasma 7d	N/A	PAS 7d	PAS 7d	Plasma 5d	N/A	Plasma 7d	PAS 7d	N/A	N/A	N/A
	PRP WBD Plt pools	N/A	Plasma 5d	N/A	Plasma 7d	N/A	N/A	Plasma 5d	PAS 7d	N/A	N/A	N/A	Plasma 5d	N/A
Culture instrument used		BacT/ALERT VIRTUO	Bactec FX Adult bottle	BacT/ALERT 3D	BacT/ALERT 3D	BacT/ALERT 3D	BacT/ALERT 3D	Bactec FX Paediatric bottle	Bactec FX Adult bottle	BacT/ALERT 3D	BacT/ALERT 3D	BacT/ALERT 3D and VIRTUO	BacT/ALERT 3D	BacT/ALERT VIRTUO
Apheresis sample source		Daughter bags	Mother bag	Daughter bags	Mother bag	Mother bag	Mother bag	Mother bag	Daughter bags	Daughter bags	Mother bag	Daughter bags	Daughter bags	Daughter bags
WBD Plt pool sample source		Pooling bag	Pooling bag	Pooling bag	Pooling bag	Pooling bag	Pooling bag	Pool of 3–1 mL unit samples	Pooling bag	Pooling bag	Pooling bag	N/A	Pooling bag	N/A
Culture inoculation volume		8–10 mL each BPA/BPN	8 mL Aerobic	10 mL each BPA/BPN	8–10 mL each BPA/BPN	8–10 mL BPA	8 mL each BPA/BPN	3 mL Aerobic	10 mL Aerobic	8 mL each BPA/BPN	8 mL each BPA/BPN	8 mL each BPA/BPN	8 mL each BPA/BPN	8 mL each BPA/BPN
Inoculation timing after collection (hours)		≥36	≥24	≥36	≥48	≥Day 1	≥36	Apheresis: ≥12 WBD Plts: ≥16	≥Day 1	≥36	≥36	≥48	Apheresis: ≥48 WBD Plts: ≥36	≥48
Hold time before product release (hours)		None	12	6	12	None	None	12	None	6	6	12	12	12
Implementation year		2021	2015	2017	2015	2012	2009	2013	2016	2011	2007	2021	2021	2021

Abbreviations: BPA, BacT/ALERT aerobic bottle; BPN, BacT/ALERT anaerobic bottle; N/A, not applicable; PAS, platelet additive solution; Plt, platelet; PRP, platelet-rich plasma; QC, quality control; WBD, whole blood-derived.

TABLE 3 Blood collection establishment procedures for donor and product management related to incubator alarms.

Country	Australia	Canada-1	UK-England	USA-1	USA-2	Canada-2	Denmark	UK-Scotland	Brazil	New Zealand	Oman	Sweden	USA-3	%
Annual Plt distributions	≥120,000				20,000–120,000				<20,000					
Timeframe for hospital transfusion service notification of involved co-components	X	X	X	X	X	X		X	X	X	X	X	X	92
When/what types of culture alarm scenarios must be evaluated by a blood centre physician	X	X	X	X	X	X	X		X	X	X		X	85
Required investigation elements by each combination of bottle/product culture results	X	X	X	X	X	X	X		X	X	X			77
Required medical assessments of donors for continued donation	X	X	X	X		X		X			X		X	62
Mechanisms to identify/notify suspected source among donors associated with contaminated pools	X		X	N/A				X	X	X	X		N/A	55
Mechanisms to identify donors associated with any/some type of past alarm event		Some	Some	Any	Any	Any		Any			Any			54
Prescribed donor communications for various confirmed-positive organisms	X	X	X	X									X	38
Specific donor procedures/tests mandated for specific organisms	X													8

Abbreviations: N/A, not applicable; Plt, platelet.

Of the 10 potential procedural elements queried, high-volume collectors (≥120,000 units per year) noted they had written procedures for 80%–90% these, while all others reported <7 of 10. The Scandinavian centres reported the fewest written procedures at 3 or 4 of 10. All high-volume BCEs prescribe donor communications for certain organisms, while only one of the nine lower volume BCEs do so.

Alarm evaluation requirements

We provided five scenarios to blood collectors, requesting that they identify important elements of investigations for each (Table 1). We described (1) generally non-contaminant, common pathogens isolated from Plt components or only in alarming bottles; (2) unusual environmental opportunistic pathogens isolated from Plt components or only in alarming bottles but associated with a transfusion reaction; (3) low-pathogenicity or pathogenic skin organisms isolated from Plt components or only in alarming bottles but associated with a transfusion reaction; (4) skin or environmental pathogens in alarming bottles, but not in products or associated with a transfusion reaction and (5) machine error false positives.

There was some disagreement with assignment of various bacterial species to the rubric categories, which likely prompted a few evaluators to pursue investigations more or less aggressively. For example, as specified in some free text comments in responses, a few centres felt that some organisms classified as environmental could also be considered common pathogens (such as *Acinetobacter* or *Serratia* species) and that not all skin organisms are as easily relegated to a 'pathogenic skin organism' category, as they may be common pathogens (*Staphylococcus aureus*) or even bacteremic in the right setting (coagulase-negative *Staphylococci*).

Figure 1 summarizes elements felt to be important within each type of investigation. Agreement was high that blood collector physician oversight and involvement are key for definite and likely contamination events with potentially bacteremic organisms (both common pathogens and some environmental organisms). Almost 40% of centres (5 of 13) required donor HCP referral and evaluation in the setting of confirmed or likely contamination with a unit containing a dental, ear, nose and throat (ENT)/respiratory or gastrointestinal (GI)/genitourinary (GU) pathogen. Two of these five centres also require specific testing for donors to continue donating. At least three other collectors not among the five stating the need for referral in this setting noted that they did have procedures for HCP referral. One centre not requesting donors be specifically evaluated by their HCP mentioned that blood centre staff often conduct a phone interview that determines additional donor actions. Although it is sometimes difficult to know if community HCPs have conducted a thorough search for a source of intermittent bacteraemia, blood collector physician involvement in decision making about donor eligibility may underlie some of these answers, particularly if donors are deferred during investigations of certain organisms. In less than half of centres, a surveillance flag is placed in donors' records in the setting of definite/likely contamination

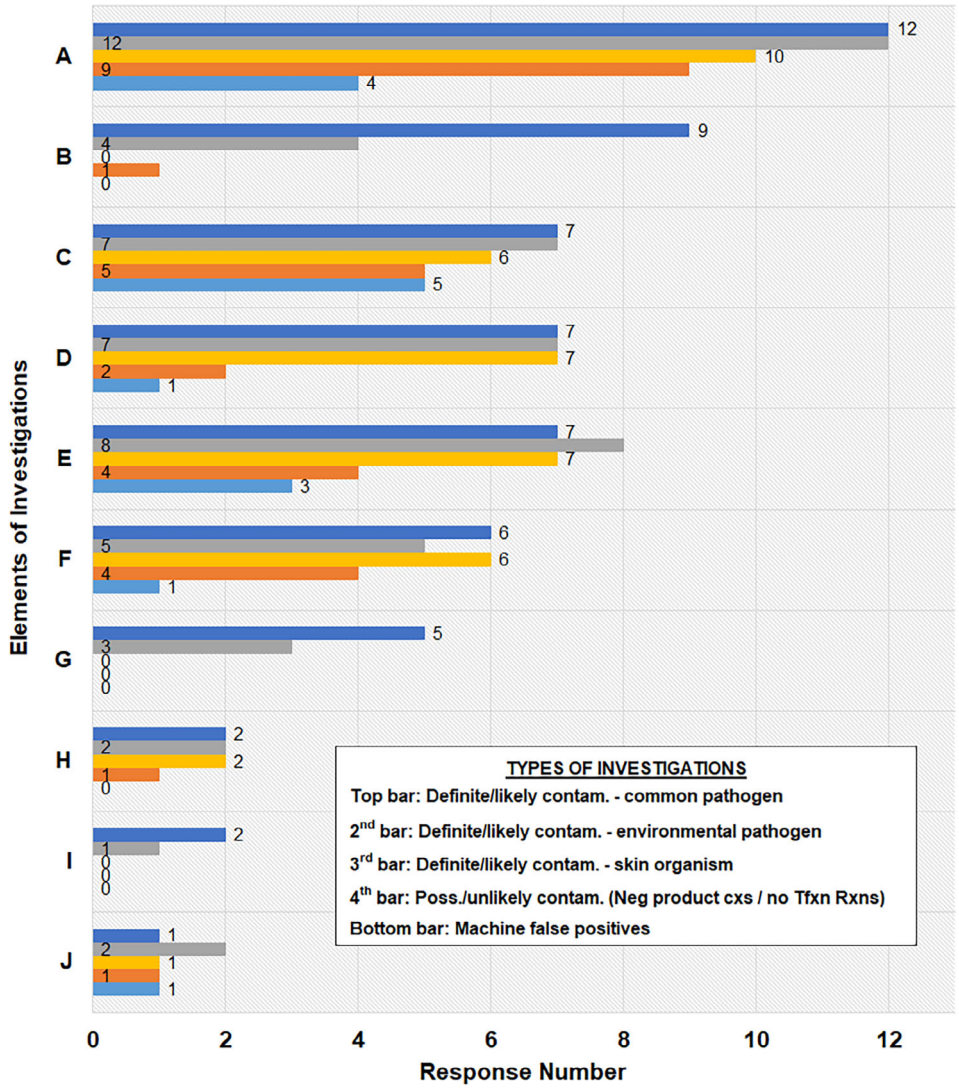

FIGURE 1 Important elements within various types (inset) of investigations. A: Blood collector physician direction/evaluation of alarm investigation. B: Blood collector physician calls donor to take complete medical history & provide preliminary donor advice. C: Sterility chain review (apheresis kits, sterile welders, incubators, etc.). D: Phlebotomist observation for proper skin decontamination technique. E: Technologist observation for proper sampling and inoculation technique. F: Place a surveillance flag on non-deferred donors' records should they be associated with another alarm. G: Donor healthcare practitioner referral and evaluation. H: Donor arm inspection by blood collector staff for difficult skin decontamination. I: Specific testing is required for approval to continue to donate. J: Enhanced inspection of bags for microtears and leaks only observable under pressure.

should they be associated with another alarm, but permanent deferral of identified donors was not reported by any participant under any of the five presented scenarios. Concerns that might determine donor deferral are discussed later under 'Considerations in Determination of Future Donor Eligibility'.

