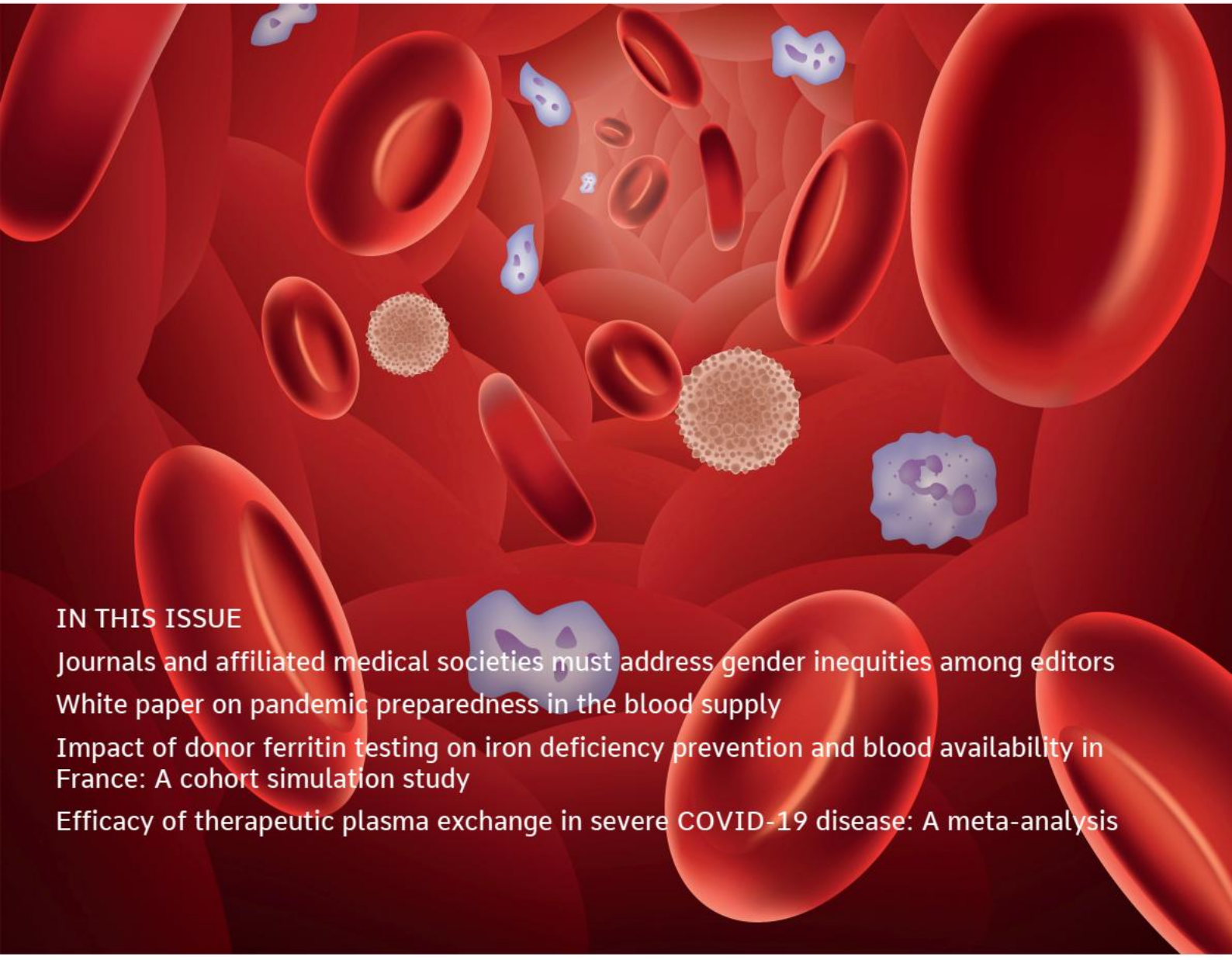
ISSN 0042-9007 (Print)
ISSN 1423-0410 (Online)

Volume 119 Number 7 JULY 2024

Vox Sanguinis

The International Journal of Transfusion Medicine



IN THIS ISSUE

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Vox Sanguinis

International Journal of Blood Transfusion

Official Journal of the International Society of Blood Transfusion

Founded 1956 by J. J. van Loghem, L. P. Holländer, J. Dausset, A. Hässig and J. Julliard (formerly Bulletin of the Central Laboratory of the Blood Transfusion Service of the Dutch Red Cross, founded 1951)

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 eight main sections:

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

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VOX SANGUINIS (Online ISSN: 1423-0410 Print ISSN: 0042-9007) is published monthly. Postmaster: Send all address changes to VOX SANGUINIS, Wiley Periodicals LLC, C/O The Sheridan Press, PO Box 465, Hanover, PA 17331, USA.

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Printed in the UK by Hobbs the Printers Ltd.

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Vox Sanguinis (2024) **119**, 631–770

119/7/2024

Contents

Received: 28 September 2023 | Revised: 16 January 2024 | Accepted: 27 February 2024

DOI: 10.1111/vox.13615

COMMENTARY

Vox Sanguinis ISBT International Society of Blood Transfusion

Economic performance of an accredited laboratory for cell manipulation in a public health setting

Cell manipulation is currently required to ensure standard therapies in clinical haematology in which cell transplants and cell transfer are applied for long-lasting disease control or correction of congenital diseases involving haematopoiesis. Since 1998, the Joint Accreditation Committee ISCT-Europe & EBMT (JACIE)/the Foundation for the Accreditation Cellular Therapy (FACT) accreditation system has introduced voluntary international standards for haematopoietic transplantation and related therapies [1]. Several scientific societies recommend these, and some countries have used them as a model for mandatory institutional accreditation. They guarantee high quality and standardization for cell product exchange and maximum safety for patients [1].

Although they are voluntary, JACIE/FACT standards comply with national, continental and international rules, including those of Italy and several other European countries [2, 3]. JACIE/FACT standards are the sole unified body of rules applicable to laboratories that support autologous/allogeneic transplants, send and receive cell products worldwide and support CAR-T cell therapy.

Another complication for cell processing laboratories, not fully referable to JACIE/FACT or other accreditation systems, is the somewhat confusing laboratory requirements for carrying out minimal cell manipulations. Thus, the basic prescriptions of competent authorities are often upgraded to a grade C environment, rather than grade D [3, 4] (Note: cleanroom grade C requires meeting standards ISO 7 at rest and ISO 8 in operation, while grade D requires ISO 8 at rest and is not defined for in operation) [5].

The introduction of high quality standards and improved environmental control increases safety and efficacy but also costs in human, material and technology resources. International standards and continental directives cannot provide criteria for calculating reimbursements, as this goes beyond their scope. Still, recommendations for building a set of suitable reimbursements for countries adopting standards would be appropriate. This is true also for the European community where European directives [2] define safety, quality and general laboratory organization, leaving each country free to choose public or private facilities and the nature of reimbursements. Necessarily, each country that recommends quality standards (and/or has promulgated an institutional accreditation system) should define proper and balanced forms of reimbursement, based on local criteria and on the public or private nature of its health system. For instance, in Italy, human blood and haematopoietic stem cells for clinical use are public goods, but reimbursements cover only the costs of collection and production [6]. On the other hand, costs of production in Italy, and likely in some other countries, do not include the following additional expenses of cell manipulation and storage:

1. Organizational costs (laboratory personnel for planning, maintaining continuous relationships with transplant and collection units, donor registry, regulatory authorities, international accrediting bodies, suppliers, and for the proper control and functioning of the laboratory technologies). Also included are costs of clinical-grade technological resources (the laboratory area, processing reagents and devices) and quality assurance (a specific laboratory person must be in charge of quality assurance).
2. Long-term storage of cryopreserved products beyond the first year from collection.
3. Costs of product registration, coding and labelling.
4. Costs to maintain an active and effective disaster plan. In case of disaster, the contractor company must ensure immediate transportation of stored products, reagents and disposables to an appropriate alternative area, and must make available a functioning (and quality-assured) replacement processing laboratory.

Owing to the lack of certain reimbursements, the risk of gross imbalance between costs and incomes is very high, affecting the affordability of processing laboratories in our country and, likely, elsewhere as well. To accurately evaluate costs and incomes, we have analysed the overall performance of our laboratory at San Camillo Forlanini Hospital, Rome. This laboratory has an area of 180 m^2, which includes a large grade C room, an analytical area (equipped with a blood counter, a flow cytometer and a system for blood cultures), a registration/labelling area and a freezing/storage area with a capacity of 2000 frozen bags. Full-time laboratory personnel comprise three biologists and two laboratory technicians. The laboratory supports three different transplant programs of the Lazio region. The activity in the year 2019 (chosen as a pre-pandemic year, since during the pandemic the activity was altered by permanent freezing of allogeneic products) was as follows:

1. One-hundred and thirty-three freezing procedures for paired autologous haematopoietic stem cell collections (i.e., 266 frozen bags), including registration, pre-freezing processing and labelling;
2. Seven freezing procedures for allogeneic haematopoietic stem cell units (14 frozen bags);

3. Seventy registration and labelling procedures for allogeneic haematopoietic stem cell collections for immediate reinfusion;
4. Three-hundred and ninety-nine blood cultures for autologous haematopoietic stem cell units;
5. One-hundred and forty-seven blood cultures for allogeneic haematopoietic stem cell units;
6. Two-hundred and sixty-six blood counts/cytometric counts for autologous haematopoietic stem cell collections;
7. Three hundred blood counts/cytometric counts for allogeneic haematopoietic stem cell collections.

TABLE 1 Annual expenses (in euros) of the processing laboratory in 2019.

(a)	Laboratory maintenance (including quality assurance)	132,100.00 (20.40%)
(b)	Liquid nitrogen/carbon dioxide supply	76,559.00 (11.80%)
(c)	Personnel	322,862.00 (49.90 %)
(d)	Reagents and sterile plastic materials	75,865.72 (11.70%)
(e)	Disaster plan maintenance	40,000.00 (6.20%)
	Total	647,386.72

Note: Percentages are approximated to obtain the total sum of 100%.

Work of the laboratory may be subdivided into nine major lines:

1. Product registration and labelling
2. Management of unrelated donor units, cooperating with donor registries
3. Microbiological testing (blood cultures)
4. Cell counts (blood and cytometric counts)
5. Pre-freezing manipulations
6. Freezing procedures

TABLE 2 (a) Annual incomes of the processing laboratory in 2019; (b) simulation of incomes after the introduction of long-term storage and non-cryopreserved product registration charge; (c) income values as percentages for the simulated set of reimbursements; (d) simulation of incomes increasing the charge for long-term storage to breakeven; (e) income values as percentages for the simulation at breakeven.

	(a) Current reimbursement (euros) [6, 7]	(b) Simulated reimbursement (euros)	(c) % values of simulated reimbursement[a]	(d) Simulated reimbursement at breakeven (euros)	(e) % values of simulated reimbursement at breakeven[a]
1. Product registration and labelling	NA	14,000.00 (70 procedures × 200 euros[b])	2.40	14,000.00 (70 procedures × 200 euros[b])	2.10
2. Management of unrelated products from national donor registry	113,004.00	113,004.00	20.00	113,004.00	17.50
3. Microbiological testing (blood cultures)	4476.00	4476.00	1.00	4476.00	0.70
4. Cell counts (blood and cytometric counts)	18,323.00	18,323.00	3.20	18,323.00	2.80
5. Pre-freezing manipulations	2100.00	2100.00	0.40	2100.00	0.30
6. Freezing procedures	148,734.20	148,734.20	26.20	148,734.20	23.00
7. Long-term storage of additional or non-used frozen units	NA	150,000.00 (1000 frozen bags × 150.00 Euros per year; i.e., 12.50 Euros per bag/month[c])	26.30	229,849.52 (1000 frozen bags × 229.84 euros per year; i.e., €19.15 per bag/month)	35.60
8. Product release	116,900[d]	116,900[d]	20.60	116,900[d]	18.00
Total	403,537.20	567,537.20		647,386.72	
Balance incomes/costs	−243,849.52	−79,849.52		0	

Abbreviation: NA, not applicable.

[a]Percentages are approximated in order to obtain the total sum of 100%.

[b]200 euros is the simple sum of costs for personnel (around 2 h of work of a qualified operator), materials and software licence (the annual cost of software licence has been divided by the number of units registered annually).

[c]This value has been calculated adding the 35% of cost for annual laboratory maintenance (the storage area is around 35% of the whole surface of the laboratory) to the 90% of annual cost of liquid nitrogen and to the 80% of annual cost of disaster plan maintenance, divided by 1000 (i.e., the number of long-term-stored frozen bags).

[d]This value (i.e., €668 [6]) represents the cost that is reversed to the transplant unit at release of transplantable bags; see also in the text (i.e., one or more bags that contain an adequate number of CD34+ cells/kg).

7. Short-term storage of transplantable units
8. Long-term storage of additional or non-used frozen units
9. Product release

As anticipated, our country reimburses only the activities 2–6 and 9, as stated by the national rules [6, 7]. The 2019 costs and incomes of the laboratory are shown in Tables 1 and 2. Our economic loss per year is more than €200,000; thus, the laboratory spends €1.60 for each euro gained, which is far from economic parity. This economic disparity is unaffordable for a public, non-profit institution, but in any case, resources should be allocated based on economic affordability. Starting from these considerations and from reimbursements already set in our country, we can hypothesize a sort of tariff integration, introducing charges for activities that lack economic recognition as follows:

1. Storage of frozen products from the 1st until the 10th year from collection (our national authority allows the discarding of such products after 10 years of storage [8]) for an annual reimbursement of €150 per frozen bag (€12.5 per month). We obtained this figure by adding 35% of annual laboratory maintenance (the storage area is around 35% of the whole laboratory area) to 90% of the annual cost of liquid nitrogen and 80% of the disaster plan maintenance, divided by 1000 (i.e. the number of long-term stored units in our laboratory).
2. Product registration and labelling of products for immediate reinfusion are to be reimbursed at €200 [this value is the simple sum of costs for personnel (around 2 h of work of a qualified operator), for materials and for software licence (the annual cost of software licence has been divided by the number of units registered annually)].

Recent reports show that most frozen autologous collections are reinfused within the first year from harvesting and that those remaining beyond the first year are likely to remain in storage for at least 5 years (reinfusion after 5 years is exceptional) [9, 10]. Our analysis incorporates economic figures that support laboratory incomes, as shown in Table 2, attributing reimbursements for the above points 1 and 2. This economic postulate shows that an important income for the laboratory consists of charges for the storage of non-used products. As shown, a monthly charge of €12.5/bag for long-term storage does not reach the laboratory's break-even point even though it is not far from that reported by a German group (€13.0/bag) [9]. The calculation of charge for the storage of unused bags may vary according to the characteristics of an institution or its laboratory, or in different countries.

A group [11] from the United States indicates a charge of $34 (around €31 at the current exchange) per bag/month. Our analysis shows that the breakeven of our laboratory may be reached by increasing the monthly charge to €19.15/bag (Table 2). This value is substantially less than that adopted by the U.S. group but higher than that of the German group. To break even, or better, the storage charge for unused bags should be calculated on the basis of local characteristics of the laboratory. In any case, to achieve affordability of the laboratory, a careful policy of product disposal is necessary, as recently proposed [12]. The calculation of costs should be tuned to the health policy of a given country and based on the political and social views that apply to the national health system.

Our analysis leads us to some interesting conclusions: (1) Given the current policies of unused product disposal [8], the lack of specific reimbursement for products stored beyond the first year from collection does not lead to the affordability of processing laboratories. (2) The calculation of a charge for long-term storage of unused bags is unexpectedly complex. It should be based on the local characteristics of the laboratory, allowing the laboratory to break even if possible. (3) Applying a cost/income simulation (column d, Table 2), our laboratory reached a break-even point after 10 years of activity, when unused (and chargeable) bags per year were not less than 1000. This aspect should be taken into account when planning the size of a processing laboratory, in order to guarantee a proper 'critical mass' that may translate into quality, safety and affordability. (4) The income shown for point 8 in Table 2 (product release) is a matter of debate. In fact, it is unclear whether these charges should be applied at collection or at product release. However, it seems reasonable that reimbursement for a product is given by the transplant unit to the collection facility, and in turn the processing facility charges the transplant unit for product registration, laboratory controls, labelling and, when appropriate, freezing and long term-storage. (5) Following this reimbursement system, the income shown for product release should be erased and break-even reached by increasing by 51% the charge for long-term storage (i.e., around €28.90/bag/month, not far from the charge adopted by the U.S. group [11]).

Finally, the future of cell therapies is related to their affordability as well as quality and efficacy, as promoted by the JACIE/FACT standards in recent years. Haematopoietic stem cell transplantation is a suitable model to attempt a basic evaluation of economic issues related to cell therapies. The affordability of new cell therapies should be evaluated with complex and dedicated modelling strategies [13].

FUNDING INFORMATION

The authors received no specific funding for this work.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The authors declare that the data used for the writing of the present manuscript are fully available.

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How to cite this article: Pierelli L, Vacca M. Economic performance of an accredited laboratory for cell manipulation in a public health setting. Vox Sang. 2024;119:635–8.

Received: 15 May 2023 | Revised: 3 January 2024 | Accepted: 15 March 2024

DOI: 10.1111/vox.13638

REVIEW

Vox Sanguinis ISBT International Society of Blood Transfusion

A scoping review of sociology of voluntary blood donation

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Funding information
Open access publishing facilitated by The University of Melbourne, as part of the Wiley - The University of Melbourne agreement via the Council of Australian University Librarians.

Abstract

Background and Objectives: Maintaining stable blood supplies presents an increasing challenge for blood collection agencies (BCAs). Novel and multidisciplinary approaches and research have been called for to understand the mechanisms underlying the trends. The current body of sociological research on blood donation is a potentially valuable resource, but it is dispersed over different publications and theoretical frameworks.

Materials and Methods: We conducted a scoping review of sociological research on voluntary, non-remunerated blood donation to identify insights and practical applications for researchers and BCAs.

Results: Four organizing themes were identified: donated blood, blood donors, organizations and blood service systems. Key challenges associated with the organization of voluntary blood donation exist at the institutional and systems levels, and they may not be readily resolved by interventions focussed solely on the individual donor level. We identified opportunities for organizations to build trust with donors and the public through communications and working with communities to promote inclusion in blood donation.

Conclusion: The results support a multidisciplinary approach and research for BCAs to move forward and find novel ways to ensure safe, resilient blood service systems.

Keywords

blood collection, blood donation, gift, review, sociology

Highlights

- Donor barriers and motivations need to be situated within social, organizational and historical contexts.
- The meanings of 'gift' and 'altruism' are shaped by social context.
- Multidisciplinary research is recommended to address the key challenges of blood donation.