For any type of identified organism, for units definitely/likely to be contaminated, approximately half the time (6–8 of 13, 46%–62%) sterility chain review and observation of associated phlebotomy and culture inoculation technicians were considered standard. Since environmental or skin organisms may be introduced by defective supplies or suboptimal technique during phlebotomy or inoculation, it is noteworthy that fewer respondents felt these were critical investigations for skin isolates. Free text comments, however, suggest that routine quality audits of technique, environmental monitoring and supply inspection may be considered sufficient to identify staff or other issues. Still others noted that staff investigation should be triggered for individuals involved in multiple potential contaminations (i.e., not machine false-positive results). One might also predict that lesser emphasis be placed upon staff technique evaluations for organisms likely to have been introduced during transient donor bacteraemia (i.e., common dental, ENT/respiratory and GI/GU pathogens), but changing accepted routines may require more effort to individualize management than is justifiable.

Few centres (≤2, 15%) felt strongly about conducting formal donor arm inspections. Although routine arm inspections for potential drug use and obvious skin conditions precluding effective disinfection are performed at every donation, this may not be focused enough for donors

associated with more than one likely Plt unit contamination with skin or other organisms. Operationalizing the inspection of a specific donor's arm is difficult, and centres may have to rely on routine arm inspection rather than calling donors in for an off-schedule examination. The role of identifying donors associated with more than one culture alarm assumes greater importance in this setting. Less than half of centres do this, however.

Two collectors conduct enhanced bag examination when there is concern about external introduction of bacteria during manufacture or storage. Most centres, therefore, rely upon routine product inspection for obvious leaks or tears.

Finally, in the case of machine alarms in the absence of culturable bacteria (false positives), while four centres noted that no investigations are required (data not shown), six collectors stated that supply review and/or technologist observation may be important, likely for proper bottle and machine use practices. Known causes of false-positive alarms include temperature changes during bottle loading and incubator electrical supply spikes, including the potential for bottle lot issues. Three collectors still required the centre physician sign-off on false-positive alarms.

BCE actions with reverse notification from transfusion services

While collector 'reverse notification' of Plt bacterial contamination by a transfusion service might occur in the setting of a suspected septic transfusion reaction, this question posed a different scenario: that is, routine hospital re-assessment of un-transfused products culture-negative at blood collector distribution. That could be by secondary culture, bacterial antigen testing or surrogate measures like pH, or it could be as simple as a concerning visual inspection. Two respondents were hospitals not routinely performing secondary testing, as they are both the collector and the transfusion service provider. In these facilities, other collectors' Plts suspected of harbouring bacteria would trigger a collector notification.

Most BCEs, after third-party notification of product sterility concerns, described variations of co-component control, product and co-component re-culture and management as a false alarm (negative cultures) or a false-negative contamination event (positive cultures). At least two collectors specifically commented that secondary testing of any type is not conducted by hospitals in their country.

Monitoring and trending of culture data

Although all collectors monitor overall alarm rates and the outcomes of their investigations, two of three U.S. centres reported periodic instead of real-time monitoring and trending.

Considerations in determination of future donor eligibility

While three collectors did not report any key donor management considerations, almost all remaining collectors felt that identifying donors with difficult-to-disinfect arms or with the potential for intermittent bacteraemia is important and that they should be deferred unless a reversible cause of contamination can be identified and corrected, particularly if donors are involved in prior (not false-positive) alarm events. The need for case-by-case transfusion medicine expert assessment of the appropriate extent of this search was mentioned by 5 of 10 of respondents. Interestingly, 6 of 13 BCEs noted that if an HCP cleared the donor, they would be accepted regardless of work-up details. Only two collectors felt that certain organisms should result in automatic donor deferral, although one can conceive that the combination of organism and reversibility of its source could easily identify donors who are not appropriate for Plt donation (e.g., an odontogenic organism in a donor unable to address poor dentition).

Donor management with alarming WBD Plt pools

Themes emerging regarding management of donors of alarming pooled WBD Plts included the need to contact and interview all donors in many circumstances, lesser concern about *Cutibacteria* and less pathogenic skin contaminants, measured responses in proportion to the potential for underlying donor health issues and intermittent bacteraemia (prompting HCP referrals) and culture results of available co-components. Of 11 collectors producing WBD Plts, 2 do not generally investigate donors contributing to pooled Plts.

Management of donors associated with prior alarm events

Although not very common outside false-positive alarm involvement, case-by-case evaluation appears warranted when collectors are able to identify donors associated with prior potential or confirmed contamination events. The need to defer donors involved in credible repeat events was noted by several centres, including those with multiple skin organism contaminations and scarred arms.

DISCUSSION

This International Forum was designed to understand the various Plt bacterial culture strategies being employed by blood collectors internationally and specifically focus on how culture results impact donor evaluation and ongoing donation eligibility decisions.

Overall, the scope of this International Forum was not to define what the 'best practice' or ideal response in a particular setting should be but simply report on the overarching extant response patterns for donor evaluations. Although we saw general consensus in the management for two extremes of scenarios (definite/likely contamination events with bacteraemic organisms and machine error false positives), for the other scenarios there was notable variability in responses and divergence in practices. Some of these could be due to subjectivity in interpretation of the proposed scenarios.

Bacterial contamination of Plts stored at room temperature represents the highest risk for infection as well as fatality among all transfusable blood components [1]. Standards have been proposed that require blood collection and transfusion service members to detect and limit bacterial contamination in all Plt components. From a hospital transfusion service perspective, protocols are generally in place to help clinicians to recognize and manage transfusion reactions, including those potentially caused by bacterial contamination [14]. Guidance exists for BCEs regarding Plt bacterial culture procedures, immediate management of donors and assessment of future donation eligibility, but adoption remains inconsistent [14, 15]. This International Forum highlights significant within-country as well as between-country variability and heterogeneity with regard to specific scenarios for donor evaluation and future eligibility. These divergent BCE practices underscore the need for standardization and protocolization for these approaches and the need for adoption of more proactive approaches to managing donors with culture-positive Plt donations.

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INTERNATIONAL FORUM

Vox Sanguinis ISBT International Society of Blood Transfusion

International Forum on Management of Blood Donors with Culture-Positive Platelet Donations: Responses

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UNITED STATES—COMMUNITY CENTRE

Ruchika Goel

1. Type of blood collection service:
   Community
2. Annual whole blood and automated red cell collection volume:
   50,000–199,999
3. Annual distribution of platelet doses:
   60,000–119,999
4. Type(s) of bacterially cultured leuko-reduced platelet offered:
   Apheresis platelets in plasma
   Platelet-rich plasma whole blood-derived pools in plasma
   Platelet-rich plasma whole blood-derived individual donor units in plasma
5. Percentage of non-pathogen-reduced units cultured:
   100
6. Culture instrument(s) used:
   BacT/ALERT
   Biomerieux
7. Culture sample source:
   Apheresis individual products (mother bags) are sampled. Pooled platelets are individually sampled. Whole blood-derived individual split units are pooled and sampled at the transfusing facility.
8. Total volume removed for culture:
   16 mL from each product
9. Timing of sample inoculation after collection:
   Forty-eight hours after collection for apheresis platelets and 36 h after filtration for whole blood-derived pooled platelets
10. Culture inoculation practices:
    Aerobic culture:
    One bottle is inoculated per product: For example, if single product per donor, one bottle is inoculated; if double product, two bottles are inoculated and if triple product, three bottles are inoculated.
    Aerobic inoculation volume:
    8 mL
    Anaerobic culture:
    One bottle is inoculated per product: For example, if single product per donor, one bottle is inoculated. If double product, two bottles are inoculated, and if triple product, three bottles are inoculated.
    Anaerobic inoculation volume:
    8 mL
11. Hold time before product release:
    Twelve hours after BacT sampling before release to hospitals
12. Longest allowable platelet expiration:
    Pooled platelets are BacT-sampled at 1.5 days (36 h) post filtration and expire 5 days from when the whole blood-derived platelet was collected. Apheresis platelet products that are BacT-sampled at 48 h post collection expire after 7 days of collection.
13. Implementation year of current culture method:
    2021