INTRODUCTION

The need for blood for transfusion and medicines presents an ongoing challenge for blood collection agencies (BCAs) responsible for maintaining stable blood supplies. Recently, scholars [1, 2] have suggested adopting a multidisciplinary social sciences approach to inform fresh approaches for donor recruitment and retention policies. While most research and review articles on blood donors are informed by psychological or medical frameworks, sociological research has occupied a more marginal role in the research agendas and key journals of the blood service field. Existing sociological articles on blood donation can be difficult to access due to their spread across journals and

application of a wide range of social theory and methods to study blood donation as a social practice. However, we suggest that sociology of blood donation has much to contribute to the understandings of blood donation and donors' experiences as well as the role of BCAs.

Sociology is a social science that focuses on the study of social life and its institutions, such as the family, religion or blood donation [3]. While it is not always easy to distinguish sociological research from other social sciences [3], one key difference is that social theory considers how human activity produces and changes institutions over time and how existing institutions structure individual behaviour. For example, sociology considers how meanings of donation are produced across different times and places, while much research focussing on individual donor motivations risks portraying blood donor behaviour as outside of history, culture, location, organizational context and the broader political and social contexts [4]. This is clearly not the case because BCA constantly adapt their donor management strategies according to changes in their social, regulatory and political environments. We included articles from social anthropology and science and technology studies that aligned with our definition and add to the understanding of the sociological approach to blood donation.

We take inspiration for this review from the work of Titmuss [5], Piliavin [6] Charbonneau [7] and Healy [8] who were interested to understand the varying social arrangements in which blood donation becomes possible and meaningful to people, that is, the ways in which blood collection is socially organized, including the knowledge, technology and expertise that the practice requires as well as the norms and roles that inform a blood donor identity. Their perspective broadens the scope from the individual characteristics of blood donors to the social and cultural characteristics that shape its processes. For example, Titmuss' landmark study argued that blood donated in a welfare state formed a gift given altruistically to help an anonymous patient, as distinguished from blood as a market good, or commodity, to be traded [5]. Titmuss' Gift theory offers an example of a social theory that can be used to analyse how donors understand the meanings of donating blood as well as the idea of blood as gift of life becoming institutionalized in the policies and practice of blood services. The ideals of altruism and the gift continue to serve as the BCA standard for how blood donation should be carried out and as theoretical concepts that inform much social sciences research [6, 8].

In this scoping review, we aim to identify key research insights and applications from sociological research on blood donation to make this knowledge accessible for a diverse readership, and we also aim to inform a research agenda to assist with maintaining stable blood supplies through voluntary non-remunerated (VNR) donation. It is beyond the scope of this article to review the social theories or methodologies used in the articles. Rather we hope to create interest in the reader to explore these articles further.

METHODS

Articles focussing on the social context of VNR blood donation and employing social theories were sought to be included in the review. A scoping review is a method to map evidence from a diverse field of research using different methods including qualitative, quantitative and theoretical studies [9]. This review was conducted in accordance with PRISMA ScR review guidelines [10] using the framework adapted from Arksey and O'Malley [9].

We devised a work plan (see Supplementary Materials) that details our review strategy and the protocol followed. We adapted an integrative scoping review strategy that comprised a systematic search, two-tier screen and selection process, thematic analysis and narrative synthesis to identify key insights and applications [9–11].

Search and two-tier screening process

We constructed a two-tier systematic article search and selection strategy (Supplementary Figure 1). In August 2019, we conducted a reference database: Web of Science, Sociological Abstracts and EBSCOhost and PubMed search on title, abstract, keyword and subject, using search terms such as 'blood donation' or 'blood donor' (Supplementary Table 2) We supplemented the database search with citation searches and articles from our own libraries. We limited the search to peer-reviewed journal articles on blood donation published between January 2000 and July 2019. For all the resulting articles, we conducted a title/key word/abstract screening by criteria detailed in Supplementary Table 1 to select articles to import into a shared Mendeley library (Supplementary Figure 1). In this first-tier screen, we excluded articles that clearly represented a natural or medical science, humanities, or other social science, for example, psychology and economics approach (Supplementary Table 1). After first-tier screening and removing duplicates, 285 articles were selected for second-tier screening.

Second-tier screening involved each author conducting full-text review of half of the articles to evaluate whether the article matched our eligibility criteria (Supplementary Table 3). To ensure alignment, both authors initially reviewed the same four articles and discussed their decisions and any modifications needed to the screening tool. We constructed a review sheet to be used for both second-tier screening and narrative synthesis and thematization (Supplementary Table 3) [9].

Eligibility assessment and narrative synthetizing

For each article, one author constructed a narrative summary, which included the research question, social theory approach, methods and data analysis approach, key sociological insights and research application, and applied a quality appraisal tool to systematically assess eligibility [11]. After the second-tier screen, 78 articles were selected to be included in the review (Supplementary Figure 1).

Thematic analysis

We developed four inductive themes to organize the articles based on their contextual perspective on the social context of blood donation

(Supplementary Table 4) [12]. Articles focussed on meanings associated with blood were allocated to the 'Meanings of donated blood' theme; those focussed on blood donor experience were allocated to Theme 2, 'Blood donors'; those considering the local organizational arrangements of donating blood were allocated to the Theme 3, 'Organization'; and articles considering blood donation systems and principles were allocated to Theme 4, 'Blood service systems'. The authors then analysed the article summaries to develop sub-themes within each theme. Some sub-themes fitted into more than one theme. As a team, we decided which theme to allocate these sub-themes into.

RESULTS

This review includes 68 articles, with 10 in the theme Donated Blood, 18 in Blood Donors, 22 in Organizations and 18 in Blood Service Systems. We decided to only include a selected example (5) of studies on the social impacts of the deferral rule for men who have sex with men (MSM) policy as we felt this topic is worthy of a separate review.

Theme 1: Meanings of donated blood

This theme includes studies addressing questions about how blood as both a material and a symbolic substance that is donated and processed into different products is perceived by donors in different cultural and historical contexts.

Donated blood as a gift and a commodity

Papers in this sub-theme focus on the narrative often employed by BCAs, that blood is donated as a 'gift' that passes directly from donor to recipient. They argue that this narrative does not accurately represent the processing of blood that takes place between collection and eventual use or address new meanings that may emerge as a result of the processing of blood. They discuss the social implications [13–19].

In a case study from Belgium, Wittock and colleagues show that BCA practices of considering blood as a gift in interactions with donors and a commodity when manufactured and sold to hospitals may optimize workflows but also lead to sub-optimal communication about blood processing between donors and BCAs [13]. Moreover, the narrative of blood as a gift may not coincide with how donors understand their donations [14]. Lynch and Cohn's observations of staff and donors in donor centres in the United Kingdom provide evidence that BCAs could broaden the narratives employed in communications with donors to include explanations of how blood is processed or used for research.

Many BCAs now provide donors the opportunity to voluntarily 'gift' blood to health and genetic research. An interview study with donors of Indian background living in Houston found that while participants might be happy to donate blood for research to advance 'the greater good', how donors interpret this as benefitting the community can differ from the understandings of researchers [15]. An interview study with Finnish donors found them often prioritizing the use of donated blood for transfusions [16]. The articles highlight the need to build understandings of how donors understand the concrete social benefits of their donations for research, and to consider how their perspectives can best be addressed [15, 16].

Studies about 'alienability' of blood address to what extent different stakeholders consider that blood, once donated, is still theirs and forms part of their identity. In an Australian interview study [17], most participants, composed of blood donors, recipients and hepatitis C-positive people, did not consider blood to retain identity once donated, contrary to what previous studies suggest is common with a gift. Rather than gift, blood recipients conceptualized donated blood as a form of medication, while donors perceived their donations as given to the organization rather than to another person. Studies conclude that previous experience with blood and with blood-borne viruses shaped participants' perceptions of the risks associated with blood exchange [17].

An ethnographic study with UK donors suggests taking account of how informing donors about new biological aspects of blood, like viruses, or biotechnological processes such as large-scale genomic testing, can change donors' views about blood's relation to self, risks and identity, adding new and contesting old motifs to donated blood [18]. A further UK study of the use of cultured blood found that even transfused blood is never just a universal scientific product, but through its use becomes part of the social and political context of donation [19].

Cultural differences in meanings of donated blood

Articles in this sub-theme demonstrate that donor perspectives on what donated blood means to them are diverse and informed by local cultures. Moreover, studies show that donors may hold beliefs about blood donation as an altruistic act simultaneously with more symbolic, contextualized beliefs that challenge dominant medical notions of donated blood and the body [20–27].

A Canadian interview study found that members of minority ethnic groups more often associated donated blood with gift, identity and religion while those in the majority commonly described it a medical substance [20]. Negotiating acceptability of donating blood more frequently, interviewed UK donors drew upon their own experiences and ideas about regeneration of blood, and the impact of donation on different body types instead of only relying on formal medical guidance [21]. A Brazilian study also found ideas about donation as beneficial to the donor co-existed with organizationally derived beliefs about altruism [22].

A study from Pakistan suggests that notions of voluntary blood donation as an altruistic gift is based on European/North American values and does not necessarily translate directly to other cultures [23]. For example, qualitative studies among ethnic minority groups in Europe/North America demonstrate how in some communities the ideal of donating blood for a stranger can mean blood crossing

sacred boundaries of family, clan, nationality or other affinity in ways that is subject to a taboo or contamination of some kind. For those who hold these beliefs, the organization of blood donation in Western countries may not be considered a normal practice. Other studies have explored ways to work with communities and with donors' own understandings to encourage donation as an everyday practice in local settings [24–27].

Theme 2: Blood donors

The question of who donates blood and why continues to intrigue academics and is of practical concern for BCAs. In this review, we focus on research that considers donors' perspectives and experiences of blood donation as a social role, rather than on donor demographics.

Blood donation as a social experience

These articles argue that the rationale for blood donation is not only a matter of individual motives but it is also informed by shared experiences of becoming and being a donor [28, 29], and maintaining this practice [30]. Studies from Australia [29] and Italy [28] found donors' shared conceptualizations of blood donation were more complex than non-donors' who focussed more on the practical aspects of the donation process [29]. Donors were more responsive to BCA campaigns because of their positive experiences and knowledge of donation [28]. A Norwegian survey study suggests that invitations from other donors seems to be the best way to encourage younger people to donate [31]. An Austrian survey study observed that information and invitations to donate were more effective for first-time donors when shared by close social networks using an appropriate communication style [32]. Little is yet known about the ways in which social media influences these interactions and experiences of donation [33]. Interview studies with deferred donors from Australia [30] and Canada [34] conclude that providing clear reasons for the deferral, while also considering the costs to the donor, can also help improve donor perceptions of being deferred, improve retention rates and reputation.

Differences between experiences

Research investigating the low participation rates of minority groups in blood donation, particularly with Black [35–37] and indigenous [25, 26], communities, report mistrust and suspicion towards health care systems, including blood collectors, which can be traced back to past events of structural discrimination. These studies from Australia, Canada and United States provide knowledge of different cultural viewpoints on blood donation that shapes how it is experienced. Studies suggest that future research could address differing personal and community experiences of blood and health service systems. They argue that having a contextualized understanding of these differences between social groups can assist BCAs to create better targeted measures to increase inclusivity in donation [25, 26, 35–37]. For example, an interview study with donors and representatives of Montreal's Black communities recommends communicating more specifically the community's need for donated blood [35]. Where there has been a history of exclusion by race, another Canadian study found it to be preferable for organizations not to racially profile migrant communities when calling for them to donate [38].

Theme 3: Organizations

This theme considers how the local organization of blood donation, at the level of the BCA, shapes blood donation practices. The key point is well captured by Healy's finding that altruism does not help explain regional differences between blood donation rates. His analysis of Eurobarometer data demonstrated that these differences result from different ways of organizing blood donation in different institutional contexts. Success in reaching blood collection targets thus depends on the institutional ability to make optimal use of these local structures from a donor recruitment perspective [39].

BCAs channelling social resources into blood donation

The social capital lens illustrates that not everyone is structurally in a similar position to choose to 'give back' to their community through blood donation. The theory of social capital considers social relationships as a resource in life. Social capital accrues when individuals engage in behaviours that their social networks endorse [40]. However, donating blood may not accrue social capital for all. In contrast, studies from Australia, Canada and the Netherlands demonstrate how the interplay between the organization of blood collection, the donor's place in social networks, their location and access to shared resources influence whether donating blood is perceived as rewarding or costly [41–44]. In a Canadian study, Smith and colleagues encourage research to further explore how existing and new social capital created through blood donation can be used as a recruitment tool and in creating local donor cultures [40, 45].

An interview study from Canada illuminates the influence of family on how new generations learn to donate blood [46]. The concept of a 'donor career' brings attention to the social dynamics of donors' relationship with BCAs, which changes due to life events, life-stage or social situations [47–49]. An Italian survey study proposes that blood donation may compete, but also have synergies with, other voluntary contexts for being a 'donor' [48]. Australian research also suggests that donors' approval of a lateral career shift between donation types depends upon consistency in institutional and donor roles regarding this shift [49].

Blood donation as a practice and space of inclusion and exclusion

BCAs tend to frame blood donation as a private, voluntary choice for altruistic and responsible citizens, while imposing eligibility criteria

that exclude certain groups from donating or constructing some bodies, like women's, as riskier than others [50] The qualitative studies in this sub-theme provide Danish [51], Japanese [52] and Australian [53] perspectives to these practices of exclusion and how they—even when not intended—can create categories of 'good' and 'bad' in relation to citizens, bodies and blood. They discuss the ways in which these practises can be experienced as stigmatizing for groups seen to embody those 'bad' qualities that exclude them from acting 'good' like others. Moral categorization can continue to have a negative influence on public views of excluded groups even after eligibility criteria have changed [51–53]. BCAs are therefore encouraged to continue investing in risk assessment technologies and educating the public about their purposes [51]. Part of the rhetorical work performed by BCAs around the world is promoting the act of VNR blood donation as a 'universal social good' [54]. However, ethnographic studies conducted in Sri Lanka found that in countries with diverse religions, beliefs and conflicts, the aims of VNR blood donation organized by a national blood service can be difficult to separate from other identity-related rhetorics [54, 55], In other settings, such as in Singapore, representations of VRN blood donation have been purposefully harnessed in the government's political projects [56].

The issue of inclusion of ethnic minority donors in countries in which migrant communities are underrepresented in donor panels is a growing focus of research. A sense of belonging in society and perceived racial discrimination were found to be the most important factors explaining blood donation among African migrants in Australia [36, 37]. The wave of migration can also impact the relationship with the local BCA [38]. Studies in this theme encourage BCAs to undertake programmes with minority groups and to utilize their knowledge to facilitate inclusion in blood donation.

These articles collectively demonstrate how BCAs operate as part of the local cultural and political structures and set out an agenda to improve their understanding of the complexity of group identities held by diverse groups of blood donors [36–38, 51–56].

Expanding the practice and purpose of blood donation

Articles in this sub-theme consider how institutional statements promoting donation do not fully account for variations in how the worth of blood donation is viewed. Two focus group studies from Spain [57] and United States [58], with donors and non-donors, suggest that donors do not always recognize blood donation to be the exceptional act sometimes depicted in promotional materials [57, 58]. For some non-donors, donating blood may be perceived as too impersonal to be defined as volunteering [58]. Dalsgaard's ethnographic study from Denmark observes BCAs' use of 'strategies of reciprocity' by which they make sure donors receive care, gratefulness, food and other things of value in return for donations [59]. The articles identify the potential for BCAs to demonstrate the tangible life-improving value of donation to donor communities [57–59].

BCAs in many countries have expanded to utilize blood donor panels as baseline populations for biobanking or public health research [60–63]. To achieve this successfully the consent of donors, extra time, and effort are required [60]. A focus group study from Finland reports that willingness to participate in biobanking depends upon the trust BCAs have acquired, while ethnographic work from the United Kingdom shows it also depends on how donors imagine the uses, and recipients, of their blood and related health data [61, 62]. Also considering UK donor views, Busby argues that successfully expanding the uses of blood while maintaining public trust requires public knowledge of present-day blood banking, which includes for-profit collaborations [63]. Research shows that BCAs can successfully extend their services if they maintain a dialogue with donors about the uses of blood for research and the social benefits of these activities [60–63].

Theme 4: Blood service systems

BCAs' primary responsibility is to protect the health of patients and donors and to maintain stable blood supplies. However, the articles reviewed noted that constructing this kind of blood service system is a social process of communication and of value choices. They show how common goals of blood product availability and safety still require decisions on correct solutions to get there and of the acceptable level of risks. They trace how policies that structure blood donation are negotiated in the context of information and tools available, and why they should be implemented considering their broader societal impacts.

Social constructions of blood safety

The studies under this sub-theme argue that blood safety should be addressed as a social process as well as a medical and technological matter. These studies demonstrate how the criteria for medical safety, acceptable risks and precautionary measures vary by country and over time, and thus depend on local resources, institutions and cultures [64–66]. These studies aim to provide tools for adapting shared principles on blood safety to contextual policy decisions with social implications [65, 66]. For example, O'Neill's analysis of policy documents about 'mad cow disease' identifies tensions between blood donation as community action, and risks managed on a national basis, when blood components, donors and infections travel through global networks in ways that require coordination of safety at a global level [64].

After the HIV/AIDS crises of the 80s, many countries introduced the so-called 'MSM ban', which refers to sex between men as a permanent or temporary exclusion criterion for blood donation, as a blood safety measure. Research looking at media documents from Canada [65] and Belgium [67, 68], demonstrate that the justifications of the MSM policy have since been much publicly debated. Research provides good case examples from Hong Kong, United Kingdom, United States [69] and Sweden [70] that encourage BCAs to work with gay communities to prioritize both inclusivity and safety [70].

TABLE 1 Summary and research agenda.

Theme	Sub-theme	Insight	Research agenda	Reference
Meanings of donated blood	Donated blood as a gift and a commodity	The gift/commodity binary is unnecessary when explaining the processing and uses of blood.	Identify productive ways to communicate how blood gets processed and used.	[13–19]
	Cultural differences in meanings of donated blood	Ideas about blood and related risks continue to be associated with cultural representations and situated experiences.	Work with communities to develop shared understandings of blood donation and developments in biomedicine.	[20–27]
Blood donors	Blood donation as a social experience	Reasons for donating come from social discourses and experiences of blood donation.	Research the social embedding of blood donation knowledge.	[28–34]
	Differences between experiences	Representation of different social groups among blood donors can reflect differences in collective experience.	Successful collaboration with ethnic and migrant communities requires BCAs to hear how people understand factors influencing inclusion, and reflective attention to their own approaches.	[25, 26, 35–38]
Organizations	Blood collection agencies (BCAs) channelling social resources to blood donation	BCAs moderate the connection between blood donor status, social belonging and access to resources.	How can motives and barriers be integrated into a broader understanding of institutional factors that make blood donation a viable choice.	[39–49]
	Blood donation as a practice and space of inclusion and exclusion	Practices of inclusion and exclusion create categories of blood and donors.	How does the historical/cultural context of blood donation affect donor inclusion.	[36–38, 51–56]
	Expanding blood donation practice and purpose	Promotions of donation do not account for the variation in modern practices of BCAs nor public views.	Research and communicate the tangible social value of donation and research participation.	[57–63]
Blood Service Systems	Social constructions of blood safety	Criteria for blood safety vary by country and over time.	How to conceptualize and organize risk management at local and global levels.	[64–70]
	Rethinking the gift system	Distinctions between market-based and gift systems distract from facts-based dialogue.	How to re-frame the donor–organization contract moving forward?	[71–80]

Rethinking the gift system

Studies in this sub-theme reflect on Titmuss' question of whether donation is viewed as gift or commodity exchange, as well as on the safest blood systems and types of donations [71–80]. Farrugia and colleagues argue that global differences in blood safety levels between countries reflect unequal resourcing of national health care systems rather than donor type, that is, the reliance of developing countries on family replacement or paid donors, while wealthy countries are able to rely more often on VNR donors. The authors argue that the policy emphasis on the link between safe blood and altruistic motives shifts focus away from global inequality in relation to health care services and access to safe blood [71]. Evidence from other studies also points to a need for strategies that recognize the effects of inequalities in local conditions on VNR donation [72, 73].

New ways of using blood for research, to extract information and in production, attributes blood with new values and expectations of profits [74]. With most European countries reliant on commercial plasma often sourced from paid US donors, the demarcation between market and gift exchange systems appears increasingly artificial. Studies in this theme suggest that a realistic, efficient health policy starts from open discussion of these facts [75, 76]. In a critical analysis of EU regulations and policy, Farrell shows that as a safety policy and risk management tool, VNR donation by principle of gift is insufficient; and that endorsing it in principle but not as full practice discourages Member States to take responsibility for issues of self-sufficiency and fairness in blood procurement [75].

In a theoretical paper that references European research, Steiner argues that as BCAs expand more into market production, the more they position blood donation as an altruistic gift [77]. However, the altruism that motivates 'the free gift' is a complex construct [78], and one not detached from the broader context in which blood is processed into products, sold, bought and used [74]. A UK study argues that continuing to frame blood collection/donation as a gift system

separate from blood component production blurs and limits donors' knowledge of how and by whom their donations are used and whether this remains compatible with their altruistic intents and interest in patient health [74]. Recognizing evidence that new framings of blood systems are needed, Mahon-Daly suggests framing blood donation as a donor–organization contract that reflects social reciprocity as its underlying principle. For this, citizens need to be provided with information about how blood supply systems work [79]. A Danish interview study shows that blood donors are able to adapt their views on the complexities of the blood donation systems, such as the need for BCAs to import and sell plasma, when this is clearly explained to them. This study argues that a transparent, dialogic approach is best to manage the risks of losing donors as BCAs diversify their activities [80].

DISCUSSION

Our aim in undertaking this scoping review was to identify key research insights from the sociological literature on blood donation and inform a research agenda (see Table 1). By analysing the literature at four thematic levels, we hope to have highlighted the benefits of researching blood donation as a dynamic practice that is shaped at the local, social, organizational and regulatory contexts. Further, as many of the reviewed articles employed qualitative and inductive methodologies, findings represent participant views that often challenge traditional ways of thinking about donation and propose new ones through processes of co-construction. How can these insights and approach be used by researchers and BCAs to address problems with donor recruitment and retention?

We argue that BCAs need to stay informed of people's everyday perspectives on blood donation and its outcomes, and what changes their behaviour or opinions, if they wish donation to be perceived as a worthy commitment [8]. To do this, we recommend that researchers adopt a broad model of donation that situates individual barriers and motivations within their social, institutional and historical contexts [2]. For example, our findings suggest that BCAs need to be aware of how social context shapes what the 'gift' means, such as when the request is addressed to a minority group, or if it refers to a biobank sample and data. Adopting a broad model can keep BCAs and researchers informed of the impacts of political, technological and economic changes on the willingness and ability of people to engage in voluntary activities such as blood donation.

Technological developments, such as digital cultures, generative AI and continuing global expansion in both tissue and health data economies are re-defining what a BCA is, but still very little is known about the actual workings of these new donor relationships [81]. Research could explore how best to explain how blood gets processed and used in healthcare systems, and how through donation, blood donors contribute to public health. Research into these topics could have practical benefits for BCA in terms of growing organizational trust, modernizing understandings of the donor–organization relationship, and what it means to be a donor.

We also suggest a collaborative effort between researchers, blood donors and BCA professionals to establish how using multidisciplinary social sciences approaches can contribute to a long-term research agenda. This may be a challenging endeavour as qualitative, critical and context-specific research methodologies may seem unfamiliar and slower than other research methods. However, from the reviewed articles, we found examples of strategies for mutually productive ways to engage with stakeholders and local communities not just on blood donations but in the work to make donating blood a relevant choice for many kinds of people. This approach has been applied successfully in Canada to contribute to evidence for changing donor eligibility criteria for MSM [82]. We also found ideas about how to move forward from the ideal of self-sustained national systems and gift solidarity to address structural questions of the inequity of global blood systems. This is a topic that urgently needs to be researched.

This review has limitations and biases. The selection of articles with sociological insights was a difficult process, and synthesizing insights from articles with multiple theoretical lenses a challenging exercise that does not do justice to the richness they represent. Nevertheless, we hope this review reads as invitations to discover the original articles.

ACKNOWLEDGEMENTS

We are grateful to the Finnish Red Cross Blood Service staff for insight and to Dr Heta Tarkkala, Professor Barbara Masser and Dr Alison Gould for feedback. Open access publishing facilitated by The University of Melbourne, as part of the Wiley - The University of Melbourne agreement via the Council of Australian University Librarians.

V.R. and R.T. wrote the original draft, reviewed and edited the manuscript and contributed to conceptualization; V.R. designed the methodology and supported the investigation; R.T. supported designing the methodology and led the investigation.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

No original data sets were used for this review article. The original articles and data that support the findings will be available in: Web of Science at https://www.webofscience.com/wos/woscc/basic-search, Sociological Abstracts at https://about.proquest.com/en/products-services/socioabs-set-c/, EBSCOhost at https://www.ebsco.com/products/research-databases/academic-search-premier, PubMed at https://pubmed.ncbi.nlm.nih.gov.

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SUPPORTING INFORMATION

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

How to cite this article: Raivola V, Thorpe R. A scoping review of sociology of voluntary blood donation. Vox Sang. 2024;119:639–47.

Received: 18 December 2023 | Revised: 19 March 2024 | Accepted: 21 March 2024

DOI: 10.1111/vox.13629

ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Increasing the upper age limit for blood donation: Perspectives from older donors

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Funding information
Sanquin 'Product and Process Development - Cellular Products' grant, Grant/Award Number: PPOC20-01

Abstract

Background and Objectives: In the Netherlands, as of April 2018, the upper age limit for blood donation has been raised from 69 to 79 years, providing an opportunity to study older donors' perspectives regarding donating at older age. This study aims to explore whether older donors agree with the increase of the age limit, if they feel obliged to continue donating, to identify their motivators and barriers for donating blood and describe donation-related experiences and complications.

Materials and Methods: An online survey was distributed among Dutch blood donors aged 68–73 years. The survey contained questions regarding the increase of the upper age limit, motivations and barriers for donating, donation-related experiences and obligatory feelings to continue donating.

Results: Six hundred sixty donors (55%) were included in the analyses, including 38 stopped donors. Most donors (92%) agreed with the increase of the upper age limit. Approximately 63% of participating donors felt obliged to continue donating, especially women with high education. Donors indicated they felt healthy enough to keep donating (95%), and 72% thought it is good for their health to keep donating. Few donors reported that they found it hard to keep donating (5%) or indicated that they did not feel healthy enough to donate or thought it was not safe for them anymore (3.4%).

Conclusion: Most of the older donors agree with the increase of the upper age limit for blood donation, report only few and minor donation-related experiences or complications and are highly motivated to continue their donor career at an older age.

Keywords

barriers, blood donation, blood donor, motivation, perspectives, safety

Highlights

- Blood donors in the Netherlands agree with the increase of the upper age limit for blood donation.
- Older donors report limited and minor donation-related experiences or complications.
- Older donors are highly motivated to continue their donation career at an older age.

INTRODUCTION

To protect the safety of blood donors, various blood establishments have instated an upper age limit for donating blood. This upper age limit is not evidence-based, but often instated to be risk aversive and to prevent donation-associated adverse reactions that are expected to more likely occur in older donors. However, multiple studies show that donating at a higher age is safe. In a biomedical excellence for safer transfusion Collaborative study involving Canada, the United States, the United Kingdom and New Zealand, no increase in vasovagal reactions was found in donors aged >71 years compared with a cohort of donors aged 24–70 years [1].

As of April 2018, Sanquin Blood Bank in the Netherlands increased the upper age limit for donating from 69 year to 79 years. This decision was made after several complaints from donors who disagreed with the mandatory ending of their donation career. In addition, an increase of the upper age limit allows an additional 4000 donors annually to continue to donate blood. Because older donors are overrepresented, loyal and generally donating with a high donation frequency, retaining these donors is valuable, especially because recruiting new donors is challenging [2].

Knowledge about the safety of donating blood at older age is accumulating [3], but not much is known about how donors themselves perceive donating at higher age. An interview study conducted in donors aged >50 years found that donors stated that they rather be deemed eligible for blood donation based on their health than on their age [4]. However, increasing the upper age limit might make donors feel obliged to continue donating. In order to best guide this group of donors, information regarding their motives and intentions is essential. Therefore, this study aims to determine (1) whether older donors agree with the increase of the upper age limit, (2) whether older donors feel obliged to continue donating, (3) the motivations for continuing or discontinuing their donor career and (4) donation-related experiences and complications reported by older donors.

METHODS

Setting

By law, Sanquin Blood Bank is the only organization authorized to collect, process and distribute blood products in the Netherlands. Blood donation is voluntary and non-renumerated. Prior to a donation, a donor's eligibility to donate is evaluated using a donor health questionnaire and an onsite assessment including a haemoglobin (Hb) and blood pressure measurement. As of April 2018, donors are allowed to donate until they reach the age of 80 years. In order to donate, donors need to be in good health and free from transfusion-transmissible infections. New donors can only register as blood donors if they are aged 18–64 years. This study was reviewed and approved by Sanquin's Ethical Advisory Board.

Study design and exclusion criteria

In this cross-sectional study, we invited 1200 donors aged 68–73 years old by email to participate in an anonymous online questionnaire programmed in Qualtrics (Provo, Utah, USA). Per year of age, 200 donors were randomly selected and invited for the study. We excluded donors who did not have sufficient knowledge of the Dutch language, donors who already had a permanent medical deferral before reaching the age of 70 and those that made an official complaint concerning the upper age limit. Due to ethical concerns, we did not invite donors who were deregistered as blood donor because of the earlier age criteria and were not eligible for re-entry.

Online questionnaire

The questionnaire started with general questions about the donor's sex, age, education and occupancy. We asked about their blood donor career, whether they still donated blood, and if so, how many plasma or whole blood donations they estimated they have made. Furthermore, the questionnaire assessed their opinion about the increase of the upper age limit, their motivations and barriers to donate and whether they experienced donation-related experiences. Donors who stopped donating were questioned as to why they had stopped donating, whereas donors who still donate were questioned about if they ever ignored an invitation to donate, and, if so, why.

Measures

To investigate whether donors agreed with the increase of the upper age limit, donors were provided with the following statement: 'What do you think about Sanquin raising the upper age limit for blood donation?' Answers could be given on a five-point scale ranging from 'very bad idea' to 'very good idea', and were dichotomized, combining 'very good idea' and 'good idea' to 'agree' and 'no opinion', 'bad idea' and 'very bad idea' to 'disagree'. Feelings of obligation were surveyed by providing donors with the following statement: 'I feel obligated to continue donating blood or plasma'. Questions about barriers and motivations to donate were formulated as statements. Statements could be answered according to a five-point scale, ranging from 'completely disagree' to 'completely agree'. If suggested options did not apply, the donor could use free text space to write down their opinion. Answer options were dichotomized, combining 'completely agree' and 'agree' to 'agree', and 'totally disagree' and 'disagree' to 'disagree'. We defined donors as 'experienced donors' if they reported to have donated blood or plasma over 20 times. Furthermore, educational level consisted of six options, which were recoded into three categories: low (no school, primary school or secondary school), middle (secondary vocational education) and high (higher professional education or university). We classified donors who reported only donating whole blood throughout their donor career as 'whole

blood only' donors. Donors were asked about donation-related experiences with the question 'Could you indicate how often you experienced the following experiences/complaints after or during a donation'. Several donation-related experiences were proposed, and the donor could indicate the frequency of occurrence on the following scale: 'never', 'almost never', 'sometimes', 'often', 'always'. Proposed donation-related experiences were bruising, fainting, tiredness, painful arm, feeling sick for a longer period (days), dizziness, bleeding from the puncture site, scarring at puncture site, headache, feeling fit or full of energy after a donation and feeling thirsty.

Registry data

In addition to the self-reported donation-related experiences, we extracted registered donation-related complications from the donor database from donors aged 68–73 years who donated in the period 1 January till 31 December 2021. These are registered according to international established guidelines [5, 6]. Donation-related complications can not only occur at the donation centre but also longer after a donation. If donation-related complications, such as fainting, occur at a donation site, the event is recorded by a blood bank staff member. If occurring off-site, the affected donor can report complications by phone or at a next donation. These complications are then registered retrospectively. The blood bank does not register all donation-related complications. In this study, we report registry data on fainting (loss of consciousness), bruising and/or haematoma, pain at puncture location and bleeding, with the aim to see whether registry data on donation-related complications is similar to self-reported data on this topic. Registry data were grouped in their respective category (fainting, bruising/haematoma, pain, bleeding), regardless of severity, duration or time of occurrence of the complication.

Statistical analyses

Analyses were performed using SPSS® version 28 (IBM, Armonk, New York, USA). Descriptive statistics for continuous variables will be reported as mean and standard deviation or median and interquartile range in case of a non-normal distribution. Dichotomous or categorical variables will be reported as total numbers of cases and proportions. Predictors were estimated using logistic regression and adjusted for sex, donation type, donation experience and educational level. If outcome data were missing, those donors were excluded from that specific analysis.

RESULTS

Baseline characteristics

A total of 1200 donors were invited for the survey, of which 729 (60.8%) responded. All records that contained more than 40% missing data were excluded for analysis, as well as records with missing data on age or sex ($N = 65$, 8.8%). The final sample consisted of 660 donors (Table 1). The mean age of the invited sample was equal to those who were included in the final sample (70.6 ± 1.7). Of these donors, 30.6% were aged ≤69 years. The majority of participants were male (60%), and over 95% were experienced donors.

TABLE 1 Baseline characteristics.

	Males	Females
Total (%)	396 (60)	264 (40)
Age (mean, SD)	70.8 (1.8)	70.4 (1.6)
Stopped donors (%)	20 (5)	18 (7)
Experienced donors (>20 donations) (%)	386 (97)	247 (94)
Education[a]		
Low (%)	68 (17)	104 (39)
Middle (%)	124 (31)	81 (31)
High (%)	198 (50)	77 (29)
Whole blood only donor[b] (%)	256 (65)	170 (64)

Abbreviation: SD, standard deviation.
[a]Low (no school, primary school or secondary school), middle (secondary vocational education) and high (higher professional education or university); eight donors did not want to report their education level.
[b]Donors who solely donated whole blood in their donor career.

Agreement with the upper age limit

We asked donors whether they agreed with the increase of the upper age limit from 69 to 79 years of age. In both stopped and active blood donors, 92.1% agreed with the increase. In active donors, we found no significant predictors for agreeing with the upper age limit (Table 2).

Feeling obliged to donate

Approximately 63% of donors aged ≥68 reported feeling obliged to continue donating. Significant predictors for feeling obliged to keep donating were being a woman and having high education (Table 3). After stratifying by sex, having a high education remained a significant predictor for feeling obliged to keep donating in women.

Motivators and barriers to continue donating

Most donors (>95%) agree with the statements 'I feel healthy enough to keep donating', 'A reason to donate is the good atmosphere at the blood bank' and 'If I am invited for a blood or plasma donation, I think it is logical to donate' (Figure 1).Overall, 72% of the donors think that it is good for their health to keep donating. Only 5% of the donors find it hard to keep donating. Compared with women, men more often would feel guilty if they would stop donating blood or plasma, thought friends and family would want them to keep donating as long they were healthy and found the good atmosphere at the blood bank a

TABLE 2 Predictors for agreeing with the upper age limit (only active donors).

	OR	CI 95%	*p*-value
Age	0.884	0.742–1.052	0.165
Female sex	1.073	0.575–2.002	0.825
Experienced donor (>20 donations)	0.623	0.136–2.842	0.541
Education[a]			
Low	Ref	-	-
Middle	1.138	0.494–2.622	0.761
High	1.408	0.649–3.054	0.386
Whole blood only donor[b]	0.868	0.475–1.588	0.646

Abbreviations: CI, confidence interval; OR, odds ratio.
[a]Low (no school, primary school or secondary school), middle (secondary vocational education) and high (higher professional education or university).
[b]Donors who solely donated whole blood in their donor career.

TABLE 3 Associations with feeling obliged to keep donating (only donors who still donate).

	OR	CI 95%	*p*-value
Age	0.913	0.826–1.008	0.071
Female sex	**1.477**	**1.033–2.113**	**0.033**
Experienced donor (>20 donations)	1.051	0.370–2.989	0.926
Education[a]			
Low	Ref	-	-
Middle	1.211	0.760–1.931	0.421
High	**2.027**	**1.307–3.143**	**0.002**
Whole blood only donor[b]	1.107	0.779–1.573	0.571
Females			
Age	0.871	0.738–1.028	0.103
Experienced donor	0.977	0.261–3.659	0.973
Education[a]			
Low	-	-	-
Middle	1.535	0.803–2.933	0.195
High	**2.350**	**1.241–4.450**	**0.009**
Whole blood only donor[b]	0.841	0.484–1.461	0.538
Males			
Age	0.940	0.829–1.065	0.329
Experienced donor	1.142	0.201–6.503	0.881
Education[a]			
Low	-	-	-
Middle	0.885	0.449–1.746	0.726
High	1.649	0.893–3.046	0.110
Whole blood only donor[b]	1.364	0.860–2.162	0.187

Note: Values in bold indicate a *p*-value < 0.05.
Abbreviations: CI, confidence interval; OR, odds ratio.
[a]Low (no school, primary school or secondary school), middle (secondary vocational education) and high (higher professional education or university).
[b]Donors who solely donated whole blood in their donor career.

reason to donate. Additionally, they more often thought it was good for their health to keep donating and more often felt healthy enough to keep donating.