14. Culture protocol references:
    No
15. Written procedures:
    Overall management of screening platelet culture instrument alarms
    Immediate product actions to be taken upon culture alarm
    Timeframe for hospital transfusion service notification of involved co-components
    Required investigation elements (donor assessment, staff observations) by each combination of bottle/product culture results
    When/what types of culture alarm scenarios must be evaluated by a blood centre physician
    Mechanisms to identify donors associated with any type of past alarm event
16. Evaluations required in definite or likely contamination with a common pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Blood collector physician calls donor to take a complete medical history and provide preliminary donor advice
    Donor arm inspection by blood collector staff for difficult skin decontamination
    Phlebotomist observation for proper skin decontamination technique
    Technologist observation for proper sampling and inoculation technique
    Other: Defer the donor until PCP evaluation and clearance
17. Evaluations required in definite or likely contamination with a low-pathogenicity or pathogenic skin organism:
    Phlebotomist observation for proper skin decontamination technique
    Technologist observation for proper sampling and inoculation technique
18. Evaluations required in definite or likely contamination with an environmental opportunistic pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Blood collector physician calls donor to take a complete medical history and provide preliminary donor advice
    Phlebotomist observation for proper skin decontamination technique
    Technologist observation for proper sampling and inoculation technique
19. Evaluations required in possible but unlikely contamination:
    Phlebotomist observation for proper skin decontamination technique
    Technologist observation for proper sampling and inoculation technique
20. Evaluations required in unlikely contamination (e.g., false positives):
    Phlebotomist observation for proper skin decontamination technique
    Technologist observation for proper sampling and inoculation technique
21. Policy for managing co-components, confirming results and investigating your potential false-negative results:
    We quarantine and discard associated products (co-components). We send the positive bottle out for additional gram stain and further culturing. We investigate with Biomerieux to see if there were temperature issues, electrical issues, too many bottles loaded or cell issues if there is a false positive. Cells are also calibrated if they have a positive bottle as per Biomerieux instructions to identify false positives.
22. Monitor and investigate screening culture results in real time:
    Yes
23. Factors weighed in donor management:
    Whether a source of infection was identified and definitively treated
    Whether underlying risks for intermittent bacteraemia or sub-optimal skin disinfection exist
    Whether donors' healthcare providers have cleared them, regardless of workup
    Whether a search for infection or risk factors has been completed to the satisfaction of the blood collector's physician
    Whether the donor has been involved in prior alarm events (excluding false-positive alarms)
24. Donor management for definite or likely contamination:
    If a pooled unit has definite or likely contamination, we would contact all donors and advise evaluation/testing and assess for future donation eligibility.
25. Management of donors with repeat alarm events:
    We maintain a spreadsheet of donors who have had an alarm event. If they have more than one alarm event, the blood centre physician will contact and evaluate the donor and advise evaluation/testing as needed.

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OMAN

Arwa Z. Al-Riyami and Zakariya Al-Muharrmi

1. Type of blood collection service:
   Hospital-based
2. Annual whole blood and automated red cell collection volume:
   50,000–199,999
3. Annual distribution of platelet doses:
   2000–19,999
4. Type(s) of bacterially cultured leuko-reduced platelet offered:
   Apheresis platelets in plasma

Platelet-rich plasma whole blood-derived individual donor units in plasma

Buffy coat whole blood-derived pools in plasma

5. Percentage of non-pathogen-reduced units cultured:
   100
6. Culture instrument(s) used:
   BACTEC FX blood culture system
   Becton Dickinson
7. Culture sample source:
   For platelet-rich plasma and buffy coat platelet units, pooled samples from individual platelet units (pooled samples from three platelet units) are inoculated into one culture bottle. If positive BACTEC FX results on the pooled sample, repeat sampling from individual units is performed for repeat BACTEC. For apheresis units, sampling is performed per unit without pooling.
8. Total volume removed for culture:
   3 mL of the pooled sample or apheresis unit
9. Timing of sample inoculation after collection:
   Minimum of 12 h after donation or 16–24 h after processing the blood unit (whenever feasible)
10. Culture inoculation practices:
    Aerobic culture:
    One aerobic paediatric culture bottle
    Aerobic inoculation volume:
    3 mL
    Anaerobic culture:
    None
    Anaerobic inoculation volume:
    None
11. Hold time before product release:
    Minimum of 12 h after incubation of the blood culture bottle. If no alarms, the units can be issued upon request. Incubation is performed for a total of 5 days on all inoculated culture bottles. Our procedure necessitates repeat testing from single units by BACTEC FX if the pooled sample BACTEC FX result is positive. In addition, a gram stain is performed on the pooled culture bottle and, on any positives, BACTEC FX results on individual samples. All results need to be reviewed by a microbiologist to determine the clinical significance of the identified organism. Decision on the use of the units and how to manage the donor is made based on the results of the repeat testing, gram stain/culture results and on the clinical suspicion of any pathogenic organism. If the organism was found to be pathogenic, the microbiologist and the transfusion medicine physician will work out a plan on how to manage the donor.
12. Longest allowable platelet expiration:
    The expiration date for all types of platelet units is 5 days. At times of platelet shortages, the expiration can be extended up to 7 days with authorization by the transfusion medicine physician if the platelet units were tested negative by BACTEC FX after 5 days.
13. Implementation year of current culture method:
    2013
14. Culture protocol references:
    No
15. Written procedures:
    Overall management of screening platelet culture instrument alarms
    Immediate product actions to be taken upon culture alarm
    Timeframe for hospital transfusion service notification of involved co-components
    Required investigation elements (donor assessment, staff observations) by each combination of bottle/product culture results
    When/what types of culture alarm scenarios must be evaluated by a blood centre physician
    Required medical assessments of donors for continued donation
    Mechanisms to identify and notify the suspected source among multiple donors associated with contaminated pools
    Mechanisms to identify donors associated with any type of past alarm event
16. Evaluations required in definite or likely contamination with a common pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Blood collector or allied health staff calls the donor to take a complete medical history and provide preliminary donor advice
    Phlebotomist observation for proper skin decontamination technique
    Technologist observation for proper sampling and inoculation technique
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
17. Evaluations required in definite or likely contamination with a low-pathogenicity or pathogenic skin organism:
    Blood collector physician direction/evaluation of alarm investigation
    Phlebotomist observation for proper skin decontamination technique
    Technologist observation for proper sampling and inoculation technique
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
18. Evaluations required in definite or likely contamination with an environmental opportunistic pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Phlebotomist observation for proper skin decontamination technique
    Technologist observation for proper sampling and inoculation technique
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
19. Evaluations required in possible but unlikely contamination:
    Blood collector physician direction/evaluation of alarm investigation
    Phlebotomist observation for proper skin decontamination technique

Technologist observation for proper sampling and inoculation technique

Sterility chain review (apheresis kits, sterile welders, incubators, etc.)

20. Evaluations required in unlikely contamination (e.g., false positives):

Blood collector physician direction/evaluation of alarm investigation

Other: If the false-positive alarms are repetitive, the blood bank performs a sterility chain review

21. Policy for managing co-components, confirming results and investigating potential false-negative results:

Most of transfused platelets in our institution are collected in-house and hence tested using the BACTEC FX system. If a septic reaction is associated with a platelet unit that is collected in-house, the related components (red cells and plasma units) are traced and cultured if still in the inventory. If these were transfused, the transfusion records of the recipients are reviewed to assess for any reactions during the transfusion. Our facility does not use point-of-issue rapid bacterial testing. If a suspected septic reaction is associated with a platelet unit(s) obtained from another blood collector, the blood collector is notified with the reaction to trace the donor for investigation.

22. Monitor and investigate screening culture results in real time:

Yes

23. Factors weighed in donor management:

Whether a source of infection was identified and definitively treated

Whether underlying risks for intermittent bacteraemia or suboptimal skin disinfection exist

Other: The decision is made by the microbiologist and the transfusion medicine physician based on the type of the organism identified

24. Donor management for definite or likely contamination:

Decision to call the donor is decided on an individual basis by the transfusion medicine physician and the microbiologist. This is dependent on the results of the repeat investigations on the individual units and the type of the organism identified. The donor contact information is available in our electronic system in case they need to be contacted.

25. Management of donors with repeat alarm events:

All records of platelet testing include donation identification numbers, which can be used to trace the donor's past donations if required. Our electronic system captures all in-house past donations information and contact information of all donors.

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UNITED STATES—HOSPITAL CENTRE

Cyril Jacquot

1. Type of blood collection service:

Hospital-based

2. Annual whole blood and automated red cell collection volume:

<50,000

3. Annual distribution of platelet doses:

<2000

4. Type(s) of bacterially cultured leuko-reduced platelet offered:

Apheresis platelets in plasma

5. Percentage of non-pathogen-reduced units cultured:

100

6. Culture instrument(s) used:

BacT Virtuo

BioMerieux

7. Culture sample source:

Individual split units

8. Total volume removed for culture:

16 mL per platelet unit, so 32 mL for a double

9. Timing of sample inoculation after collection:

Forty-eight hours after collection (large-volume delayed sampling [LVDS] 48h pathway)

10. Culture inoculation practices:

Aerobic culture:

1

Aerobic inoculation volume:

8 mL

Anaerobic culture:

1

Anaerobic inoculation volume:

8 mL

11. Hold time before product release:

At least 12 h after inoculation

12. Longest allowable platelet expiration:

Up to 7 days

13. Implementation year of current culture method:

2021

14. Culture protocol references:

No

15. Written procedures:

Overall management of screening platelet culture instrument alarms

Immediate product actions to be taken upon culture alarm

Timeframe for hospital transfusion service notification of involved co-components

When/what types of culture alarm scenarios must be evaluated by a blood centre physician
Required medical assessments of donors for continued donation
Prescribed donor communications (oral or written) with various confirmed-positive organisms

16. Evaluations required in definite or likely contamination with a common pathogen:
Blood collector physician direction/evaluation of alarm investigation
Blood collector physician calls donor to take a complete medical history and provide preliminary donor advice
Donor arm inspection by blood collector staff for difficult skin decontamination
Phlebotomist observation for proper skin decontamination technique
Technologist observation for proper sampling and inoculation technique
Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
17. Evaluations required in definite or likely contamination with a low-pathogenicity or pathogenic skin organism:
Blood collector physician direction/evaluation of alarm investigation
Phlebotomist observation for proper skin decontamination technique
Technologist observation for proper sampling and inoculation technique
Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
18. Evaluations required in definite or likely contamination with an environmental opportunistic pathogen:
Blood collector physician direction/evaluation of alarm investigation
Phlebotomist observation for proper skin decontamination technique
Technologist observation for proper sampling and inoculation technique
Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
19. Evaluations required in possible but unlikely contamination:
Blood collector physician direction/evaluation of alarm investigation
20. Evaluations required in unlikely contamination (e.g., false positives):
Blood collector physician direction/evaluation of alarm investigation
21. Policy for managing co-components, confirming results and investigating your potential false-negative results:
We would quarantine and culture any available co-components on the BacT Virtuo if there was any concern. Our blood bank rarely performs Pan Genera Detection testing.
22. Monitor and investigate screening culture results in real time:
Periodic monitoring only
23. Factors weighed in donor management:
Whether a source of infection was identified and definitively treated
Whether underlying risks for intermittent bacteraemia or sub-optimal skin disinfection exist
Whether donors' healthcare providers have cleared them, regardless of workup
Whether a search for infection or risk factors has been completed to the satisfaction of the blood collector's physician
Whether the donor has been involved in prior alarm events (excluding false-positive alarms)
24. Donor management for definite or likely contamination:
Emphasize the need for donor to be evaluated by their physician in order to complete any necessary treatment. Future eligibility is contingent on resolution of the issue.
25. Management of donors with repeat alarm events:
Has not occurred but would rely on donor's physician evaluation to determine whether deferral is warranted. If in doubt, would lean towards deferral to ensure patient safety.