When asked to answer statements about experienced barriers for donating, most donors did not report many (Figure 2). However, 10% of female donors and 7% of male donors agreed that they were often not eligible for donation. Additionally, male donors more often agreed that they already help people in other ways (additionally to donating) compared with females (17% vs. 9%, $p < 0.01$). Although this might not be a direct barrier for donating, it might play a role if a donor

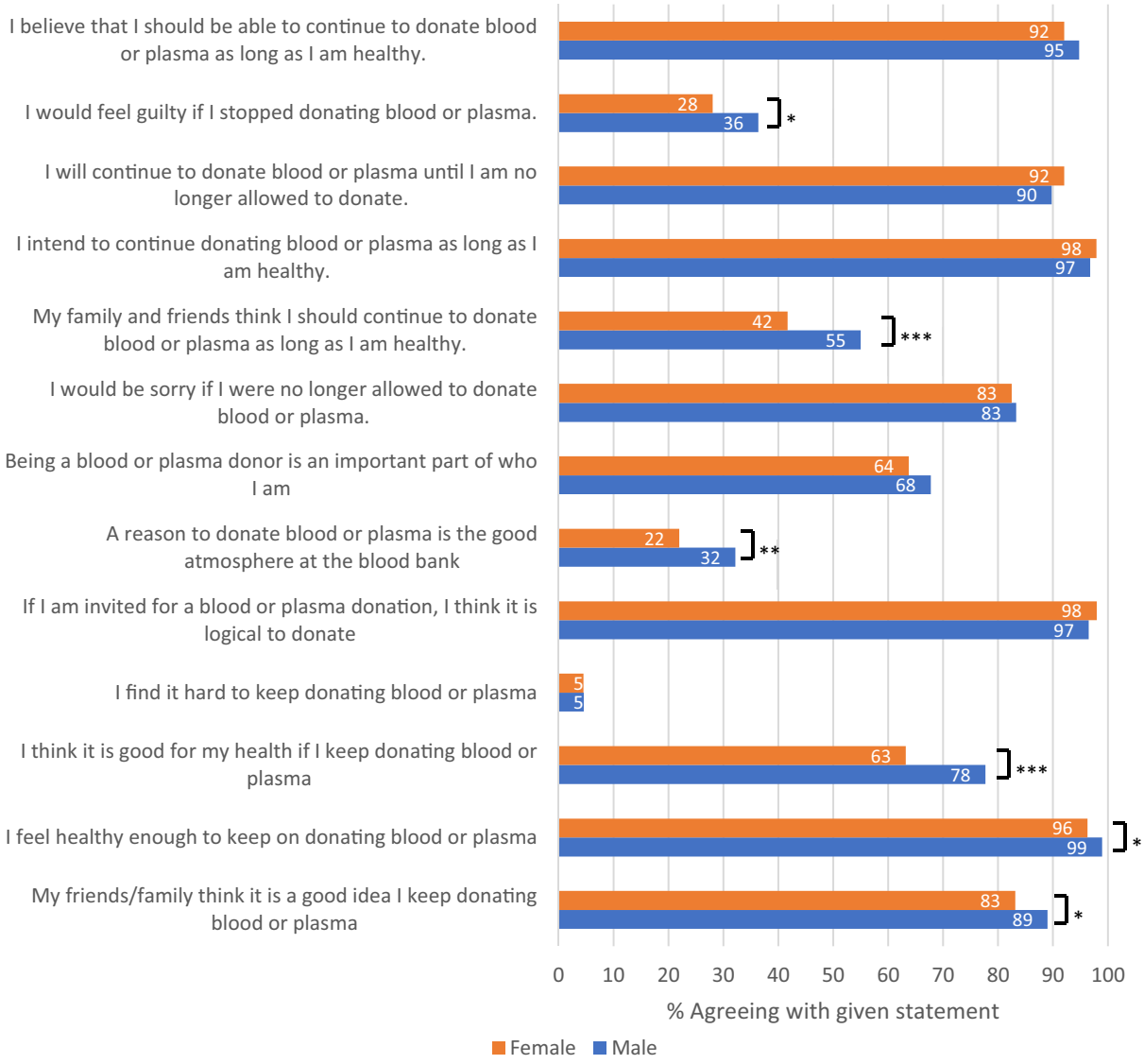

FIGURE 1 Percentage of donors who agree with statements about motivators and barriers to donate blood or plasma. *$p < 0.05$ **$p < 0.01$ ***$p < 0.001$.

considers to stop donating. Only 5% of donors indicated that they found it hard to keep donating blood or plasma (Figure 1).

When asked whether donors sometimes do not donate when they are invited to make a donation, 38% (227/602) of the surveyed donors report that this indeed occurs. Most often reported reasons for cancelling or postponing a donation were physical complaints or illness (30%) or vacation or travelling abroad (27.6%). Donors who do not feel obliged to continue donating do not more often cancel or postpone a donation than donors who do feel obliged to keep donation ($p = 0.195$).

Donation-related experiences

Overall, older donors do not often report donation-related symptoms experiences, but they are more often reported by female donors compared with male donors (Table 4). The most reported negative experiences were scars, thirst and bruising after a donation. Bruising is more often reported by females (4% always/often, 16% sometimes), compared with males (6% sometimes). On a positive note, always or often feeling fit after a donation was frequently reported by both male (19%) and female donors (15%).

Looking at donation-related complications registered by the blood bank (Table 5), haematoma is most often reported (0.95%), followed by fainting (0.31%). Women more often report fainting (0.57% vs. 0.20%) and arm pain (0.18% vs. 0.06%) compared with men ($p < 0.001$).

Stopped donors

Thirty-eight donors who stopped donating participated in the questionnaire. The most frequently reported reason to stop donating was medical reasons, including medication use that prohibits them from

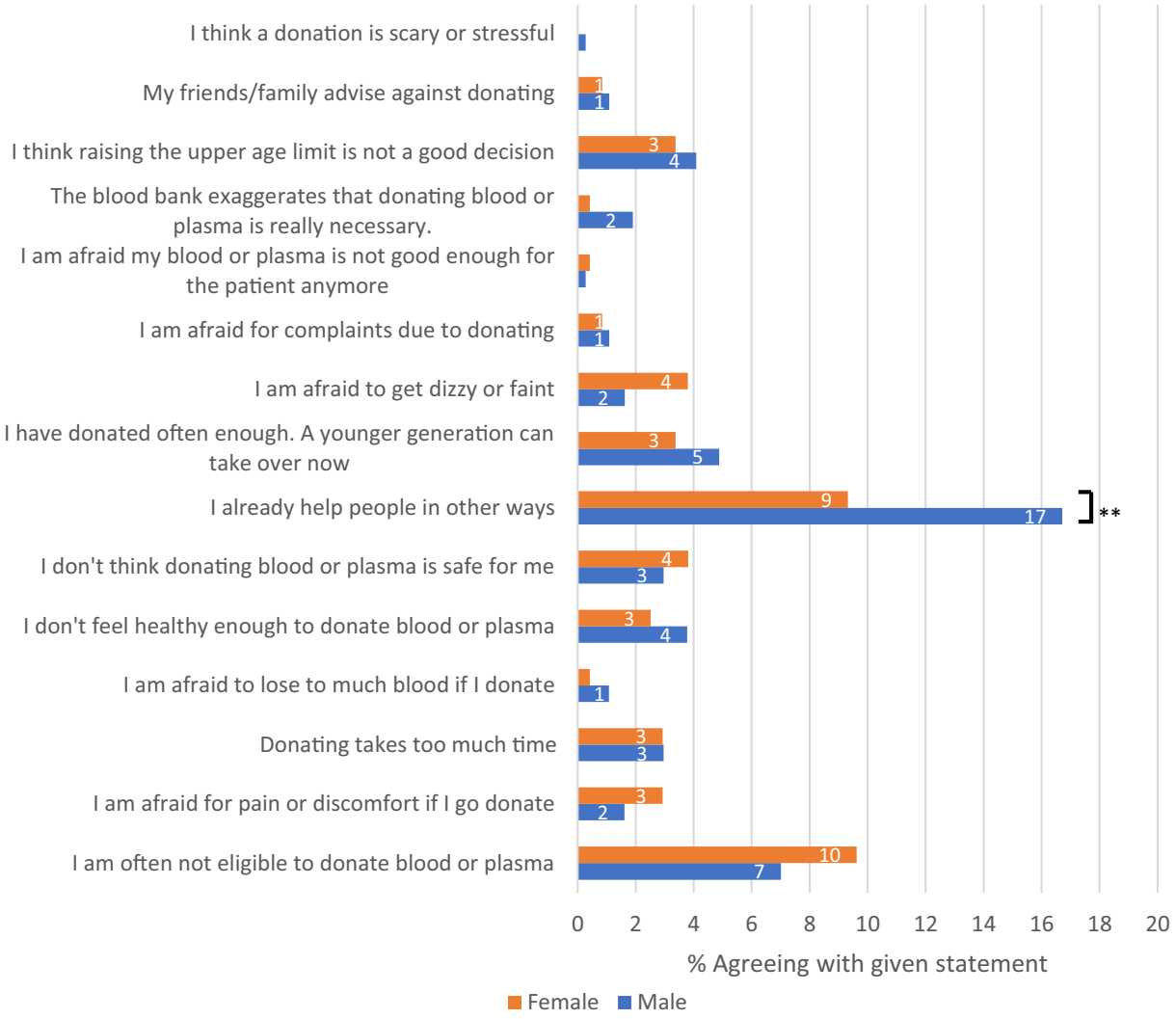

FIGURE 2 Percentage of donors who agree with statements about barriers to donate blood or plasma. *$p < 0.05$ **$p < 0.01$ ***$p < 0.001$.

TABLE 4 Self-reported donation-related experiences.

	Male			Female		
	Always/often	Sometimes	Rarely/never	Always/often	Sometimes	Rarely/never
Bruising	0%	6%	94%	4%	16%	81%
Fainting	0%	0%	100%	0%	3%	97%
Tired	1%	4%	95%	2%	11%	87%
Arm pain	0%	2%	98%	1%	6%	93%
Sick	0%	1%	99%	0%	1%	98%
Dizzy	0%	1%	99%	0%	9%	91%
Bleeding	0%	2%	98%	0%	3%	97%
Scars	2%	8%	90%	2%	8%	90%
Headache	0%	1%	99%	0%	2%	98%
Fit	19%	20%	61%	15%	18%	67%
Thirst	5%	18%	77%	4%	19%	77%

TABLE 5 Registered donation-related complications.

Registered complications: *N* (%)	Male	Female
Donors aged 68–73 years	41,696	19,383
Bruising/haematoma	380 (0.91)	198 (1.02)
Fainting***	82 (0.20)	110 (0.57)
Arm pain***	24 (0.06)	35 (0.18)
Bleeding	10 (0.02)	6 (0.03)

***$p < 0.001$.

donating ($N = 23$). Other reasons include low ferritin levels, not feeling like it anymore, a move or finding the age of 70 a good age to quit donating. Most stopped donors agreed with the increase of the upper age limit (92.1%), and 87.5% would like to have kept on donating if this was possible.

DISCUSSION

This study aimed to determine whether older donors agree with the increase of the upper age limit, whether they feel obliged to continue donating, what motivates these donors to keep donating and what they experience as barriers and conclusively what kind of donation-related experiences they report. We found that a large majority of donors agree with the increase of the upper age limit and that close to 65% of donors feel obliged to continue donating. Older donors are highly motivated to donate and report only limited barriers for donation and very few donation-related experiences or complications.

Older donors represent an important part of Sanquin's blood donor population. Participating donors were experienced, with more than 95% of donors having made over 20 blood or plasma donations. A vast majority of donors agreed with the increase of the upper age limit, indicating that most donors want to continue donating after their 70th birthday. This is good news for blood banks, as this group of donors consists mostly of highly experienced and very loyal donors. In 2021, around 5% of all donation attempts were made by older donors (aged ≤68 years). Because recruitment and retention of blood donors remains challenging, retaining this group is important [7].

Almost 65% of donors also indicate that they feel obliged to continue donating, mostly women with a high education level. Additionally, around 30% of donors reported that they would feel guilty if they stopped donating. This could be concerning; however, this result could be multi-interpretable. Donors might feel morally obliged to continue donating but not feel in anyway pressured by the blood bank to do so. Interestingly, women with higher education have higher odds of feeling obliged to continue donating. These findings should be further studied using interview or focus group interview studies, as these might be part of complex (social) constructs.

Most donors indicated they want to continue donating as long as they are healthy. This suggest donors are rather deemed eligible for donation based on their health than based on their age. They also reported that they thought their family and friends would support this decision. We found that donors, especially men, thought it was good for their health to continue to donate, suggesting that the pre-donation health check might be considered as a way to monitor their own health, for example, because of the regular blood pressure and Hb measurements [8].

Donors report that they experience few barriers for blood donation; however, they do report often not being eligible for blood donation (10% for women, 7% for men). This makes sense, as older donors might use more medications and are more often affected by chronic diseases [9]. However, donation-related experiences were reported by donors, especially bruising was often mentioned by older female donors. This was also seen in the registry data, where women reported more bruising or haematomas than men. Additionally, sometimes feeling tired or dizzy after a donation was mentioned by female donors. However, donors often do not report donation-related complications to the blood bank, and therefore, these symptoms are prone to underreporting, as we also see in our results. Goldman et al. [1] show higher Hb-deferral rates in older donors, especially in men, but less occurrence of vasovagal reactions. This is consistent with Paalvast et al. [10], who report that whereas overall rate of vasovagal reaction was lower, rate of moderate/severe vasovagal syncope was higher in older donors. Contrastingly, Newman et al. [11] report an increase in vasovagal symptoms in older donors, based on self-reported adverse events instead of registry data.

To our knowledge, this is the first survey study researching the perspectives of older donors towards blood donation that especially focuses on motivator and barriers for blood donation. The high response rate of almost 61% provides confidence that the surveyed group is representative of the older donor population. A limitation of this study is that it was anonymous, making it impossible to study associations with objectively assessed donation career, donation frequency, previous deferral or place of residence. Also, the number of stopped or lapsed donors who participated in the study was low. Therefore, we have only limited insights into their reasons to end their donor career. Additionally, as the increase of the upper age limit is quite recent, the oldest participating donor was 73 years old, meaning that these results might not reflect the opinions of the older donor population in the Netherlands in a few years, with donors reaching the age of 80 years.

In conclusion, the large majority of older donors agree with the increase of the upper age limit for blood donation, report only few and minor donation-related experiences or complications and are highly motivated to continue their donor career at higher age.

ACKNOWLEDGEMENTS

We thank Y.P. for extracting the donation complication data from the donor database. This work was supported by a 'Product and Process Development–Cellular Products' Grant (PPOC20-01).

F.Q., A.Z.L., B.S., S.B. and K.v.d.H. designed the study; F.Q. programmed the questionnaire, analysed the data and drafted a first version of the manuscript; F.Q., A.Z.L., B.S., S.B. and K.v.d.H. reviewed the manuscript and approved of the final version.

CONFLICT OF INTEREST STATEMENT

The authors declare 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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How to cite this article: Quee FA, Lathori AZ, Sijtsma B, Bruijns S, van den Hurk K. Increasing the upper age limit for blood donation: Perspectives from older donors. Vox Sang. 2024;119:648–55.

Received: 8 January 2024 | Revised: 26 March 2024 | Accepted: 29 March 2024

DOI: 10.1111/vox.13636

ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Improving gay, bisexual and transgender inclusion in blood and plasma donation policies and programmes in Canada: A qualitative study

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Funding information
Canadian Blood Services; Health Canada

Abstract

Background and Objectives: An increasing number of blood operators around the world, including those in Canada, have removed time-based deferral periods for gay, bisexual and other men who have sex with men and replaced them with sexual behaviour-based questions for all donors. While this marks a significant shift in screening approach, what remains unclear is how members of two-spirit, lesbian, gay, bisexual, transgender and queer (2S/LGBTQ+) communities view blood operators' initiatives to be more inclusive. As such, this study was conducted to assess the awareness of donor screening changes and other initiatives among members of 2S/LGBTQ+ communities and to explore their recommendations for blood operators' work with these communities.

Materials and Methods: Semi-structured qualitative interviews ($n = 15$) were conducted with 2S/LGBTQ+ people across Canada. Data were analysed using open inductive coding methods.

Results: Reported here are the key results on recommendations for blood operators. Three themes were identified from the data: (1) the need for increased communications with 2S/LGBTQ+ communities surrounding changes to donor policies and guidelines; (2) the need for trans-inclusive policy and procedures; and (3) the need for culturally responsive and equity-informed staff training at donor centres.

Conclusion: Results suggest that blood operators should consider 2S/LGTBQ+ communities when developing blood and plasma donation policies, screening procedures and staff training. Increased consultation with these communities is desired, and further research specific to the experiences of transgender blood donors is needed.

Keywords

blood donation, men who have sex with men, qualitative research, sexual risk screening, transgender

Highlights

- Blood operators should consider expanding their consultation processes for two-spirit, lesbian, gay, bisexual, transgender and queer (2S/LGBTQ+) communities regarding donation policies and procedures.
- Screening and registration processes for transgender donors must be improved by adding additional gender options (blood establishment computer systems must be updated to allow for this) and allowing for the use of chosen names during all stages of the donation process.
- Additional blood operator staff training is necessary to reduce stigma and to ensure 2S/LGBTQ+ donors are respected throughout the donation process.

INTRODUCTION

Globally, blood operators continue to address donor policies, particularly in relation to two-spirit, lesbian, gay, bisexual, transgender and queer (2S/LGBTQ+) populations. (The term 'two-spirit' is used to refer Indigenous people who hold diverse cultural, societal, spiritual, sexual and gender identities and roles. In this article, the umbrella term 'transgender', or 'trans', is used to refer to individuals whose sex assigned at birth does not exclusively align with their gender identity, including many two-spirit, non-binary and gender non-conforming people [1].) Historically, time-based deferral criteria for sexually active 'men who have sex with men' (MSM) have been used to mitigate the risk of transfusion-transmitted infections (MSM refers to men who have sex with men, the traditional target audience for these donation policies, and has been placed in quotations because it can exclude people who may be engaging in the behaviours, which have been deemed 'high risk' through existing blood operator policies). Many people within 2S/LGBTQ+ communities globally view these time-based deferral criteria as discriminatory [2–6]. More recently, many blood operators around the world have removed time-based deferral criteria for MSM and replaced this with individual behaviour-based screening approaches enabling some sexually active MSM, along with some transgender individuals, to donate blood [7].

Canada has two blood operators—Canadian Blood Services (CBS) and Hema-Quebec (HQ)—that rely on non-remunerated voluntary blood and plasma donors. At these blood operators, the deferral period for sexually active MSM donors has gradually decreased over the decades [8]. In fall 2021, CBS made a submission to Health Canada, requesting an end to the time-based deferral approach for MSM donors for all blood products, and replacing this with a behaviour-based screening approach for all donors. This change was implemented by CBS in September 2022, and donors of all genders and sexualities are now screened according to specific sexual behaviours rather than by sexual orientation [7]. HQ followed shortly afterwards, implementing the change in December 2022 [9].

Earlier research has indicated that the policy shift to behaviour-based screening in Canada was supported by MSM and blood donors overall, especially when the screening questions were accompanied by clear explanations and evidence-based reasoning [10–12]. Research has found that this change was preferable to prior initiatives, such as the MSM apheresis plasma donation programme previously implemented by CBS, which was deemed as discriminatory by some MSM donors [13, 14]. The policy shift to behaviour-based screening was also supported by findings indicating that screening questions would better identify MSM at risk of having recently contracted HIV, while reducing the number of MSM deferred by one third [15]. This change was intended to increase the number of blood donors who are able and willing to donate [16].

Importantly, the new donor guidelines approved by Health Canada also included updated screening procedures for transgender donors. Donors with a binary gender (male or female), including transgender people, may now self-identify their gender, instead of being registered according to the gender on their identification. Additionally, transgender donors are no longer asked questions about 'lower genital' gender-affirming surgery, a question previously posed by CBS. However, this change did not address the erasure of donors who do not identify within binary gender identities, and non-binary donors are still registered as their birth gender. These computer system or IT limitations, which exist in most blood establishment computer systems (BECS) globally, prohibit the inclusion of additional gender categories beyond the binary, meaning that blood and plasma donors must identify their gender as either male or female [17].

The literature on transgender donors to date focuses on areas for consideration with screening and registration, including BECS limitations, gender registration, gender-specific screening questions and metrics, which are influenced by biological sex and hormones (such as haemoglobin and ferritin levels and blood volume calculations) [17, 18]. There have been a variety of approaches to these issues globally, ranging from centres that defer all transgender donors indefinitely, to one donor centre in the United States, which created options for 'Transgender' and 'Other' in their BECS, developed gender-neutral screening questions, and chose to use the most restrictive measures for haemoglobin and blood volume [19]. Many blood operators continue to be limited by the BECS system provided by eProgresa, which does not allow for the customization of gender options, and changes would require the software vendor to develop additional options [18].

While negative and stigmatizing blood and plasma donation experiences have been reported in the media [20], there is a lack of peer-reviewed, primary scientific literature exploring the specific perspectives of transgender blood and plasma donors. Recent work has indicated that more research with transgender donors is needed to

ensure informed, effective and equity-conscious policymaking by blood operators [18]. In the context of the recent changes to 2S/LGBTQ+ blood donation policy in many countries, and the limited amount of literature on transgender donation experiences, this study aimed to address existing knowledge gaps by conducting semi-structured interviews with 2S/LGBTQ+ community members in Canada. Most participants were transgender ($n = 12$), with a few participants identifying as cisgender men ($n = 3$). The interviews focused on three main objectives: to assess 2S/LGBTQ+ blood and apheresis plasma donation experiences; to assess 2S/LGBTQ+ views on initiatives to make blood and plasma donation more inclusive to these communities; and to generate recommendations for blood operators wishing to address barriers to blood and plasma donation for 2S/LGBTQ+ donors. This article presents the key results of participants' recommendations for blood operators.

METHODS

Participants

Qualitative, semi-structured one-on-one interviews were conducted with 2S/LGBTQ+ people across Canada. This study was approved by Research Ethics Boards at Mount Saint Vincent University and Canadian Blood Services.

Purposive and snowball sampling were used to recruit 2S/LGBTQ+ individuals across Canada for this study. Specifically, participants were recruited through online presence among 2S/LGBTQ+ serving community organizations such as the Community-Based Research Centre's InvestiGaytors program and the AIDS Coalition of Nova Scotia. The eligibility criteria included: (1) being 18 years or older; (2) being able to speak and understand English; and (3) identifying as 2S/LGBTQ+. Participants self-declared their age, sexual and gender identity, and English language proficiency. There were no specific criteria regarding current involvement in blood or plasma donation, as the goal of the study was to assess both donor perceptions and broader community perspectives on blood and plasma donation. Participants were provided with a $25 gift card for their time.

Data collection and analysis

Each participant completed a consent form and emailed it to the research team member responsible for recruitment. Semi-structured, one-on-one interviews were conducted online using a video conferencing platform. The interviews lasted between 8 and 38 min. Participants were asked (1) if they had donated blood in the last year and what their experience was like, (2) if they had donated plasma in the last year and what their experience was like, (3) about their awareness of initiatives to make blood and plasma donation processes inclusive of 2S/LGBTQ+ communities, and (4) recommendations for how blood and plasma donation processes could be more inclusive to 2S/LGBTQ+ donors. Interviews were audio-recorded and transcribed verbatim by a professional transcriptionist.

Interview data were analysed using a thematic analytic approach based in grounded theory [21, 22]. Open inductive coding was applied, where both researchers engaged with the interview data to separately identify key themes independent of a predetermined coding framework [23, 24]. After immersing themselves in the data by reading the transcripts several times, the researcher responsible for analysis coded the transcripts. Then, the researcher examined new codes in each successive transcript and found no new recommendations emerged following 15 transcripts suggesting data saturation [25]. Codes were sorted into themes, which were then reviewed and named. Details of the themes are presented below.

RESULTS

Sample description

Of the 15 interviews, 12 participants identified as transgender, with 7 identifying as transgender men, two as transgender women and three as non-binary/agender. The remaining three participants identified as cisgender men. Most participants identified as gay or bisexual and were between the ages of 18 and 40. A detailed breakdown of participant demographics can be seen in Table 1.

An overview of the key results from the analysis is presented below. Analysis focused primarily on participants' recommendations for blood operators. The primary themes identified are as follows: (1) the need for increased communications with 2S/LGBTQ+ communities surrounding donor guidelines; (2) the need for trans-inclusive policy and procedures; and (3) the need for culturally responsive and equity-informed staff training at CBS locations.

Need for increased communications with 2S/LGBTQ+ communities regarding donor guidelines

Perceptions of blood operators differed among participants, with some reporting positive donation experiences while others expressed distrust based on previous policies, which they viewed to be discriminatory. When discussing perceptions of blood operators, many participants emphasized the importance of word-of-mouth testimonials and the impact of hearing about blood and plasma donation experiences of others in 2S/LGBTQ+ communities. They expressed a desire to see blood operators improve relationships with 2S/LGBTQ+ communities. Suggestions included establishing partnerships with trusted community-based organizations that serve 2S/LGBTQ+ populations and creating a presence for blood operators at Pride events. Several participants mentioned that social media awareness campaigns could be utilized, and one participant specifically mentioned that having campaigns showing transgender people donating blood could have a significant positive effect:

TABLE 1 Sample description.

Category	Participants
Total	15
Blood donor status	
Has donated blood	7
Attempted to donate blood, but was deferred	4
Has donated plasma	0
Has not attempted to donate blood or plasma	4
Age	
18–30	9
31–40	5
41–50	0
51–60	0
61–70	0
71–80	1
Gender	
Transgender man	7
Transgender woman	2
Non-binary/agender/gender fluid	3
Cisgender man	3
Sexual orientation	
Gay	6
Bisexual	4
Heterosexual	2
Queer	3
Race	
Black/of African descent	2
Indigenous	3
Southeast Asian	3
Caucasian/White	7
Highest education attained	
Master's degree	3
Undergraduate degree	7
Some undergraduate	4
College	1
Annual income	
Less than $10,000	2
$10,000–$19,999	5
$20,000–$49,999	4
$50,000–$69,999	4

> I noticed the guidance for trans people has changed. It makes me feel a little more secure. I still feel nervous every time I go in. And I don't know how to change that, aside from even advertising campaigns that show a visibly trans person like, "I'm giving blood."
>
> (Participant 15)

The same participant mentioned that their friends were surprised to learn that queer and transgender people are eligible to donate blood:

> I have a blood donor card because of how often I give. And so sometimes my friends who are gay men or trans people, when they see that in my wallet, they're really shocked that I'm even allowed to give. And so I have to say to them like, no, this is legal. I fall under the guidelines. And every time people are just surprised. And so I think there's this gap where people for so long have been fed this narrative that we're not allowed to give, that you don't bother looking into that further.
>
> (Participant 15)

This participant's comments demonstrate the value of community-based knowledge sharing around blood donation, and the impact of trusted source, word-of-mouth information in combatting old narratives regarding blood and plasma donation.

Several participants also suggested that clarity and transparency around nuanced guidelines, such as the four-month deferral window for those who are taking pre-exposure prophylaxis (PrEP), would increase awareness and improve the reputation of blood operators. However, participants cautioned that guidelines regarding PrEP may need to be addressed if blood operators hope to make substantive progress on their community reputation. Specifically, some participants felt that the inability to donate while taking PrEP was a significant barrier to 2S/LGBTQ+ inclusion in blood and plasma donation:

> I think really clear and targeted messaging that we want gay people to give blood. Like if you're gay, your blood is safe. It's not, you're not tainted in any way. And I think that that's still something that a lot of people carry with them. And at the same time, I think it's really hard to deliver those messages if you're saying that men who have sex with men who are not their one consistent partner within the last three months can't give, and the men who are on PrEP can't give… There's kind of a fundamental incompatibility between trying to get gay people on board with this, and then still telling us that the way that we live our lives is fundamentally incompatible with delivering this service.
>
> (Participant 15)

In addition, several participants emphasized the key role that community advocacy played in changing these policies. This signifies the importance of continued involvement with 2S/LGBTQ+ communities as policies change and evolve over time and new research evidence becomes available:

> I think definitely it feels like something that was coming from the community. And if those efforts hadn't been made, then probably nothing would have changed.
>
> (Participant 1)

Need for trans-inclusive policy and procedures

Among transgender participants, there was a desire for blood operators to implement more inclusive policies and procedures. Three sub-themes have been identified: (1) improving screening and intake processes; (2) building more intersectional policy; and (3) obtaining additional community consultation on donor policy.

1. Improving screening and intake processes: Several participants expressed frustration with the binary gender system used by blood operators, and wished there was an option to self-identify with additional gender options:

> I was very annoyed to see that they're not able to accommodate for a third gender option. Because they're like, "Oh, when our systems are not…" It's, that's so lazy. [laughs] Just saying, "Well, the computer doesn't do that."
>
> (Participant 1)

This demonstrates the need for blood operators to work with BECS developers to include more gender options in registration systems. It also highlights the importance of increased communication between blood operators and community members regarding these systems.

Another recommendation was that blood operators create space for donors to use a chosen name instead of their legal name (or 'dead name', the use of which can cause harm to transgender individuals), and to identify their pronouns to staff. Alongside this recommendation, it was noted that legal names should be available to a limited number of staff, and most staff should only have access to the chosen donor name.

Participants also identified that it would increase their comfort and reduce stigma to have unnecessary questions about gender identity and medical transition removed from the screening process. One participant stated that they avoided donating specifically due to questions they perceived as invasive:

> I just don't want to go in and then them having to question me about myself, my gender, my sexuality, and then just have a whole ordeal. Because in all honesty, the world of institutions, like those in the medical field, I don't know why, they're just very much intrusive like that.
>
> (Participant 11)

2. Building more intersectional policy: When discussing policy, the idea emerged that instead of simply removing discriminatory policies, more inclusive policies need to be built. While many participants felt that policy had improved for cisgender gay men donating blood, several noted that policy had not improved for transgender donors:

> I know a lot of trans folks in particular who have faced a lot of discrimination trying to access. So on one hand, while they're saying, you know, this category of people, we're going to reduce times, you know, make it seem like we are making an effort, a lot of trans folks have had a harder time.
>
> (Participant 2)

One participant reported that the process had improved for transgender donors who fit into the gender binary, have legally changed their name and who are generally perceived by cisgender people as their lived gender. However, the participant noted that for the many transgender people for whom this is not a reality, the process may not have improved:

> I think for me, that guidance that you can just give under your legal sex makes me feel a lot more secure when I'm giving. It makes me feel like I'm not going to face any of those types of challenges that I used to. But with the caveat that I'm going in looking like what they expect. And it's probably really different for other trans people. I'm coming from a place of being way further in my transition. I'm sure for people who are earlier in transition, I probably just wouldn't have given if my name and legal gender didn't match up.
>
> (Participant 15)

Blood operators need to better communicate information about donating blood and plasma while on gender-affirming hormones or after gender-affirming surgery, and participants noted that having these policies more widely available would ease their stress about donating.

Considerations of equity such as race and ethnicity also came up while discussing blood and plasma donation policies. Some participants felt that policies were needed to prevent racial discrimination while donating, and one participant expressed a need to decolonize blood operators and their policies affecting 2S/LGBTQ+ donors. One participant reflected on the need for increased intersectionality when working with 2S/LGBTQ+ communities:

> There are different communities that have been disadvantaged differently based on these policies. And so whether it's gay males, or whether it's trans males, whether it's people of colour, I would like to see them more specifically work towards accountability and responsibility, and not treat all the communities they disadvantaged as like a monolith.
>
> (Participant 8)

3. Engaging community consultation on policy: A common view among many participants was a desire for transgender people to be consulted on policy changes through community forums and direct contact with blood operators. Participants suggested including 2S/LGBTQ+-identified community members, organizers and researchers in these decision-making processes:

> We need queer and trans voices in there. We need people to help make the decisions on policy, and how to have those be implemented, and how to protect queer and trans workers in this field, and how to ensure that if there are people walking through the doors who are queer and trans, they feel safe and welcomed and not like they are less than.
>
> (Participant 2)

Across most interviews, participants expressed that there was a need for policy changes that made the community feel safe donating blood, going beyond tokenizing, surface-level solutions and towards structural-level change within blood operator processes.

Need for staff training

When discussing donation policy, several participants remarked that there is a need to supplement policies with additional training on implementation. This includes language sensitivity training and specifically, training for staff to use chosen names and pronouns for donors at all stages of the donation process. One participant suggested partnering with 2S/LGTBQ+ community-based organizations to deliver this training.

An additional area identified by participants was navigating gendered washrooms. While access to gender-neutral washrooms was preferred, one participant reflected on the role of staff perceptions on washroom access:

> Let's say if you ask someone where the bathroom is, they will look at you first, to kind of frame you, putting you in a box of what bathroom should be best for you. Which is not the case that everybody identifies themselves with. So maybe the staff could be trained as, "Hey, left to male bathroom, and right is to the female bathroom", that sort of thing.
>
> (Participant 13)

Further training around medical transition and blood donation was also suggested for blood operators. Experiences of participants ranged from meeting with staff who had no knowledge of medical transition to experiences, which were outwardly discriminatory. One participant described an encounter where they were asked many questions about their testosterone prescription and gender identity by a nurse during intake, and this was followed by questions from the same nurse during the donation process, in a public area:

> She came up to me and she was like, "Hi. I was just wondering more about why you take testosterone." And I was in the midst of giving blood. And so, yeah, she asked me, she was like, "Are you a woman?" And I was like, "No."
>
> (Participant 15)

This participant felt this exchange to be clearly discriminatory and stigmatizing, with the nurse asking questions they perceived to be intrusive and inquiring about their sex assigned at birth as if it was their true gender identity. This conversation occurred without consideration of donor privacy.

Participants reflected on the need for donor centre staff conducting screening for medications and recent surgeries to have knowledge of transition-related medical care and its impacts on donation to prevent stigmatization of transgender donors:

> I think more awareness of what transition-related health care looks like, and whether it actually does have any interaction with whether or not you can donate blood. Like, I know that my having undergone nipple grafts for top surgery was not actually something that was a risk. But because it is a graft, that's all they knew about it. Oh, and also the first time I gave blood, they thought that when I was taking testosterone, I meant human growth hormone. If this is a population that you've had difficulties with and performed a lot of micro aggressions against, I do think there's warrant there for people to learn more.
>
> (Participant 15)

It is important that staff at all stages of the donation process be equipped to work respectfully with 2S/LGBTQ+ donors. Participants stressed the importance of all blood operator staff being trained to have conversations in a de-stigmatizing and non-pathologizing manner and felt that this would result in an increased sense of safety during donation.

DISCUSSION

This study explored perceptions of blood and plasma donation among a sample of 2S/LGBTQ+ people in Canada, including their perceptions of inclusivity initiatives by blood operators. While there is a knowledge gap in the research examining the experiences of transgender donors, a global survey of blood operators identified that transgender blood donation is a pertinent topic in many countries [17].

In a scoping review of healthcare provider perceptions, Butler-Foster et al. recommend the implementation of inclusive intake processes [20]. Pandey et al. provide a global perspective on assessment, registration and screening questions, and highlight that the BECS used by many blood operators is a limitation when it comes to inclusive gender options for registration [17]. This study described the significance of intake and gender classification processes, including more

affirming name and gender options, and blood operators should consider working with software vendors in order to adapt BECS to be more customizable.

Similarly, Goldman et al. outline reasons for the complexity of assessment for transgender donors at CBS and indicates that increased cultural competency training is needed among CBS staff [18]. Butler-Foster et al. also found that healthcare providers across disciplines indicated a need for more comprehensive staff training on assessment and cultural competency, which the current study corroborates [20]. This aligns with findings from the current study, which recommends increased staff training.

As stated earlier, there is a paucity of peer-reviewed scientific literature specific to transgender blood donation. While CBS publicizes their guidelines on hormone usage for gender affirmation as an acceptable medication to use prior to donating blood [26], participants reported stigmatizing experiences surrounding blood donation and hormone use, and medical transition overall. These data suggest that further studies are needed to examine transgender blood donation experiences under the newly instituted donor guidelines.

Further research is also needed on the perceptions of guidelines around PrEP and blood donation, as the current study suggests these guidelines have significant implications for many potential 2S/LGBTQ+ donors. Findings from this study indicate participant discomfort regarding the inability to donate blood while using PrEP and suggested that this guideline needs to be re-evaluated. CBS suggests that there is a need for further research on how PrEP and post-exposure prophylaxis affect HIV testing [7]. Similarly, participant dissatisfaction with guidelines around anal sex with new and multiple partners should be addressed in future research on blood and plasma donation policies.

There were several limitations to this study. First, the analysis was derived from 15 interviews and, due to this low number, may be limited in its generalizability, particularly for more diverse 2S/LGBTQ+ populations. The sample, overall, had a high representation of younger and low-income 2S/LGBTQ+ people. Additionally, frequent changes to donor policy, without direct and comprehensive communication to these communities about the changes, meant that participants' perceptions of blood operators may have been based on past versions of donor screening measures. Finally, the nature of an opt-in study means that 2S/LGBTQ+ people engaged in their communities or engaged in blood donation were more likely to participate, leading to potential self-selection bias in the data.

In conclusion, the key findings of this study provide a broad overview of 2S/LGBTQ+ experiences and perceptions of blood and plasma donation in the Canadian context. For blood operators on a global scale, it is recommended that efforts are made to repair relationships with 2S/LGBTQ+ communities. Based on the findings from the current study, additional actions include increasing community consultations, changing procedures and policies to be trans-inclusive, and ensuring staff are appropriately trained to work with 2S/LGTBQ+ donors. Further, it is suggested that the blood operators should redress the equity impacts for 2S/LGBTQ+ communities through policy change and better donor experiences.