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CANADA—NATIONAL CENTRE

Sandra Ramirez-Arcos, Aditi Khandelwal and Mindy Goldman

1. Type of blood collection service:
National
2. Annual whole blood and automated red cell collection volume:
≥400,000
3. Annual distribution of platelet doses:
≥120,000
4. Type(s) of bacterially cultured leuko-reduced platelet offered:
Apheresis platelets in plasma
Buffy coat whole blood-derived pools in plasma
5. Percentage of non-pathogen-reduced units cultured:
100
6. Culture instrument(s) used:
BACT/ALERT 3D System
BioMerieux
7. Culture sample source:
Apheresis split unit and buffy coat pool
8. Total volume removed for culture:
20 mL buffy coat pool, 20 mL per apheresis dose
9. Timing of sample inoculation after collection:
Sample inoculation is done at least 36 h post collection.
10. Culture inoculation practices:
Aerobic culture:
One for each apheresis dose, one for each buffy coat pool
Aerobic inoculation volume:
10 mL

Anaerobic culture:
One for each apheresis dose and one per buffy coat pool
Anaerobic inoculation volume:
10 mL

11. Hold time before product release:
There is a hold time of at least 6 h post inoculation prior to release.
12. Longest allowable platelet expiration:
Both apheresis and buffy coat pool platelets are stored for 7 days.
13. Implementation year of current culture method:
2017
14. Culture protocol references:
Yes
https://pubmed.ncbi.nlm.nih.gov/17319821/
https://pubmed.ncbi.nlm.nih.gov/21615745/
https://pubmed.ncbi.nlm.nih.gov/28653472/
https://pubmed.ncbi.nlm.nih.gov/33140420/
15. Written procedures:
Overall management of screening platelet culture instrument alarms
Immediate product actions to be taken upon culture alarm
Timeframe for hospital transfusion service notification of involved co-components
Required investigation elements (donor assessment, staff observations) by each combination of bottle/product culture results
When/what types of culture alarm scenarios must be evaluated by a blood centre physician
Required medical assessments of donors for continued donation
Mechanisms to identify donors associated with some, but not all past alarm events
Prescribed donor communications (oral or written) with various confirmed-positive organisms
16. Evaluations required in definite or likely contamination with a common pathogen:
Blood collector physician direction/evaluation of alarm investigation
Blood collector physician calls donor to take a complete medical history and provide preliminary donor advice
Donor healthcare practitioner referral and evaluation
Specific testing is required for approval to continue to donate
Phlebotomist observation for proper skin decontamination technique
Technologist observation for proper sampling and inoculation technique
Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
Other: Check for previous test positivity or transfusion reaction associated with donor
17. Evaluations required in definite or likely contamination with a low-pathogenicity or pathogenic skin organism:
Blood collector physician direction/evaluation of alarm investigation
Donor arm inspection by blood collector staff for difficult skin decontamination
Phlebotomist observation for proper skin decontamination technique
Technologist observation for proper sampling and inoculation technique
Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
Other: If there is a phlebotomist or technologist associated with multiple positive tests, there will be quality assurance processes for follow-up of technique
18. Evaluations required in definite or likely contamination with an environmental opportunistic pathogen:
Blood collector physician direction/evaluation of alarm investigation
Donor arm inspection by blood collector staff for difficult skin decontamination
Phlebotomist observation for proper skin decontamination technique
Technologist observation for proper sampling and inoculation technique
Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
19. Evaluations required in possible but unlikely contamination:
Other: Same as per evaluations required in definite or likely contamination with an environmental opportunistic pathogen, only if unconfirmed positive.
20. Evaluations required in unlikely contamination (e.g., false positives):
Technologist observation for proper sampling and inoculation technique
Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
21. Policy for managing co-components, confirming results and investigating your potential false-negative results:
Co-components may be tested at the hospital or returned to the blood operator for testing.
22. Monitor and investigate screening culture results in real time:
Yes
23. Factors weighed in donor management:
Whether a source of infection was identified and definitively treated
Whether underlying risks for intermittent bacteraemia or suboptimal skin disinfection exist
Whether donors' healthcare providers have cleared them, regardless of work-up
Whether a search for infection or risk factors has been completed to the satisfaction of the blood collector's physician

Whether the donor has been involved in prior alarm events (excluding false-positive alarms)
Other: Case-by-case assessment looking at factors noted above

24. Donor management for definite or likely contamination:
We culture remaining co-components to try to identify which donor may be the source of contamination (e.g., red cell units). Donor advice and deferral would depend on the organism; for almost all organisms, such as skin organisms, a flag would be added to all the donor's files, if no co-components were positive on culture, but the donors would not be notified.
25. Management of donors with repeat alarm events:
This is handled on a case-by-case basis, taking into account the organism, review of arm disinfection and processes for potential causes, and questioning of the donor looking for factors. If no reversible cause can be found (usually the case), the donor will be deferred.

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UNITED KINGDOM—SCOTLAND

Katie Hands, Lorna McLintock and Heather Mitchell

1. Type of blood collection service:
National
2. Annual whole blood and automated red cell collection volume:
50,000–199,999
3. Annual distribution of platelet doses:
20,000–59,999
4. Type(s) of bacterially cultured leuko-reduced platelet offered:
Apheresis platelets in plasma
Apheresis platelets in additive solution/plasma
Buffy coat whole blood-derived pools in additive solution/plasma
5. Percentage of non-pathogen-reduced units cultured:
100
6. Culture instrument(s) used:
BACT/ALERT 3D
Biomerieux
7. Culture sample source:
Apheresis mother bag or whole blood-derived pool
8. Total volume removed for culture:
16 mL
9. Timing of sample inoculation after collection:
Samples are taken a minimum of 36 h following collection.
10. Culture inoculation practices:
Aerobic culture:
1
Aerobic inoculation volume:
8 mL
Anaerobic culture:
1
Anaerobic inoculation volume:
8 mL
11. Hold time before product release:
All platelet components (apheresis and whole blood-derived) are held for 6 h before release.
12. Longest allowable platelet expiration:
All platelet components (apheresis and whole blood-derived) have a 7-day shelf-life from donation.
13. Implementation year of current culture method:
2007
14. Culture protocol references:
No
15. Written procedures:
Overall management of screening platelet culture instrument alarms
Immediate product actions to be taken upon culture alarm
Timeframe for hospital transfusion service notification of involved co-components
Required medical assessments of donors for continued donation
Mechanisms to identify and notify the suspected source among multiple donors associated with contaminated pools
Mechanisms to identify donors associated with any type of past alarm event
16. Evaluations required in definite or likely contamination with a common pathogen:
Blood collector physician direction/evaluation of alarm investigation
Blood collector physician calls donor to take a complete medical history and provide preliminary donor advice
Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
Other: Environmental monitoring records are reviewed.
17. Evaluations required in definite or likely contamination with a low-pathogenicity or pathogenic skin organism:
Blood collector physician direction/evaluation of alarm investigation
Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
Other: Environmental monitoring record review
18. Evaluations required in definite or likely contamination with an environmental opportunistic pathogen:
Blood collector physician direction/evaluation of alarm investigation
Sterility chain review (apheresis kits, sterile welders, incubators, etc.)

Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
Other: Environmental monitoring record are reviewed.

19. Evaluations required in possible but unlikely contamination:
Blood collector physician direction/evaluation of alarm investigation
Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
Other: Environmental monitoring review
20. Evaluations required in unlikely contamination (e.g., false positives):
Blood collector physician direction/evaluation of alarm investigation
Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
Other: Environmental monitoring record are reviewed
21. Policy for managing co-components, confirming results, and investigating your potential false-negative results:
We do not have point-of-care testing or other surrogates in use. If there was concern regarding a component, for example, if a blood bank detected platelet aggregates, each case would be managed as a quality event, and depending on level of concern, co-components may be recalled.
22. Monitor and investigate screening culture results in real time:
Yes
23. Factors weighed in donor management:
Whether a search for infection or risk factors has been completed to the satisfaction of the blood collector's physician
Certain organisms should result in automatic deferral from blood donation, regardless of the donor's medical workup
Whether the donor has been involved in prior alarm events (excluding false-positive alarms)
Other: All of these events are managed on a case-by-case basis; we do not currently have a written policy for this
24. Donor management for definite or likely contamination:
The donor health check forms will be reviewed for each contributing donor, the nature of the bacteria considered and decisions regarding donor eligibility to continue to donating made following this.
25. Management of donors with repeat alarm events:
Donors will be deferred permanently if there has been a previous alarm event.

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BRAZIL—HOSPITAL CENTRE

Silvano Wendel, Patrícia Scuracchio and Roberta Fachini

1. Type of blood collection service:
Hospital-based
2. Annual whole blood and automated red cell collection volume:
<50,000
3. Annual distribution of platelet doses:
2000–19,999
4. Type(s) of bacterially cultured leuko-reduced platelet offered:
None
5. Percentage of non-pathogen-reduced units cultured:
One
6. Culture instrument(s) used:
BacT/Alert 3D System
BioMérieux
7. Culture sample source:
The sources of our culture samples are apheresis mother bag and individual units of platelet-rich whole blood-derived plasma.
8. Total volume removed for culture:
8 mL of each unit
9. Timing of sample inoculation after collection:
Pathogen reduction was implemented in our service in March 2017 through Intercept Blood System treatment for apheresis platelets in plasma and pools of platelet-rich whole blood-derived plasma; therefore, platelet bacterial culture routine was discontinued at the same time, except for quality control purposes and for all red blood cells units. Before the implementation of pathogen reduction technology, a culture for bacterial contamination was performed on the first day of storage, between 12 and 24 h after the collection of apheresis units or platelet-rich whole blood-derived plasma. Currently, we culture our products only for quality control purposes, so the time for sampling inoculation is preferably the last day of expiration (fifth day). For red blood cells units, they are cultured 12 h after production.
10. Culture inoculation practices:
Aerobic culture:
1
Aerobic inoculation volume:
8 mL
Anaerobic culture:
0
Anaerobic inoculation volume:
0
11. Hold time before product release:
Since we culture the units only for quality control, we wait for 7 days because they are not used for transfusion. The units that are submitted to pathogen reduction technology can be released for transfusion when the product is available and after the treatment by the Intercept Blood System. In relation to red blood cells, they are released 24 h after inoculation.

12. Longest allowable platelet expiration:
    As both apheresis platelets in plasma and platelets-rich whole blood-derived plasma pools are pathogen-reduced, they have a 7-day shelf-life.
13. Implementation year of current culture method:
    1997
14. Culture protocol references:
    Yes

Wendel S, Morato LE, Fontão-Wendel F, Toledo R. Double, double, toil and trouble. *Transfusion*. 2005;45:1241.

15. Written procedures:
    Overall management of screening platelet culture instrument alarms
    Immediate product actions to be taken upon culture alarm
    Timeframe for hospital transfusion service notification of involved co-components
    Required investigation elements (donor assessment, staff observations) by each combination of bottle/product culture results
    When/what types of culture alarm scenarios must be evaluated by a blood centre physician
    Required medical assessments of donors for continued donation
    Mechanisms to identify and notify the suspected source among multiple donors associated with contaminated pools
    Mechanisms to identify donors associated with some, but not all past alarm events
16. Evaluations required in definite or likely contamination with a common pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Blood collector physician calls donor to take a complete medical history and provide preliminary donor advice
    Donor healthcare practitioner referral and evaluation
    Donor arm inspection by blood collector staff for difficult skin decontamination
    Enhanced inspection of bags for microtears and leaks only observable under pressure
    Phlebotomist observation for proper skin decontamination technique
    Technologist observation for proper sampling and inoculation technique
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
    Permanently defer all such donors
    Place a surveillance flag on non-deferred donor records should they be associated with another alarm
    Other: In case of an unexplained transfusion reaction, we also report the event to the hospital risk department and to the National Agency of Sanitary Monitoring, a governmental health department.
17. Evaluations required in definite or likely contamination with a low-pathogenicity or pathogenic skin organism:
    Blood collector physician direction/evaluation of alarm investigation
    Blood collector physician calls donor to take a complete medical history and provide preliminary donor advice
    Donor healthcare practitioner referral and evaluation
    Specific testing is required for approval to continue to donate
    Donor arm inspection by blood collector staff for difficult skin decontamination
    Phlebotomist observation for proper skin decontamination technique
    Technologist observation for proper sampling and inoculation technique
    Other: In case of an unexplained transfusion reaction, we report the event to the hospital risk department and to the National Agency of Sanitary Monitoring, a governmental health department.
18. Evaluations required in definite or likely contamination with an environmental opportunistic pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Blood collector physician calls donor to take a complete medical history and provide preliminary donor advice
    Donor arm inspection by blood collector staff for difficult skin decontamination
    Enhanced inspection of bags for microtears and leaks only observable under pressure
    Phlebotomist observation for proper skin decontamination technique
    Technologist observation for proper sampling and inoculation technique
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
19. Evaluations required in possible but unlikely contamination:
    Enhanced inspection of bags for microtears and leaks only observable under pressure
    Technologist observation for proper sampling and inoculation technique
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
20. Evaluations required in unlikely contamination (e.g., false positives):
    Technologist observation for proper sampling and inoculation technique
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
21. Policy for managing co-components, confirming results and investigating your potential false-negative results:
    Because pathogen reduction routine was implemented in our service, currently we culture only red blood cells; therefore, in cases of positive results, we place the red blood cells and plasma in quarantine until receipt of results of culture bottles. If there is any bacterial growth, the component is discarded regardless of the type of bacteria isolated, that is, skin bacteria or other type of organism. The

apheresis or whole blood-derived platelet co-components are not discarded because they are submitted for pathogen inactivation.

22. Monitor and investigate screening culture results in real time:
    Yes
23. Factors weighed in donor management:
    Whether a source of infection was identified and definitively treated
    Whether underlying risks for intermittent bacteraemia or sub-optimal skin disinfection exist
    Whether donors' healthcare providers have cleared them, regardless of workup
    Whether a search for infection or risk factors has been completed to the satisfaction of the blood collector's physician
    Certain organisms should result in automatic deferral from blood donation, regardless of the donor's medical workup
    Whether the donor has been involved in prior alarm events (excluding false-positive alarms)
24. Donor management for definite or likely contamination:
    In case of a pooled unit with definite or likely contamination, the donor is called for a careful interview with the blood collector physician, and according to bacteria isolated and/or possibility of bacteraemia, the donor will be deferred definitively. Since the implementation of pathogen reduction technology, we did not have any case of septic transfusion reaction or suspicion of bacterial contamination.
25. Management of donors with repeat alarm events:
    In case of donor with a second instance of contamination with pathogenic skin organisms or common pathogens, he or she is called for an interview with the blood collector physician in order to investigate the medical history, source of infection and risk factors, besides counselling and deferring him for future donations.

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SWEDEN

Sandra M. Pettersson and Jesper Bengtsson

1. Type of blood collection service:
   Regional
2. Annual whole blood and automated red cell collection volume:
   <50,000
3. Annual distribution of platelet doses:
   2000–19,999
4. Type(s) of bacterially cultured leuko-reduced platelet offered:
   Apheresis platelets in additive solution/plasma
   Platelet-rich plasma whole blood-derived pools in additive solution/plasma
5. Percentage of non-pathogen-reduced units cultured:
   100
6. Culture instrument(s) used:
   BACTEC FX
   BD
7. Culture sample source:
   From each transfusable unit, when it is in the storage bag. If the apheresis is divided in two bags, we will take one sample of each unit. We sample the whole blood-derived pool unit after leukocyte filtration.
8. Total volume removed for culture:
   15 mL
9. Timing of sample inoculation after collection:
   Pooled platelet units are sampled after pooling on the day after the collection. Apheresis units are also sampled on the day after collection. If day of collection is a Friday, two samples are taken: the first on the same day and the second on Monday (due to staff management).
10. Culture inoculation practices:
    Aerobic culture:
    Pooled platelet units: 1 culture bottle/unit = 1 culture bottle/4–5 collections. Apheresis platelet units: as many culture bottles as the number of transfusable platelet units from that apheresis (e.g., if there are 2 units from one apheresis collection, there will be two culture bottles for that collection)
    Aerobic inoculation volume:
    10 mL
    Anaerobic culture:
    None
    Anaerobic inoculation volume:
    NA
11. Hold time before product release:
    There is no hold time; the platelet units are included in stock when release is possible due to HIV, hepatitis B, hepatitis C and syphilis.
12. Longest allowable platelet expiration:
    7 days from collection
13. Implementation year of current culture method:
    2016
14. Culture protocol references:
    No
15. Written procedures:
    Overall management of screening platelet culture instrument alarms
    Immediate product actions to be taken upon culture alarm
    Timeframe for hospital transfusion service notification of involved co-components

16. Evaluations required in definite or likely contamination with a common pathogen:
    Enhanced inspection of bags for microtears and leaks only observable under pressure
    Technologist observation for proper sampling and inoculation technique
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
17. Evaluations required in definite or likely contamination with a low-pathogenicity or pathogenic skin organism:
    Enhanced inspection of bags for microtears and leaks only observable under pressure
    Technologist observation for proper sampling and inoculation technique
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
18. Evaluations required in definite or likely contamination with an environmental opportunistic pathogen:
    Enhanced inspection of bags for microtears and leaks only observable under pressure
    Technologist observation for proper sampling and inoculation technique
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
19. Evaluations required in possible but unlikely contamination:
    Enhanced inspection of bags for microtears and leaks only observable under pressure
    Technologist observation for proper sampling and inoculation technique
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
20. Evaluations required in unlikely contamination (e.g., false positives):
    Enhanced inspection of bags for microtears and leaks only observable under pressure
    Technologist observation for proper sampling and inoculation technique
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
21. Policy for managing co-components, confirming results and investigating your potential false-negative results:
    We do not have an active surveillance of evaluating the potential false-negative results. However, we do investigate suspected transfusion reactions with a culture analysis.
22. Monitor and investigate screening culture results in real time:
    Yes
23. Factors weighed in donor management:
    None
24. Donor management for definite or likely contamination:
    We do not inform donors about the contamination
25. Management of donors with repeat alarm events:
    Normally we do not mention about the contamination in the donor record

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UNITED KINGDOM—ENGLAND

Susan R. Brailsford and Joanne Tossell

1. Type of blood collection service:
   National
2. Annual whole blood and automated red cell collection volume:
   ≥400,000
3. Annual distribution of platelet doses:
   ≥120,000
4. Type(s) of bacterially cultured leuko-reduced platelet offered:
   Apheresis platelets in plasma
   Buffy coat whole blood-derived pools in additive solution/plasma
5. Percentage of non-pathogen-reduced units cultured:
   100
6. Culture instrument(s) used:
   BacTAlert 3D
   BioMerieux
7. Culture sample source:
   Apheresis individual splits/whole blood-derived pool
8. Total volume removed for culture:
   16 mL
9. Timing of sample inoculation after collection:
   Sampled at least 36 h post collection
10. Culture inoculation practices:
    Aerobic culture:
    1
    Aerobic inoculation volume:
    8 mL
    Anaerobic culture:
    1
    Anaerobic inoculation volume:
    8 mL
11. Hold time before product release:
    6 h
12. Longest allowable platelet expiration:
    7 days
13. Implementation year of current culture method:
    2011
14. Culture protocol references:
    Yes

Brailsford SR, Tossell J, Morrison R, McDonald CP, Pitt TL. Failure of bacterial screening to detect Staphylococcus aureus: the English experience of donor follow-up. *Vox Sang*. 2018;113:540–546.