ACKNOWLEDGEMENTS

We would like to thank all study participants, members of the research team and community organizations who contributed to this study. This study received research funding support from the Canadian Blood Services MSM Plasma Program, funded by Health Canada and the provincial and territorial ministries of health. This paper does not reflect the views of the federal, provincial or territorial governments of Canada.

I.W. performed the analysis and wrote the paper. J.G. supervised the research, contributed to the conceptualization of the study and reviewed and edited the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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How to cite this article: Wright I, Gahagan J. Improving gay, bisexual and transgender inclusion in blood and plasma donation policies and programmes in Canada: A qualitative study. Vox Sang. 2024;119:656–63.

Received: 14 February 2024 | Revised: 18 March 2024 | Accepted: 28 March 2024

DOI: 10.1111/vox.13639

ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Iron status in Dutch and Finnish blood donor and general populations: A cross-cohort comparison study

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Funding information
Sanquin's Research Programming Committee, Grant/Award Number: PPOC19-02; Finnish Red Cross Blood Service

Abstract

Background and Objectives: Blood donors are at risk of developing iron deficiency (ID) (ferritin <15 μg/L, World Health Organization definition). Blood services implement different strategies to mitigate this risk. Although in Finland risk group-based iron supplementation is in place, no iron supplementation is provided in the Netherlands. We aim to describe differences in ferritin levels and ID prevalence in donor and general populations in these countries.

Materials and Methods: Six cohorts, stratified based on sex, and for women age, in the Netherlands and Finland were used to evaluate differences in ferritin levels and ID between donor populations (Donor InSight-III and FinDonor 10,000) and general populations (Prevention of Renal and Vascular End-Stage Disease [PREVEND], FinRisk 1997 and Health 2000) and newly registered Dutch donors. Multivariable logistic regression was used to quantify associations of various explanatory factors with ID.

Results: In total, 13,443 Dutch and 13,933 Finnish subjects were included. Donors, except for women aged ≤50 years old in Finland, had lower median ferritin levels compared with the general population and new donors. Dutch regular blood donors had higher or similar prevalence of ID as compared with the Dutch general population, including new donors. In contrast, Finnish donors showed similar prevalence of ID compared with the general population, except for a markedly lower prevalence in ≤50-year-old women who routinely receive iron supplements when donating.

Conclusion: Iron status in blood donors differs from that in the general population. The Finnish blood service donor management policy, for example, iron

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supplementation for risk groups, seemingly protects young female blood donors from developing ID.

Keywords
blood donation, cross-cohort comparison, ferritin, iron deficiency, iron supplementation

Highlights

- Young female donors in Finland, in contrast to the Netherlands, have a lower prevalence of iron deficiency compared with the general population.
- Differences in iron deficiency between the young female donor population and the general population within the Netherlands and Finland cannot be attributed to differences in demographic characteristics or donation history but are probably due to the iron supplementation policy for at-risk blood donors that is being followed by the Finnish blood service.
- Iron supplementation as a blood service iron management policy is likely to have large effects on the iron status of blood donors.

INTRODUCTION

Whole blood donors are at risk of developing iron deficiency (ID) due to haemoglobin (Hb)-bound iron loss [1]. To ensure blood product quality and donor health, most blood services measure Hb levels before donation and use donation intervals as a standard policy to protect donors from developing ID. The time in between two donations should enable the donor to recover the lost iron. However, in most cases, the minimum donation intervals are not sufficient to completely recover iron stores and Hb levels [2–4].

Blood banks worldwide use different strategies to manage iron levels of their donors. The Dutch National Blood Service (Sanquin) and the Finnish Red Cross Blood Service (FRCBS) are the only organizations authorized by their respective governments to collect, process and allocate blood products in the Netherlands and Finland, respectively [5]. In both countries, blood donations are voluntary and non-remunerated. Both organizations measure Hb prior to every donation using point-of-care method from skin-prick samples. Based on the threshold set by European legislation, donors with Hb levels below 7.8 mmol/L/125 g/L (female) and 8.4 mmol/L/135 g/L (male) are deferred for 3 months.

Because Hb levels do not reflect donors' iron stores, since November 2017, Sanquin has implemented ferritin measurements for new donors and at every fifth donation [6]. When ferritin levels are <15 μg/L or ≥15 and ≤30 μg/L, the subsequent donation intervals are extended to 12 or 6 months, respectively [7]. Although this policy has a beneficial effect on the iron status of the blood donor, it reduces donor availability [2]. For the present study, however, data from before implementation of this ferritin measurement policy are used, when only pre-donation Hb measurements were in place.

In Finland, ferritin monitoring has not been implemented, but since the 1980s, blood donors at risk of developing ID are issued iron supplements. Based on this policy, excluding donors with known hemochromatosis, all female donors under the age of 50 years and all donors donating every 4 months or more frequently are provided with iron supplements. Since 2021, the youngest women (i.e., 18–25 years old) are provided a higher dose of with 40 daily tablets of 50 mg ferrous bisglycinate; other risk groups are provided with 20 daily tablets. The issued iron supplements are meant to replace iron lost due to blood donation, not as treatment for low Hb values or anaemia.

The FRCBS, thus, uses a different strategy to protect donors from ID compared with the Netherlands. Yet it is unknown if the different iron management policies are reflected in the iron status of the Dutch and Finnish donor populations compared with the general population of each country. Therefore, by using data from multiple cohort studies, we aim to compare ID prevalence (ferritin <15 μg/L) in Dutch and Finnish donor and general populations.

METHODS

Setting

At the time of data collection, both Sanquin and FRCBS evaluated donors for their eligibility to donate before every donation. These pre-donation screenings consist of a health and risk behaviour assessment by means of a donor health questionnaire, an interview, blood pressure and Hb testing and blood sampling for blood typing and infectious disease testing [8]. In addition, at Sanquin, new blood donors make an additional visit for just this pre-donation screening before their first blood donation.

Donors were allowed to donate when they were in good health, at least 18 years old and not at risk for any transfusion-transmissible infections. Consistent with European legislation, Hb cut-offs for donation eligibility for male and female donors were >135 and >125 g/L (measured with the HemoCue 201, Angelholm, Sweden), respectively. Donors with Hb levels below the cut-off were not eligible to donate for 3 months. At Sanquin, blood could be donated with a minimum donation interval of 56 days, with a maximum of 5 donations per year for men and 3 donations per year for women. At the FRCBS, women could donate blood with a minimum donation interval of 91 days, women under 25 years could donate once per year and men could donate with a minimum donation interval of 61 days [9].

In addition to the aforementioned policies, the FRCBS provided at the time of data collection to donors at risk of ID (i.e., all women under 50 years of age and all donors donating every 4 months) with iron supplements consisting of 20 daily tablets containing 25 mg ferrous fumarate and 350 mg of animal-based haemoglobin, or with 30 daily tablets containing 100 mg of ferrous sulphate [10]. In contrast to current policies, neither Sanquin's ferritin-guided donation interval policy nor the FRCBS's policy of higher iron supplementation dose for the 18–25-year age group was in place during the time of data collection for the present study.

Cohort populations

Data from six population cohorts were used for the analysis. For the Netherlands, the donor population was represented by the Donor Insight-III (DIS-III, 2015–2016) cohort, the general population by the Prevention of Renal and Vascular End-Stage Disease (PREVEND, 2001–2003) cohort and a cohort of new donors (2021) who had not yet donated. The Finnish donor population was represented by the FinDonor 10,000 (FinDonor, 2015–2017) cohort, and the general population by the Health 2000 (2000–2001) and the FinRisk 1997 cohorts. All participants gave written informed consent, and the studies were conducted in accordance with the Declaration of Helsinki. The cohorts were selected for this study based on availability of ferritin data. For comparability purposes, participants from all cohorts had to meet certain criteria to be included in the analysis. Exclusion criteria for the analysis were age <18 and ≥70 years, current pregnancy and insulin-dependent diabetes mellitus. For both donor population cohorts, participants were not excluded from participation based on Hb levels that would render them ineligible for blood donation. For the final Finnish general population cohort in this current study, the FinRisk and Health 2000 cohorts were combined. Information regarding the blood samples and ferritin measurement methods for each cohort are presented in Data S1: Appendix A.

Donor Insight-III

Donor Insight is a prospective longitudinal nationwide cohort study, aimed to provide more detailed information and scientific evidence for blood service decision-making. DIS-III is the most recent round and was specifically focused on associations of lifestyle behaviours and genetic factors with blood parameters. Ethical approval was granted by the Medical Ethical Committee of the Academic Medical Center, Amsterdam, the Netherlands. The methodology of the DIS-III study has been extensively described elsewhere [11].

Prevention of Renal and Vascular End-Stage Disease

The PREVEND study is a prospective longitudinal cohort study designed to investigate the natural course of microalbuminuria in relation to renal and cardiovascular disease in the general population. All inhabitants of the Dutch city of Groningen, aged between 28 and 75 years, received a questionnaire on demographic background, disease history, smoking habits and medication use. Respondents suffering from insulin-dependent diabetes mellitus and pregnant women were excluded from participation. For the analyses of the present study, we used data from the second PREVEND survey. The PREVEND study protocol was approved by the Medical Ethical Committee of the University Medical Center Groningen, and the cohort has previously been described [12].

New donors

The new donor cohort is based on all newly registered donors who went through the donor screening in 2021. Newly registered donors' ferritin levels are measured as a part of the pre-donation screening for newly registered donors. Demographic characteristics (i.e., age, weight and height) were linked to the donor screening data using Sanquin's donor registry. To match the number of cases in the other cohort studies, a random selection of 10% of all newly registered donors was used for the analysis.

FinDonor 10,000

The FinDonor 10,000 study is a cross-sectional cohort study designed to evaluate the iron status and factors affecting iron stores in Finnish blood donors. All donors donating at one of the selected donation locations in the Helsinki metropolitan area (Kivihaka, Sanomatalo and Espoo) were informed about the opportunity to participate in the study through paper promotional material and social media. Prospective whole blood donors were also able to join the study but were not included in this study due to low participation (45 new donors). In line with the regular donation criteria, the age limit for participation was 18–70 years for experienced donors (i.e., donated at least once) and 18–59 years for first time donors. Donors with a permanent deferral were excluded from participation. The FinDonor study was ethically reviewed and approved by the Ethical Board of Helsinki University Hospital, Helsinki, Finland. The protocol of the FinDonor 10,000 study has been described in more detail [13].

FinRisk 1997

The FinRisk 1997 is a cross-sectional cohort from the FINRISK study, a prospective study in which every 5 years a new cohort of permanent Finnish residents aged 24–74 years is added based on a random sample from the National Population Information System. The FINRISK study is designed to monitor changes in major non-communicable disease risk factors and health behaviours. For the Finrisk 1997 cohort, the residents of specific Finnish regions (i.e., North Karelia, Northern Savo, Turku, Loimaa, Helsinki, Vantaa

and Oulu) were invited through a mailed postal letter. Ethical approval was obtained from the Ethics Committee of the National Public Health Institute. Further details on the methodology of the FINRISK study have already been described elsewhere [14].

Health 2000

The Health 2000 study is a cross-sectional cohort study aimed to provide an overview of the major public health problems, functional capacity and work ability in the Finnish general population. A nationally representative sample of individuals aged 18 and over, and an oversample of those aged 80 years or over, was drawn. A two-stage cluster sampling approach using the population-wide insurance database as a sampling frame from the Social Insurance Institution of Finland was used. Ethical approval for the Health 2000 study was granted by Ethical Committee for Research in Epidemiology and Public Health at the Hospital District of Helsinki and Uusimaa in May 2000. The methodology of the Health 2000 study has already been described elsewhere [15].

Statistical analysis

The statistical analyses were performed for the Dutch and Finnish cohort separately, and tables and graphs were made using summary data. Data preparation was performed using both IBM SPSS Statistics (Version 28) and RStudio (v4.0.3, RStudio, PBC, Boston). The statistical analyses were performed in R using RStudio, and the code used is available on GitHub (https://github.com/FRCBS/FIN_NL_cohort_collaboration).

Descriptive statistics are presented for all cohort populations separately in Table 1. Normally distributed continuous data are presented as mean ± standard deviation, and non-normally distributed data as median with interquartile range. Categorical data are expressed as proportions. We calculated bootstrap distributions of ID using the boot package and bootstrap confidence intervals with the rsample package. Regression models were fitted as Bayesian models using brms [16]. MCMC diagnostics (i.e., visualization of possible divergent transitions, Rhat estimation and autocorrelation) was performed using Bayesplot [17].

Regression analysis

Bayesian multivariable logistic regression analysis was used to analyse differences in ID prevalence [12]. Based on the recommendations of the World Health Organization, cut-off value for ID is ferritin level of 15 μg/L. Four different models varying in explanatory variables were used: Model 1 (M1), only including the cohort membership variables; Model 2 (M2), including the cohort membership variables and demographic variables (age and blood volume); Model 3 (M3), including the cohort membership variables, demographic variables and donation history (number of days since last donation and number of donation is the last 2 years since participation); Model 4 (M4), including the general population membership only, demographic variables, donation history, smoking, previous childbirth, hormonal contraception use and menstrual status.

For all models, the DIS-III and FinDonor were used as reference groups for the cohort variables for the Dutch and Finnish analysis, respectively. In M4 of the Dutch data analysis, only the general population cohort was included, as data on the additional variables compared with M3 were unavailable. For all general population cohorts, the number of previous donations was set at 0, and the number of days since last donation was imputed as a random value between 3 and 6 years. The latter imputation was also performed for donors with missing values for days since last donation. Blood volume was calculated using Nadler's formula: $(0.3669 \times \text{height}^3) + (0.03219 \times \text{weight}) + 0.6041$ for men and $(0.3561 \times \text{height}^3) + (0.03308 \times \text{weight}) + 0.1833$ for women. Data were stratified for sex for all analyses. Furthermore, to take menopausal status into account, data for women were also stratified by age (i.e., ≤50 and >50 years of age), whereas data on menopausal status were not available for all cohorts. An overview of the configuration of all variables is presented in Data S1: Appendix B.

RESULTS

A total of 16,578 records were available for the Dutch and 16,784 records for the Finnish population. After exclusion of cases with missing data for any of the variables used in the analysis, extreme values for ferritin (>400 μg/L) or body mass index (>50 kg/m^2), age (<17 years) and weight (<50 kg) below the donation threshold and current pregnancy or insulin-dependent diabetes mellitus, 13,443 cases were eligible of the analysis for the Dutch and 14,027 cases for the Finnish populations. An overview of the exclusions for each cohort individually is presented in Figure 1. Table 1 describes baseline characteristics split by sex and for women by age group (i.e., ≤50 years and >50 years). Blood volume and age were similar within each stratification group for both countries, except for a lower age in new blood donors. Number of donations in the past 2 years were similar between the donor population groups within the Dutch and Finnish stratification groups. However, the Dutch donor population counted more days since the last donation than the Finnish. Within all groups, both in the Netherlands and Finland, the proportion of smokers was higher among the general population compared with the blood donor population. In Finnish female blood donors aged ≤50 years, less had given birth in the past and current hormonal contraception use was higher compared with the general Finnish population. Other variables were similar across groups for both countries. Menstrual status only differed between Dutch women aged ≤50 years, with a smaller proportion of menstruating women in the blood donor population compared with the general population.

TABLE 1 Descriptive characteristics of the study populations.

	Dutch			Finnish	
	Blood donors	General population	New blood donors	Blood donors	General population
Men					
n	887	2854	1867	963	5739
Age (mean [SD])	50.9 (12.9)	54.3 (12.5)	34.3 (12.6)	46.26 (13.8)	48.2 (12.1)
Blood volume (L) (mean [SD])	5.6 (0.6)	5.5 (0.6)	5.6 (0.6)	5.50 (0.59)	5.3 (0.6)
Ferritin (μg/L) (median [IQR])	50.1 [29.1–86.4]	134.00 [82.0–204.0]	125.0 [84.0–184.0]	42.00 [25.00–67.00]	111.0 [66.6–172.7]
Iron deficiency (%)	64 (7.2)	37 (1.3)	3 (0.2)	62 (6.4)	176 (3.1)
Number of donations in the last two years (median [IQR])	4.0 [1.0–6.0]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	5.0 [2.0–7.0]	0.0 [0.0–0.0]
Number of days since last donation (median [IQR])	165.0 [97.0–499.5]	1628.0 [1355.0–1897.0]	1628.0 [1355.0–1897.5]	109.0 [77.0–193.5]	1640.0 [1370.0–1921.0]
Smoking (%)	85 (9.6)	930 (32.6)	NA	58 (6.0)	1716 (29.9)
Childbirth in the past (%)	NA	NA	NA	NA	NA
Current hormonal contraception use (%)	NA	NA	NA	NA	NA
Menstruation (%)	NA	NA	NA	NA	NA
Women (≤50 years)					
n	588	1382	2887	888	3378
Age (mean [SD])	37.5 (7.6)	42.3 (4.5)	28.6 (9.5)	33.37 (9.1)	39.09 (7.0)
Blood volume (L) (mean [SD])	4.4 (0.5)	4.3 (0.5)	4.3 (0.5)	4.20 (0.56)	4.03 (0.5)
Ferritin (μg/L) (median [IQR])	29.9 [16.1–49.9]	36.0 [19.0–63.0]	47.0 [27.0–76.0]	26.00 [16.00–40.00]	25.6 [12.7–46.5]
Iron deficiency (%)	133 (22.6)	245 (17.7)	252 (8.7)	183 (20.6)	1020 (30.2)
Number of donations in the last 2 years (median [IQR])	2.0 [1.0–4.0]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	2.0 [1.0–4.0]	0.0 [0.0–0.0]
Number of days since last donation (median [IQR])	224.00 [130.0–624.0]	1640.5 [1353.0–1918.0]	1659.0 [1375.0–1922.0]	171.0 [110.0–301.0]	1650.0 [1377.0–1907.0]
Smoking (%)	52 (8.8)	496 (35.9)	NA	49 (5.5)	778 (23.0)
Childbirth in the past (%)	359 (61.1)	940 (68.0)	NA	269 (30.3)	2565 (75.9)
Current hormonal contraception use (%)	157 (26.7)	326 (23.6)	NA	285 (32.1)	463 (13.7)
Menstruation (%)	456 (77.6)	1275 (92.3)	NA	784 (88.3)	3110 (92.1)
Women (>50 years)					
n	446	1624	617	516	2449
Age (mean [SD])	59.6 (5.6)	61.1 (8.0)	55.75 (4.0)	58.22 (5.24)	59.3 (5.4)
Blood volume (L) (mean [SD])	4.3 (0.5)	4.3 (0.5)	4.4 (0.5)	4.21 (0.52)	4.1 (0.5)
Ferritin (μg/L) (median [IQR])	40.6 [22.8–65.1]	88.0 [49.0–142.0]	80.0 [48.0–129.0]	34.00 [22.00–54.00]	53.45 [27.9–91.3]
Iron deficiency (%)	51 (11.4)	59 (3.6)	25 (4.1)	61 (11.8)	274 (11.2)
Number of donations in the last 2 years (median [IQR])	3.0 [1.0–5.0]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	4.0 [2.0–5.0]	0.0 [0.0–0.0]
Number of days since last donation (median [IQR])	189.0 [121.0–453.0]	1634.5 [1359.0–1930.0]	1629.0 [1368.0–1913.0]	147.0 [105.0–257.0]	1637.5 [1369.0–1912.0]
Smoking (%)	25 (5.6)	461 (28.4)	NA	32 (5.9)	333 (13.6)
Childbirth in the past (%)	372 (83.4)	1387 (85.4)	NA	395 (76.6)	2142 (87.5)
Current hormonal contraception use (%)	10 (2.2)	58 (3.6)	NA	42 (7.9)	21 (1.3)
Menstruation (%)	28 (6.3)	177 (10.9)	NA	66 (12.8)	390 (15.9)

Note: Data are presented as mean ± standard deviation (SD) for normally distributed continuous data, as median with interquartile range (IQR) for non-normally distributed data and proportions for categorical data.
Abbreviation: NA, not applicable.