McDonald C, Allen J, Brailsford S, Roy A, Ball J, Moule R, et al. Bacterial screening of platelet components by National Health Service Blood and Transplant, an effective risk reduction measure. *Transfusion*. 2017;57:1122–31.

15. Written procedures:
    Overall management of screening platelet culture instrument alarms
    Immediate product actions to be taken upon culture alarm
    Timeframe for hospital transfusion service notification of involved co-components
    Required investigation elements (donor assessment, staff observations) by each combination of bottle/product culture results
    When/what types of culture alarm scenarios must be evaluated by a blood centre physician
    Required medical assessments of donors for continued donation
    Mechanisms to identify and notify the suspected source among multiple donors associated with contaminated pools
    Mechanisms to identify donors associated with some, but not all past alarm events
    Prescribed donor communications (oral or written) with various confirmed-positive organisms
16. Evaluations required in definite or likely contamination with a common pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Blood collector physician calls donor to take a complete medical history and provide preliminary donor advice
    Place a surveillance flag on non-deferred donor records should they be associated with another alarm
    Other: Some of these donors will be contacted and their history taken, but the majority will be returned to panel with information placed on their medical history.
17. Evaluations required in definite or likely contamination with a low-pathogenicity or pathogenic skin organism:
    Blood collector physician direction/evaluation of alarm investigation
    Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
18. Evaluations required in definite or likely contamination with an environmental opportunistic pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Other: Cutibacteria will be recorded on donor history. But donor remains on panel, and no further action is taken unless there have been previous reactions. For pathogenic skin organisms, further investigations and discussion are carried out with the donor, which in most cases will result in deferral.
19. Evaluations required in possible but unlikely contamination:
    Blood collector physician direction/evaluation of alarm investigation
    Donor arm inspection by blood collector staff for difficult skin decontamination
    Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
    Other: Will depend on the organism
20. Evaluations required in unlikely contamination (e.g., false positives):
    Other: All initial reactive flags on the system result in donors being suspended; all false-positive results will result in donor being returned to donor panel.
21. Policy for managing co-components, confirming results and investigating your potential false-negative results:
    Any visually abnormal packs would be cultured, and depending on the outcome further investigation will be carried out, which may involve swabbing of donor and/or further investigations by their healthcare provider.
22. Monitor and investigate screening culture results in real time:
    Yes
23. Factors weighed in donor management:
    Whether a source of infection was identified and definitively treated
    Whether underlying risks for intermittent bacteraemia or suboptimal skin disinfection exist
    Whether a search for infection or risk factors has been completed to the satisfaction of the blood collector's physician
    Certain organisms should result in automatic deferral from blood donation, regardless of the donor's medical workup
    Whether the donor has been involved in prior alarm events (excluding false-positive alarms)
24. Donor management for definite or likely contamination:
    Follow-up of donors will depend on significance of the organism. In some cases, all donors will be contacted, and depending on 'risk', sent for further review.
25. Management of donors with repeat alarm events:
    If donors have had more than one positive bacterial screening result, we may need to suspend them. This is relatively rare but we have suspended some regular apheresis platelet donors who had very scarred venipuncture sites.

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BRAZIL—REGIONAL CENTRE

Luiz Amorim, Maria Esther Lopes and Margarida Pêcego

1. Type of blood collection service:
   Regional
2. Annual whole blood and automated red cell collection volume:
   50,000–199,999
3. Annual distribution of platelet doses:
   2000–19,999
4. Type(s) of bacterially cultured leuko-reduced platelet offered:

Apheresis platelets in plasma
Platelet-rich plasma whole blood-derived pools in plasma
Platelet-rich plasma whole blood-derived individual donor units in plasma

5. Percentage of non-pathogen-reduced units cultured:
   20
6. Culture instrument(s) used:
   Biotec
   Becton & Dickson
7. Culture sample source:
   Apheresis mother bag and whole blood-derived pool
8. Total volume removed for culture:
   8 mL
9. Timing of sample inoculation after collection:
   24–36 h after collection. In general, 24 h; however, if the infectious disease tests are not ready in the first 24 h, we can inoculate up to 36 h after collection.
10. Culture inoculation practices:
    Aerobic culture:
    1
    Aerobic inoculation volume:
    8 mL
    Anaerobic culture:
    0
    Anaerobic inoculation volume:
    0
11. Hold time before product release:
    12 h
12. Longest allowable platelet expiration:
    Five days for any platelet types, regardless of whether they are cultured or not. According to Brazilian regulation, even for pathogen-reduced platelets the expiration time is 5 days.
13. Implementation year of current culture method:
    2015
14. Culture protocol references:
    No
15. Written procedures:
    Overall management of screening platelet culture instrument alarms
    Immediate product actions to be taken upon culture alarm
    Timeframe for hospital transfusion service notification of involved co-components
    Required investigation elements (donor assessment, staff observations) by each combination of bottle/product culture results
    When/what types of culture alarm scenarios must be evaluated by a blood centre physician
    Mechanisms to identify and notify the suspected source among multiple donors associated with contaminated pools
16. Evaluations required in definite or likely contamination with a common pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Blood collector physician calls donor to take a complete medical history and provide preliminary donor advice
    Phlebotomist observation for proper skin decontamination technique
    Technologist observation for proper sampling and inoculation technique
17. Evaluations required in definite or likely contamination with a low-pathogenicity or pathogenic skin organism:
    Blood collector physician direction/evaluation of alarm investigation
    Phlebotomist observation for proper skin decontamination technique
    Technologist observation for proper sampling and inoculation technique
18. Evaluations required in definite or likely contamination with an environmental opportunistic pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Phlebotomist observation for proper skin decontamination technique
    Technologist observation for proper sampling and inoculation technique
19. Evaluations required in possible but unlikely contamination:
    Blood collector physician direction/evaluation of alarm investigation
20. Evaluations required in unlikely contamination (e.g., false positives):
    None of the above
21. Policy for managing co-components, confirming results and investigating your potential false-negative results:
    The co-components are discarded if the culture is positive, regardless of the level of suspicion or the bacterial species. If co-components are available, they are also cultured. If not, we warn the hospital to which the co-components were shipped and we ask them to send back the co-components to us; if the result is false negative, we investigate the collection methods and procedures for the specific case.
22. Monitor and investigate screening culture results in real time:
    Yes
23. Factors weighed in donor management:
    Whether a source of infection was identified and definitively treated
    Whether underlying risks for intermittent bacteraemia or sub-optimal skin disinfection exist
    Whether the donor has been involved in prior alarm events (excluding false-positive alarms)
24. Donor management for definite or likely contamination:
    We call all the involved donors for a donor healthcare physician evaluation. The decision about deferral (or not) and future eligibility is taken according to the physician evaluation.
25. Management of donors with repeat alarm events:
    Donors are permanently deferred if they have a confirmed second instance of a definite or likely contamination.

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CANADA—REGIONAL CENTRE

Marc Germain and Christian Renaud

1. Type of blood collection service:
   Regional
2. Annual whole blood and automated red cell collection volume:
   200,000–399,999
3. Annual distribution of platelet doses:
   20,000–59,999
4. Type(s) of bacterially cultured leuko-reduced platelet offered:
   Apheresis platelets in plasma
   Platelet-rich plasma whole blood-derived pools in plasma
5. Percentage of non-pathogen-reduced units cultured:
   100
6. Culture instrument(s) used:
   BacT/Alert
   Biomerieux
7. Culture sample source:
   Apheresis mother bag or pooled platelet if derived from whole blood
8. Total volume removed for culture:
   20 mL
9. Timing of sample inoculation after collection:
   At least 48 h
10. Culture inoculation practices:
    Aerobic culture:
    1
    Aerobic inoculation volume:
    8–10 mL
    Anaerobic culture:
    1
    Anaerobic inoculation volume:
    8–10 mL
11. Hold time before product release:
    At least 12 h after inoculation
12. Longest allowable platelet expiration:
    Seven days after collection, both for apheresis and whole blood-derived platelets
13. Implementation year of current culture method:
    2015
14. Culture protocol references:
    Yes

Delage G, Bernier F. Bacterial culture of platelets with the large volume delayed sampling approach: a narrative review. *Ann Blood*. 2021;6:30.

15. Written procedures:
    Overall management of screening platelet culture instrument alarms
    Immediate product actions to be taken upon culture alarm
    Timeframe for hospital transfusion service notification of involved co-components
    Required investigation elements (donor assessment, staff observations) by each combination of bottle/product culture results
    When/what types of culture alarm scenarios must be evaluated by a blood centre physician
    Required medical assessments of donors for continued donation
    Mechanisms to identify donors associated with any type of past alarm event
16. Evaluations required in definite or likely contamination with a common pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Blood collector physician calls donor to take a complete medical history and provide preliminary donor advice
    Donor healthcare practitioner referral and evaluation
    Specific testing is required for approval to continue to donate
    Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
17. Evaluations required in definite or likely contamination with a low-pathogenicity or pathogenic skin organism:
    Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
    Other: Procedures have recently been downsized. A second positive result will trigger a complete medical evaluation
18. Evaluations required in definite or likely contamination with an environmental opportunistic pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Blood collector physician calls donor to take a complete medical history and provide preliminary donor advice
    Donor healthcare practitioner referral and evaluation
    Specific testing is required for approval to continue to donate
    Donor arm inspection by blood collector staff for difficult skin decontamination
    Enhanced inspection of bags for microtears and leaks only observable under pressure
    Phlebotomist observation for proper skin decontamination technique
    Technologist observation for proper sampling and inoculation technique
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
    Place a surveillance flag on non-deferred donors' records should they be associated with another alarm

19. Evaluations required in possible but unlikely contamination:
    Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
20. Evaluations required in unlikely contamination (e.g., false positives)
    None
21. Policy for managing co-components, confirming results and investigating your potential false-negative results:
    Depending on the situation, co-components might be recalled and tested for sterility
22. Monitor and investigate screening culture results in real time:
    Yes
23. Factors weighed in donor management:
    Whether a source of infection was identified and definitively treated
    Whether underlying risks for intermittent bacteremia or sub-optimal skin disinfection exist
    Whether donors' healthcare providers have cleared them, regardless of workup
    Whether a search for infection or risk factors has been completed to the satisfaction of the blood collector's physician
    Whether the donor has been involved in prior alarm events (excluding false-positive alarms)
24. Donor management for definite or likely contamination:
    It varies depending on the pathogen; if it suggests an underlying systemic infection, the suggested investigation will be more aggressive and extensive; much less so if it is a skin contaminant.
25. Management of donors with repeat alarm events:
    Donor will be permanently deferred unless there is a very convincing explanation that exonerates the donor as the source of contamination.

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NEW ZEALAND

Sarah L. Morley and Ronald So

1. Type of blood collection service:
   National
2. Annual whole blood and automated red cell collection volume:
   50,000–199,999
3. Annual distribution of platelet doses:
   2000–19,999
4. Type(s) of bacterially cultured leuko-reduced platelet offered:
   Apheresis platelets in additive solution/plasma
   Buffy coat whole blood-derived pools in additive solution/plasma
5. Percentage of non-pathogen-reduced units cultured:
   100
6. Culture instrument(s) used:
   BacT/Alert 3D
   bioMerieux
7. Culture sample source:
   Apheresis mother bag and whole blood derived pool
8. Total volume removed for culture:
   16 mL
9. Timing of sample inoculation after collection:
   The sample is collected for testing at or after 36 h from post collection. Inoculation generally occurs between 36 and 48 h post collection.
10. Culture inoculation practices:
    Aerobic culture:
    1
    Aerobic inoculation volume:
    8 mL
    Anaerobic culture:
    1
    Anaerobic inoculation volume:
    8 mL
11. Hold time before product release:
    The product is released following inoculation.
12. Longest allowable platelet expiration:
    Both apheresis platelets in additive solution/plasma and buffy-coat-derived platelets in additive solution/plasma have a maximum expiration of 7 days from collection.
13. Implementation year of current culture method:
    2009
14. Culture protocol references:
    Yes

Dickson M, Dinesh D. Bacterial contamination of platelet concentrates produced in New Zealand. *N Z Med J*. 2013;126:12–21.

NZBS. NZBS Haemovigilance Report (Produced Annually). Available from: https://www.nzblood.co.nz/clinical-information/haemovigilance-programme

15. Written procedures:
    Overall management of screening platelet culture instrument alarms
    Immediate product actions to be taken upon culture alarm
    Timeframe for hospital transfusion service notification of involved co-components
    Required investigation elements (donor assessment, staff observations) by each combination of bottle/product culture results
    When/what types of culture alarm scenarios must be evaluated by a blood centre physician
    Mechanisms to identify and notify the suspected source among multiple donors associated with contaminated pools
16. Evaluations required in definite or likely contamination with a common pathogen:

Blood collector physician direction/evaluation of alarm investigation.

Phlebotomist observation for proper skin decontamination technique

17. Evaluations required in definite or likely contamination with a low-pathogenicity or pathogenic skin organism:
    Blood collector physician direction/evaluation of alarm investigation
    Phlebotomist observation for proper skin decontamination technique
18. Evaluations required in definite or likely contamination with an environmental opportunistic pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Phlebotomist observation for proper skin decontamination technique
    Other: This may require inspection of bags and sterility chain review if the physician requires it
19. Evaluations required in possible but unlikely contamination:
    Blood collector physician direction/evaluation of alarm investigation
20. Evaluations required in unlikely contamination (e.g., false positives):
    None
21. Policy for managing co-components, confirming results and investigating your potential false-negative results:
    All platelet components, plasma components and any red cell components from the same collection/unit with an initial positive result will be traced and quarantined. If available, the platelets/red cells associated with a BacT/ALERT Initial Positive will be sent to a local accredited microbiology laboratory for gram stain, culture and identification. If the initial positive result confirms a false-positive result, the associated plasma will be directed to fractionation. If the initial positive result returns an indeterminate or confirmed positive result, the associated plasma will be discarded. The definition a confirmatory test and the classification of true positive and true negative follows the Association for the Advancement of Blood and Biotherapies guidelines as described in the Association Bulletin # 04-07.2: False-Negative investigation. If the transfusion reaction included a temperature rise of 1.5°C or more, a gram stain is requested immediately. If the gram stain and/or culture is positive, all platelet components, plasma components and any red cell components associated are quarantined. The transfusion medicine specialist will determine whether the other components should be released from quarantine, discarded or sent for culture
22. Monitor and investigate screening culture results in real time:
    Yes
23. Factors weighed in donor management:
    Other: In general, donor investigations are not triggered unless the transfusion medicine specialist determines that this is required
24. Donor management for definite or likely contamination:
    In general, New Zealand Blood Service does not trigger a donor investigation in the event that a pooled unit has definite or likely contamination.
25. Management of donors with repeat alarm events:
    New Zealand Blood Service does not routinely identify donors associated with past positive culture events.

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UNITED STATES—NATIONAL CENTRE

Hany Kamel, Mary Townsend and Courtney Hopkins

1. Type of blood collection service:
   National
2. Annual whole blood and automated red cell collection volume:
   ≥400,000
3. Annual distribution of platelet doses:
   ≥120,000
4. Type(s) of bacterially cultured leuko-reduced platelet offered:
   Apheresis platelets in plasma
5. Percentage of non-pathogen-reduced units cultured:
   100
6. Culture instrument(s) used:
   Bact 3D and Virtuo
   BioMerieux
7. Culture sample source:
   Individual split units
8. Total volume removed for culture:
   16 mL
9. Timing of sample inoculation after collection:
   ≥48 h
10. Culture inoculation practices:
    Aerobic culture:
    One per individual split unit
    Aerobic inoculation volume:
    8 mL
    Anaerobic culture:
    One per individual split unit
    Anaerobic inoculation volume:
    8 mL
11. Hold time before product release:
    12 h
12. Longest allowable platelet expiration:
    Seven days, using large volume delayed sampling protocol

13. Implementation year of current culture method:
    2021
14. Culture protocol references:
    No
15. Written procedures:
    Overall management of screening platelet culture instrument alarms
    Immediate product actions to be taken upon culture alarm
    Timeframe for hospital transfusion service notification of involved co-components
    Required investigation elements (donor assessment, staff observations) by each combination of bottle/product culture results
    When/what types of culture alarm scenarios must be evaluated by a blood centre physician
    Required medical assessments of donors for continued donation
    Mechanisms to identify donors associated with any type of past alarm event
    Prescribed donor communications (oral or written) with various confirmed-positive organisms
16. Evaluations required in definite or likely contamination with a common pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Blood collector physician calls donor to take a complete medical history and provide preliminary donor advice
    Donor healthcare practitioner referral and evaluation
    Phlebotomist observation for proper skin decontamination technique
    Technologist observation for proper sampling and inoculation technique
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
    Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
17. Evaluations required in definite or likely contamination with a low-pathogenicity or pathogenic skin organism:
    Blood collector physician direction/evaluation of alarm investigation
    Donor arm inspection by blood collector staff for difficult skin decontamination
    Phlebotomist observation for proper skin decontamination technique
    Technologist observation for proper sampling and inoculation technique
    Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
18. Evaluations required in definite or likely contamination with an environmental opportunistic pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Blood collector physician calls donor to take a complete medical history and provide preliminary donor advice
    Donor healthcare practitioner referral and evaluation
    Technologist observation for proper sampling and inoculation technique
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
    Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
19. Evaluations required in possible but unlikely contamination:
    Blood collector physician direction/evaluation of alarm investigation
    Blood collector physician calls donor to take a complete medical history and provide preliminary donor advice
    Technologist observation for proper sampling and inoculation technique
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
    Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
20. Evaluations required in unlikely contamination (e.g., false positives):
    Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
21. Policy for managing co-components, confirming results and investigating your potential false-negative results:
    Co-components are retrieved and quarantined. If available, platelets co-components are sent for culture.
22. Monitor and investigate screening culture results in real time:
    Periodic monitoring only
23. Factors weighed in donor management:
    Whether a source of infection was identified and definitively treated
    Whether underlying risks for intermittent bacteremia or suboptimal skin disinfection exist
    Whether donors' healthcare providers have cleared them, regardless of workup
    Whether a search for infection or risk factors has been completed to the satisfaction of the blood collector's physician
    Whether the donor has been involved in prior alarm events (excluding false-positive alarms)
    Other: Decisions are made by medical director on a case-by-case basis when scenario is not addressed by SOP (standard operating procedure).
24. Donor management for definite or likely contamination:
    Not applicable. We do not produce pooled platelets.
25. Management of donors with repeat alarm events:
    This is a rare occurrence. Donor eligibility is determined by the medical director.