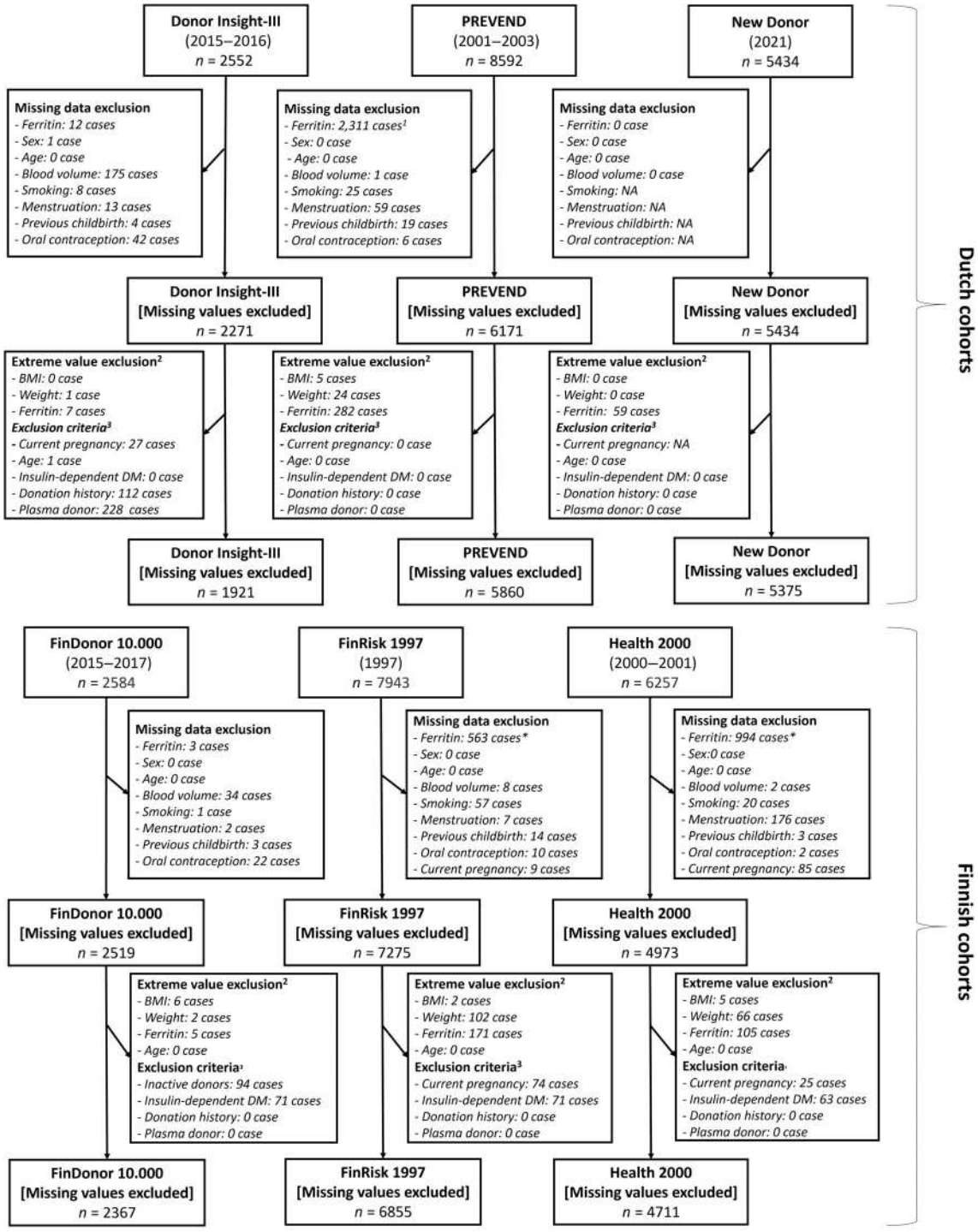

FIGURE 1 Flow diagram of Dutch and Finnish donor and general population cohorts. PREVEND, Prevention of Renal and Vascular End-Stage Disease. NA, not applicable. [1]Study visit 2 was missing for all participants. [2]Extreme values included body mass index (BMI) >50 kg/m^2, weight <50 kg, ferritin <1 μg/L or >400 μg/L. [3]Exclusion criteria: current pregnancy, insulin-dependent diabetes mellitus, age <18 years and inactive donors (no donations in the past 3 years). *No blood samples available.

Ferritin distribution

Ferritin levels were consistently lower in both Dutch and Finnish blood donors compared with the general population and new donors, with the only exception being Finnish women aged ≤50 years. The distribution of ferritin levels among participants is presented in Figure 2 by country and stratification group separately. When visualized, the distributions for the general populations appear wider, with higher median values and longer left tails. This is noticeable particularly in men and postmenopausal women. For women ≤50 years of age, a larger proportion of the left tails of ferritin distribution was below the ID cut-off value (ferritin <15 μg/L).

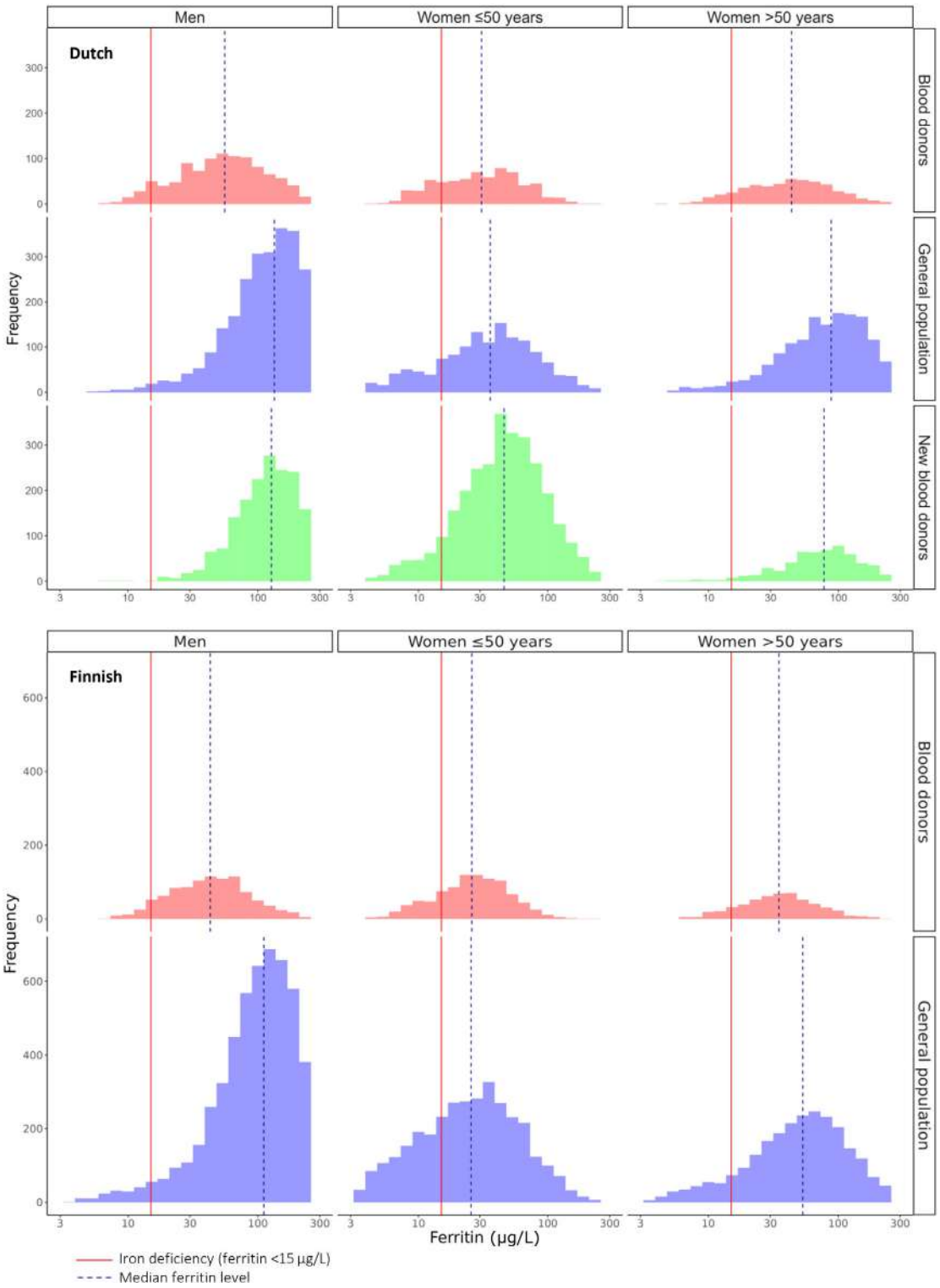

FIGURE 2 Distribution of ferritin levels among Dutch and Finnish donor and general populations by sex and menopausal status (divided at age 50 years), presented on a log-scale. The cut-off for iron deficiency, ferritin <15 µg/L, is represented by the solid red lines. Median ferritin levels for the different subgroups are represented by the dashed blue lines. Distributions of ferritin levels in the original two Finnish general population cohorts, Health 2000 and FinRisk 97 are available in Data S1: Appendix D.

Prevalence of iron deficiency

Prevalence of ID in Dutch and, although to a lesser extent, in Finnish men was higher for blood donors compared with the respective general population cohorts across all age groups (Figure 3). Similarly, for the Dutch women, across all age groups prevalence of ID was lower in the general population and new donor cohort compared with the blood donors. In contrast to the Dutch women, prevalence of ID

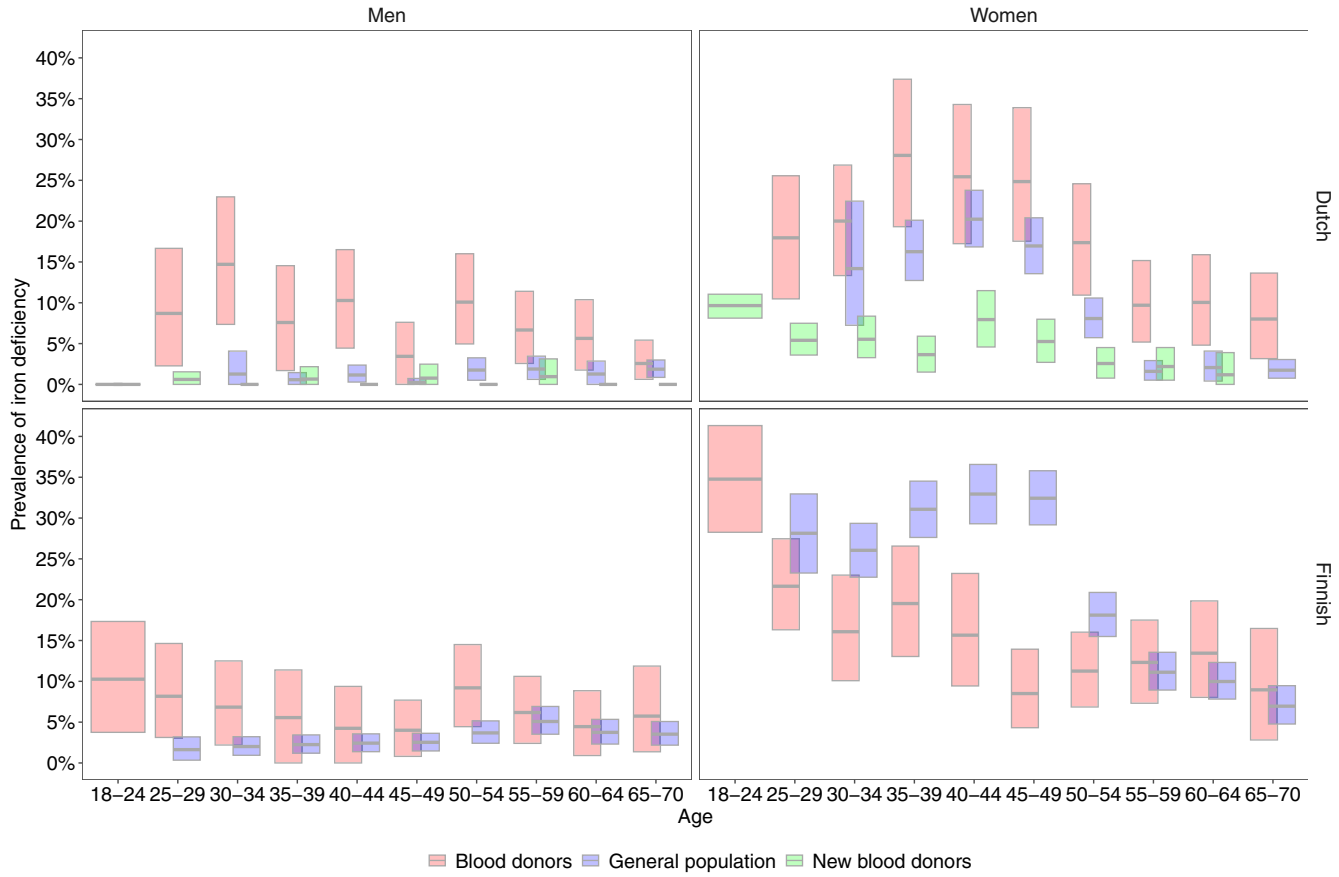

FIGURE 3 Prevalence of iron deficiency among Dutch and Finnish donor and general populations, grouped by sex and 5-year age groups. Prevalence estimates are presented with bootstrapped 95% confidence intervals. Due to lack of power for the bootstrapped confidence intervals, data on Dutch donors in the age group 18–24 were not included in the graph.

in Finnish women in the age group 25–54 was lower in the blood donors compared with the general population. No differences between donor and general populations were observed in the age group 55–70 in Finnish women.

Regression analysis

To evaluate differences in prevalence of ID between the different populations within each country, we performed Bayesian logistic regression modelling as described in methods (Figure 4). The numeric values of odds ratio's and their 95% credible intervals per country, group, model and variable are presented in Data S1: Appendix C.

Both Dutch and Finnish men and Dutch women aged >50 years in the general population had significantly lower odds of ID compared with the donor population. This was not the case for women aged ≤50 years. In contrast, Finnish general population women aged >50 years showed significantly higher odds of ID compared with the donor population. New blood donors had significantly lower odds of ID for all stratification groups compared with the blood donors.

Adjusting for age and blood volume did not change the associations of donor versus general populations with ID, neither did the additional adjustment for smoking, childbirth, menstruation and hormonal contraception. Further adjustments for days since last donation and number of donation in the past 2 years resulted in an inversed effect for the general population and new donor cohort membership in both countries compared with the previous models. For Finnish women aged >50 years in the general population, who already had higher odds of ID, the shift to the right caused the estimate to become larger compared with previous models.

DISCUSSION

With this study, we aimed to evaluate differences in ferritin levels and ID prevalence in donor and general populations in the Netherlands and Finland, countries that both have national blood services, which have chosen different policies regarding iron supplementation. The present study shows that, in contrast to the Dutch population, young Finnish female donors (i.e., aged below 50 years) have a lower prevalence of ID compared with the general population. Logistic regression modelling showed that this could not be attributed to differences in demographic characteristics nor donation history between the two countries.

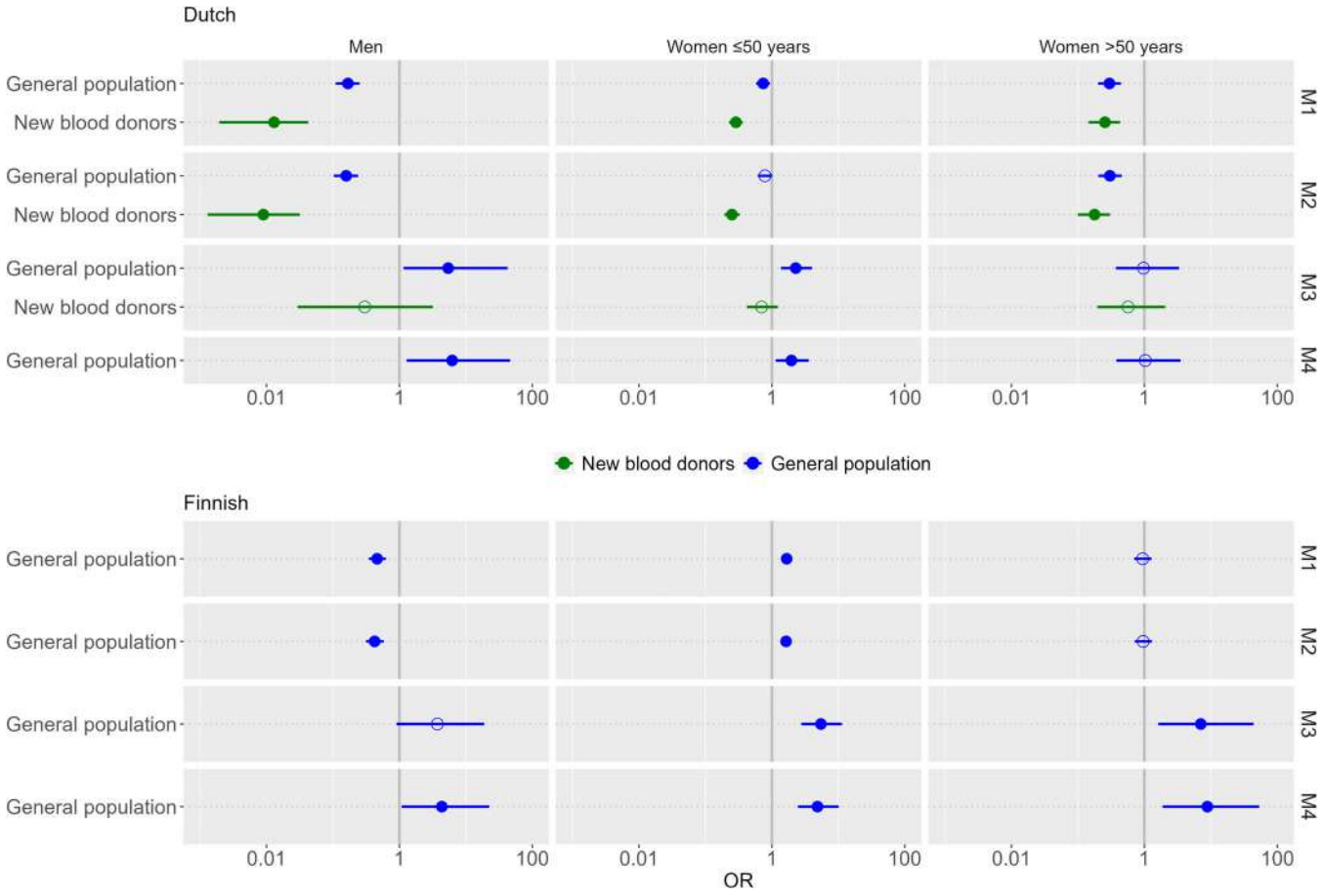

FIGURE 4 Bayesian logistic regression models showing associations of cohort membership with iron deficiency. Circles represent significant (filled) or non-significant (blank) standardized coefficients, with 95% credible intervals (horizontal thick lines). Model 1 (M1), only cohort membership; Model 2 (M2), M1 + demographic variables (age and blood volume); Model 3 (M3), M2 + donation history (number of days since last donation and number of donation is the last 2 years since participation); Model 4 (M4), M3 + smoking and female reproductive health variables (previous childbirth, hormonal contraception use and menstrual status).