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DENMARK

Lene H. Harritshoej and Christian Erikstrup

1. Type of blood collection service:
   National
2. Annual whole blood and automated red cell collection volume:
   50,000–199,999
3. Annual distribution of platelet doses:
   20,000–59,999
4. Type(s) of bacterially cultured leuko-reduced platelet offered:
   Apheresis platelets in plasma
   Apheresis platelets in additive solution/plasma
   Buffy-coat whole blood-derived pools in additive solution/plasma
5. Percentage of non-pathogen-reduced units cultured:
   100
6. Culture instrument(s) used:
   BACTALERT
   BIOMERIEUX
7. Culture sample source:
   Whole blood-derived pool and apheresis mother bag
8. Total volume removed for culture:
   8–10 mL
9. Timing of sample inoculation after collection:
   One day after collection
10. Culture inoculation practices:
    Aerobic culture:
    One per pool and one per apheresis
    Aerobic inoculation volume:
    8–10 mL
    Anaerobic culture:
    Not applicable
    Anaerobic inoculation volume:
    Not applicable
11. Hold time before product release:
    We do not use a hold time after culturing
12. Longest allowable platelet expiration:
    Seven days for both pooled and apheresis platelets
13. Implementation year of current culture method:
    2012
14. Culture protocol references:
    No
15. Written procedures:
    Overall management of screening platelet culture instrument alarms
    Immediate product actions to be taken upon culture alarm
    Required investigation elements (donor assessment, staff observations) by each combination of bottle/product culture results
    When/what types of culture alarm scenarios must be evaluated by a blood centre physician
16. Evaluations required in definite or likely contamination with a common pathogen:
    Blood collector physician direction/evaluation of alarm investigation:
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
17. Evaluations required in definite or likely contamination with a low-pathogenicity or pathogenic skin organism:
    Blood collector physician direction/evaluation of alarm investigation
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
18. Evaluations required in definite or likely contamination with an environmental opportunistic pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
19. Evaluations required in possible but unlikely contamination:
    Blood collector physician direction/evaluation of alarm investigation
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
20. Evaluations required in unlikely contamination (e.g., false positives):
    Blood collector physician direction/evaluation of alarm investigation
    Sterility chain review (apheresis kits, sterile welders, incubators, etc.)
21. Policy for managing co-components, confirming results and investigating your potential false-negative results:
    Culture from culture bottle and quarantine of co-RBC components until culture bottles are negative. If sample from the culture bottle is positive, we also culture from the quarantined RBC co-components
22. Monitor and investigate screening culture results in real time:
    Yes
23. Factors weighed in donor management:
    None
24. Donor management for definite or likely contamination:
    Donor is contacted only if a bacteremia of donor is the most possible source of contamination. Therefore, we almost never contact a donor when we detect a culture-positive pool/apheresis. The sterility of the production process and sample drawing process is primarily evaluated.
25. Management of donors with repeat alarm events:
    No response provided

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AUSTRALIA

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1. Type of blood collection service:
   National
2. Annual whole blood and automated red cell collection volume:
   ≥400,000
3. Annual distribution of platelet doses:
   ≥120,000
4. Type(s) of bacterially cultured leuko-reduced platelet offered:
   Apheresis platelets in additive solution/plasma
   Buffy-coat whole blood-derived pools in additive solution/plasma
5. Percentage of non-pathogen-reduced units cultured:
   100
6. Culture instrument(s) used:
   BacT/ALERT VIRTUO bacterial contamination screening system bioMerieux
7. Culture sample source:
   Apheresis and pooled platelets from integrated sampling pouches
8. Total volume removed for culture:
   Pooled platelets: 16–20 mL; apheresis platelets 16–20 mL/unit (can be 1, 2 or 3 units)
9. Timing of sample inoculation after collection:
   BCS bottles are inoculated 36–72 h after collection
10. Culture inoculation practices:
    Aerobic culture:
    1
    Aerobic inoculation volume:
    8–10 mL
    Anaerobic culture:
    1
    Anaerobic inoculation volume:
    8–10 mL
11. Hold time before product release:
    We use large volume/delayed sampling. Platelet components are held until samples are taken 36–72 h after collection, upon which the platelets are released (assuming the mandatory testing is also not reactive). Note answer to 10 above: This does not cover two types of Platelets Pooled: one aerobic and one anaerobic bottle, 8–10 mL/each bottle, that is, 16–20 mL/apheresis: one aerobic and one anaerobic bottle for each unit of which there are one, two or three 8–10 mL/each bottle and 16–20 mL/unit.
12. Longest allowable platelet expiration:
    All platelet products: 7 days (all platelet components have an expiry of 7 days after collection).
13. Implementation year of current culture method:
    2021
    We implemented full LVDS 21 March 2021; large-volume sampling was implemented 30 November 2020.
14. Culture protocol references:
    Yes

Thyer J, Perkowska-Guse Z, Ismay SL, Keller AJ, Chan HT, Dennington PM, et al. Bacterial testing of platelets: has it prevented transfusion-transmitted bacterial infections in Australia? *Vox Sang*. 2017;113:13–20.
Borosak M, Wood E. Bacterial pre-release testing of platelets: the Australian Red Cross Blood Service clinical experience. *Transfus Med Hemother*. 2011;38:239–241.

15. Written procedures:
    Overall management of screening platelet culture instrument alarms
    Immediate product actions to be taken upon culture alarm
    Timeframe for hospital transfusion service notification of involved co-components
    Required investigation elements (donor assessment, staff observations) by each combination of bottle/product culture results
    When/what types of culture alarm scenarios must be evaluated by a blood centre physician
    Required medical assessments of donors for continued donation
    Specific procedures or tests mandated for specific organisms (e.g., colonoscopy for *S. bovis* group isolates)
    Mechanisms to identify and notify the suspected source among multiple donors associated with contaminated pools
    Prescribed donor communications (oral or written) with various confirmed-positive organisms
16. Evaluations required in definite or likely contamination with a common pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Blood collector physician calls donor to take a complete medical history and provide preliminary donor advice
    Donor healthcare practitioner referral and evaluation
    Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
    Other: Many of these are routine or covered in audits, not triggered by positive blood culture testing, some are not absolutes: for example, specific testing is required for approval to continue to donate.
17. Evaluations required in definite or likely contamination with a low-pathogenicity or pathogenic skin organism:
    Blood collector physician direction/evaluation of alarm investigation
    Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
    Other: We do not agree *S. aureus* and coagulase-negative Staphylococci are considered similarly in the rubric. *S. aureus* can cause occult bacteremia as well as being a skin colonizer, so is regarded by Lifeblood as highly pathogenic, that is, your category 4a not category 2. In addition, we treat *Cutibacterium* spp. and

coagulase-negative *Staphylococci* differently: Cutibacteria do not get donor follow-up, coagulase-negative *Staphylococci* get followed up. As per other questions above about pathogenic species, we routinely inspect bags and equipment, train the staff on donor arm disinfection and audit this routinely.

18. Evaluations required in definite or likely contamination with an environmental opportunistic pathogen:
    Blood collector physician direction/evaluation of alarm investigation
    Blood collector physician calls donor to take a complete medical history and provide preliminary donor advice
    Donor healthcare practitioner referral and evaluation
    Place a surveillance flag on non-deferred donors' records should they be associated with another alarm
    Other: We regard Acinetobacter and Serratia as potentially pathogenic, so follow up the donor in this way.
19. Evaluations required in possible but unlikely contamination:
    Blood collector physician direction/evaluation of alarm investigation
    Other: Lifeblood struggles with this separation of confirmed/unconfirmed/indeterminate because the lack of confirmation by culturing other bags or the donor or patient does NOT mean the bacterium was not there in the first place; it could be a low inoculum or the other bags do not have good conditions to support that species of bacteria, or the bacteremia in the donor or patient might have been transient and thus relevant cultures are negative. This artificial separation is on final reports that go to hospitals and they find it very confusing. Our regulator has also expressed this concern. With relation to donor follow-up, there is none in this scenario if it is a *Cutibacterium* spp., but the doctor follows up for any other organism, that is, group 2, 3, 4a and 4b organisms.
20. Evaluations required in unlikely contamination (e.g., false positives):
    Other: We answered this for false positives, as we still follow up indeterminate as per species isolated; for this, we do not do any of the above.
21. Policy for managing co-components, confirming results and investigating your potential false-negative results:
    This seems to relate to point-of-care testing in hospitals prior to transfusion, which does not occur in Australia. The only bacterial testing that is done is in the context of investigating transfusion reactions, and if reported to Lifeblood, would be managed as a suspected transfusion-transmitted bacterial infection. This scenario has not occurred at Lifeblood in the last 5 years at least. However, if it does, we would manage it as if it were a transfusion-transmitted infection near-miss, that is, recall all products, clinician notification if associated product transfused, culture all available products, contact implicated donors, enact deviation or corrective and preventative actions and conduct root cause analysis which would include extensive investigation, notify our executive, and so on.
22. Monitor and investigate screening culture results in real time:
    Yes
23. Factors weighed in donor management:
    Whether a source of infection was identified and definitively treated
    Whether underlying risks for intermittent bacteremia or suboptimal skin disinfection exist
    Whether donors' healthcare providers have cleared them, regardless of workup
    Whether a search for infection or risk factors has been completed to the satisfaction of the blood collector's physician
    Whether the donor has been involved in prior alarm events (excluding false-positive alarms)
    Other: With respect to automatic permanent deferral, Lifeblood does not do this but considers donors on a case-by-case basis.
24. Donor management for definite or likely contamination:
    Lifeblood follows up all four donors, as it is not possible to know which donor was implicated. We would not do this for *Cutibacterium* spp. or coagulase-negative *Staphylococci*.
25. Management of donors with repeat alarm events:
    Not aware of any such donors, but if this happened, the donor would be considered on a case-by-case basis by consultation with the medical microbiologists; this scenario would probably result in permanent deferral.

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See also https://www.isbtweb.org/events/hvwebinars.html

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