The finding that young Finnish female donors have a lower ID prevalence than those in the general population contradicts the consensus that young female blood donors have an increased risk of developing ID due to pregnancy, childbirth and menstrual blood loss [18]. This may be explained by the iron management policy in place at the FRCBS. Whereas Sanquin only measured Hb before donation at the time of data collection, with similar deferral rates compared the FRCBS, the latter provided all young female donors with iron supplements after every donation [19]. Maghsudlu et al. showed through a clinical trial in young female donors (i.e., below 45 years of age) that short periods of post-donation iron supplementation decreased the prevalence of ID [20]. In contrast, the prevalence of ID substantially increased with repeated donations in the placebo group. Yet whereas multiple studies have shown the beneficial effect of post-donation iron supplementation on iron store recovery in donors, an earlier publication using FinDonor data showed that iron supplementation is not associated with ferritin levels [21–23]. Although these findings would suggest that iron supplementation would not be effective in preventing ID among donors, this study was underpowered in many respects. For example, the study could not detect the association for smoking. With the current study including three times more participants, this association could also be detected. Furthermore, the differences between the donor- and general populations within the countries was not observed for any of the groups that are less likely to be provided with iron supplements by the FRCBS.

In addition to evaluating the prevalence of ID, logistic regression modelling was performed to quantify the differences in odds of ID between the donor and general populations within the different stratification groups for each country and to account for potential confounding factors. In line with the differences in ID prevalence among the different groups (Figure 3), only young Finnish females from the general population showed higher odds of ID compared with the donor population (Figure 4). When the logistic regression model was adjusted for donation history, the odds of ID in all Dutch and Finnish general population cohorts became similar or greater than the donor population. This shows that although there are policy differences regarding donation intervals between the two countries, the differences in ID in the young female donors compared with the general population between the Netherlands and Finland is not associated with differences in donation intervals.

Fertility rates have been on the decline in Europe for the past 20 years, which can be reflected in the significantly lower birth rates and higher use of hormonal contraception in young Finnish donors compared with the general population [24]. Hormonal contraception decreases the volume of menstrual blood loss and is used as treatment for abnormal uterine bleeding [25]. As such, hormonal contraception provides protection against ID due to excessive iron loss. Although this protective effect, in combination with the lower birth rate, could explain the lower prevalence of ID in young Finnish blood donors, the addition of menstruation, childbirth and hormonal contraception use in the logistic models did not change the associations of donor versus general populations with ID.

One of this study's strengths is that country-based differences in ID prevalence are accounted for by including data from donor and general populations. This allowed us to determine if differences in prevalence of ID in the donor populations from both countries might be due to geographical differences [26]. Iron management policies vary greatly among international blood services. Research evaluating the effect of different iron management policies on ID prevalence in donors is limited. Although previous studies assessed differences in iron management policies between countries, the effects of such policies on ferritin levels have not been evaluated [10, 27]. However, this information is important for blood services to guide their decision-making for iron management policy making services to guarantee donor health.

There are, however, additional differences between donor and general populations. Donors are screened for their eligibility to start and continue with donation, which might have resulted in selecting a relatively healthy subset of the population: the healthy donor effect [28]. Although Hb screening was not utilized as an exclusion criterion for the donor cohorts in this study, the practice of screening in general might explain why, after adjusting for donation history, the prevalence of ID was similar or lower among donors than in the general population. Yet the results not adjusted for donation history showed higher ID prevalence in all donor populations except young Finnish females. Furthermore, by including a new donor cohort in the analysis of the Dutch populations, we were able to look into the biased health status upon registration as a new donor. In line with what we expected, new donors had a significantly lower prevalence of ID compared with the donor population. However, although this analysis could not be performed for the Finnish population, these findings should be interpreted with caution. Furthermore, as different sampling and measurement methods were used by the different cohorts, some measurement variance in ferritin levels is probably present [29]. However, we see similar patterns in all but young Finnish donors, that is, all inside of the FinDonor population, which used similar measurements during the specified time, we do not expect this to explain our finding.

This study demonstrates that young female donors in Finland, in contrast to the Netherlands, have a lower prevalence of ID compared with the general population. Unlike the Dutch blood service iron management policy (i.e., pre-donation Hb measurements), the Finnish blood service policy (i.e., pre-donation Hb measurements and iron supplementation for risk groups) seems to protect young female blood donors from developing ID. These findings provide evidence that iron supplementation as a blood service iron management policy is likely to have large effects on the iron status of blood donors. The results from this study can support international blood services with their decision-making for an effective iron management policy.

ACKNOWLEDGEMENTS

Dr. Lyanne Kieneker, Professor Stephan J.L. Bakker and Professor Ron T. Gansevoort provided the PREVEND data. THL Biobank (project THLBB2020_19) provided FinRisk 1997 and Health 2000 data. We would like to thank all study participants for their generous participation, blood service and biobank staff, the general sponsor of the PREVEND study (Dutch Kidney Foundation) and Professor Seppo Koskinen and Dr. Satu Männistö of THL for commenting on the manuscript. This study is part of a research project supported by the Product and Process Development Cellular Products Grant (PPOC19-02) granted to Katja van den Hurk by Sanquin's Research Programming Committee, the PhD Study Track of the Faculty of Medicine of the University of Helsinki and the research leading to these results was supported by the Research Fund of the Finnish Red Cross Blood Service granted to Jan H. M. Karregat.

J.H.M.K., S.E., J.C., K.v.d.H. and M.A. designed the research study; J.H.M.K., S.E. and M.A. performed the research; J.H.M.K. and S.E. wrote the first draft of the manuscript; J.C., K.v.d.H. and M.A. reviewed and edited the manuscript; all authors of this article contributed significantly to the study and manuscript preparation and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

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

How to cite this article: Karregat JHM, Ekroos S, Castrén J, Arvas M, van den Hurk K. Iron status in Dutch and Finnish blood donor and general populations: A cross-cohort comparison study. Vox Sang. 2024;119:664–74.

Received: 5 November 2023 | Revised: 15 February 2024 | Accepted: 25 March 2024

DOI: 10.1111/vox.13634

ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Platelets retain function and can be stored following disruption of human leucocyte antigens

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Funding information
Australian Governments

Abstract

Background and Objectives: Antibodies to human leucocyte antigen (HLA) Class-I antigens can lead to refractoriness to platelet transfusion. Although this can be overcome by transfusion of HLA-compatible platelets, they are not always available. Disruption of HLA antigens on platelets by acid treatment may be a suitable alternative when no other components are available. The aim of this study was to assess the effect of HLA disruption and subsequent storage of platelet components.

Materials and Methods: Platelet components were treated with 0.9% saline or citric acid solution (pH 3.0), and then stored until expiry (Day 7). HLA and platelet glycoprotein expression, platelet viability, activation and sialylation were measured by flow cytometry. Release of soluble factors was measured by ELISA and metabolism by biochemistry analyser. Reactivity to patient anti-sera containing anti-HLA antibodies was measured using platelet immunofluorescence tests (PIFTs) and monoclonal antibody immobilization of platelet antigen (MAIPA) assays. Platelet function was measured using aggregometry and thromboelastography (TEG).

Results: Acid treatment reduced detection of HLA Class-I on platelets by 75%, with significant reductions in reactivity to patient anti-sera. Acid treatment reduced platelet content and viability, increased platelet activation and accelerated metabolism. Glycan cleavage was increased by acid treatment. Treatment reduced platelet activation following agonist stimulation by ADP and TRAP-6, but platelets remained functional, displaying increased aggregation response and reduced time to clot formation by TEG.

Conclusion: Although HLA disruption had some detrimental effects, acid-treated platelets remained functional, retaining their capacity to respond to agonists and form clots, and with further development could be used to support refractory patients.

Keywords
blood components, function, human leucocyte antigen, platelets, quality, refractoriness

Highlights

- Citric acid treatment significantly disrupted human leucocyte antigen (HLA) Class I antigens, and this disruption was maintained during platelet storage.
- HLA-disrupted platelets retained their ability to aggregate and form clots over storage despite increased activation and platelet loss.
- With further development, HLA-disrupted platelet components may be used to support transfusion needs when HLA-compatible components are unavailable.

INTRODUCTION

The primary recipients of platelet components are haematology and oncology patients, who require regular platelet transfusions to maintain their platelet counts and reduce the risk of bleeding. Platelet refractoriness, defined as the repeated failure to achieve an appropriate platelet count post-transfusion, can arise over time as patients receive multiple platelet transfusions as part of their treatment. Between 4% and 25% of patients become refractory to platelet transfusions [1, 2].

While non-immune factors are the most common causes of platelet refractoriness [3], immune factors also contribute. The formation of antibodies directed against the human leucocyte antigen (HLA) system is the most frequent immunological cause of platelet refractoriness [4]. The classical HLA Class-I antigens HLA-A, HLA-B and HLA-C are expressed on platelets [5]. Immunological refractoriness is most often caused by antibodies to HLA-A and HLA-B [4], with some reports of refractoriness due to HLA-C antibody formation [6].

One of the most common strategies for overcoming immunological refractoriness due to HLA antibody formation is provision of HLA-A, -B compatible platelets [4, 7], which involves identifying donor platelets compatible with a patient's HLA phenotype or genotype. Other strategies include the antibody specificity prediction method [7], epitope matching [8] and platelet cross-matching [4, 9]. Each strategy is hindered by the limited availability of compatible components to support specific patients [10]. However, it has been estimated that as many as 40% of transfusions with HLA-compatible platelets are unsuccessful [11].

An alternative method investigated sporadically over the past 30 years is treating platelets with a citric acid solution to selectively denature and disrupt HLA Class-I but not Class-II antigens on the cell surface [12, 13]. Although there is evidence that intact HLA class-I may also be removed from the platelet surface following acid treatment [14], the general consensus is that the primary mechanism by which HLA antigenicity is reduced is through dissociation of β_2M from the HLA heavy chain, thus disrupting the native trimolecular conformation of HLA class-I [15]. Reports describing the effects of citric acid treatment on platelets have yielded variable results, possibly due to differences in the type of platelet product studied and variations in treatment methodology.

While several studies have investigated HLA disruption [14, 16–19], the impact of storing platelets following HLA disruption by citric acid treatment has been rarely studied. As such, this study was undertaken to investigate the characteristics of apheresis platelets following HLA disruption and subsequent storage.

MATERIALS AND METHODS

Ethics approval was obtained for this study from the Australian Red Cross Lifeblood Research Ethics Committee. All donations were collected from eligible, voluntary, non-remunerated donors.

Experimental design

Double-apheresis platelet components were collected using Trima Accel v7.01 in 40% plasma/60% SSP+. On Day 1 post collection, platelet components were pooled, split into two components into the original collection bags and randomly assigned to either acid or saline (control) treatment ($n = 10$ pairs). The components were centrifuged (1350*g*, 10 min, room temperature [20–24°C; RT]) and the supernatant was expressed using a manual plasma expressor and set aside. Each platelet pellet was gently resuspended in residual supernatant followed by incubation with either 15 mL citric acid solution (1:1 mix of 263 mM citric acid/123 mM disodium phosphate, pH 3.0; both from Merck, Darmstadt, Germany) or 15 mL 0.9% NaCl (control; Baxter International, Deerfield, IL, USA) at 4°C for 10 min. Treatment was stopped by diluting the platelets in 500 mL PAS-E (SSP+; Macopharma, Mouvaux, France). The platelets were centrifuged (1350*g*, 10 min, RT) and resuspended in the original supernatant.

Platelet count and metabolism

Platelet components were rested for 1 h at RT without agitation, and then stored for a further 6 days at RT with agitation. Samples were removed aseptically on Days 1, 5 and 7 post collection. Platelet counts and mean platelet volume (MPV) were measured using a haematology analyser (CELL DYN Emerald, Abbott Laboratories, Abbott

Park, IL, USA). The pH was measured using a pH meter at RT (Mettler-Toledo GmbH, Greifensee, Switzerland). Platelet metabolism was determined from measurements of supernatant glucose and lactate using a Vitros XT 3000 biochemistry analyser (Ortho Clinical Diagnostics, Raritan, NJ, USA).

Detection of HLA removal

HLA disruption was measured by flow cytometry using a FACSCanto II flow cytometer (BD Biosciences, Becton Dickinson, NJ, USA). For each experiment, 10,000 gated single platelet events were collected. The platelet population was identified and gated based on forward versus side scatter. Antibody isotype controls were used to set the lower boundaries of the positive gates. Platelets were stained with antibodies directed against HLA Class-I native conformation (HLA-A, -B, -C; G46-2.6; monoclonal), β_2-microglobulin subunit (β_2M; TÜ99; monoclonal; BD Biosciences, San Jose, CA, USA) and HLA free heavy chain (A4; monoclonal; Thermo Fisher Scientific, Waltham, MA, USA). Median fluorescent intensity (MFI) and percentage positive events were recorded.

Antigenicity of acid-treated platelets

Reactivity of platelets to a panel of anti-sera of in-house patients was measured on Day 5 of storage using platelet immunofluorescence test (PIFT) and monoclonal antibody immobilization of platelet antigen (MAIPA) assays. Anti-sera were from multiparous females who had delivered infants with foetal and neonatal alloimmune thrombocytopaenia (FNAIT). Negative controls were non-reactive sera or plasma from male group AB donors who had tested negative in all antibody screening. In PIFT, platelets were incubated with patient anti-sera containing either anti-HLA class-I antibodies only, or various combinations of anti-HPA (either anti-HPA-1a, anti-HPA-5a or anti-HPA-5b) and anti-HLA class-I antibodies. After incubation, platelets were washed, incubated with PE-conjugated anti-human IgG, washed again and then analysed by flow cytometry.

MAIPA was performed using the above anti-sera, according to the 'Modified Rapid MAIPA Protocol' from the National Institute for Biological Standards and Control [20]. Platelets were incubated with anti-sera, followed by incubation with anti-HLA class-I or anti-HPA specific murine monoclonal IgG antibodies. The platelets were lysed, releasing serum antibody–antigen–monoclonal antibody triplexes into solution. The triplexes were bound to 96-well plates coated with goat-anti-mouse IgG and then incubated with peroxidase-conjugated goat-anti-human IgG. Plates were developed with *o*-phenylenediamine dihydrochloride substrate solution (Dako, Glostrup, Denmark), and the optical density (OD) was measured at 490 nm. PIFT and MAIPA ratios were calculated by dividing the test result by the negative control result as measured in MFI or OD, respectively.

Platelet glycoproteins and activation

The effect of acid treatment on platelet glycoproteins CD41a, CD42b (GPIbα), CD61 and GPVI was measured using the following antibodies: CD61-FITC (Y2/51; Dako), CD41a-FITC (HIP8) and CD42b-PE (HIP1) (BD Biosciences) as well as GPVI-eFluor660 (HY101; Thermo Fisher Scientific) or isotype controls including IgM_K-FITC, IgG_{1K}-FITC, IgG_{1K}-PE, IgG_{1K}-APC (BD Biosciences), IgG_{2aK}-RPE (Dako), IgG_{1K}-eFluor660 and IgG_{1K}-APC (Thermo Fisher Scientific).

Platelet viability was measured using calcein-AM and FM® 4-64 (Thermo Fisher Scientific) [21]; mitochondrial membrane potential (MMP) was measured using tetramethylrhodamine ethyl ester (TMRE; Abcam, Cambridge, UK) [22]; and phosphatidylserine (PS) exposure was determined by annexin V binding (AnnV; BioLegend, San Diego, CA, USA) [23] by flow cytometry.

Changes in platelet glycans were measured by flow cytometry using fluorescein-conjugated *Sambucus nigra* (SNA/EBL) and *Ricinus communis* agglutinin-1 (RCA-1) lectins (Vector Laboratories, Burlingame, CA, USA) [24].

Platelet activation was determined by flow cytometry, measuring PAC-1 antibody (BD Biosciences) binding to the GPIIb/IIIa receptor complex following stimulation with 20 μM adenosine diphosphate (ADP; Sigma-Aldrich, St. Louis, MI, USA) [23], or CD62P (P-selectin) following stimulation with 20 μM thrombin receptor-activating peptide 6 (TRAP-6; Sigma-Aldrich) [25]. Basal activation of CD42b was measured using AN51-RPE antibody (Dako).

Aggregation and clot formation

Platelet aggregation was measured in response to 10 μg/mL collagen (Helena Laboratories, Beaumont, TX, USA) using light transmission aggregometry as previously described [23]. Aspects of coagulation and clot formation were measured using thromboelastography (TEG) following exposure to kaolin in the presence of 0.01 M $CaCl_2$ using a TEG 5000 analyser (Haemonetics Corporation, Braintree, MA, USA).

Soluble factor analysis

Platelet supernatants were collected at each time point by centrifugation at 1600*g* for 20 min followed by 12,000*g* for 5 min at RT, and stored at −80°C. The release of soluble CD40 ligand (sCD40L), soluble CD62P (sCD62P), RANTES and platelet factor 4 (PF4) was measured using ELISA DuoSet kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Data were analysed using SPSS version 23.0 (IBM Corporation, Armonk, New York, USA). Graphs were prepared using GraphPad

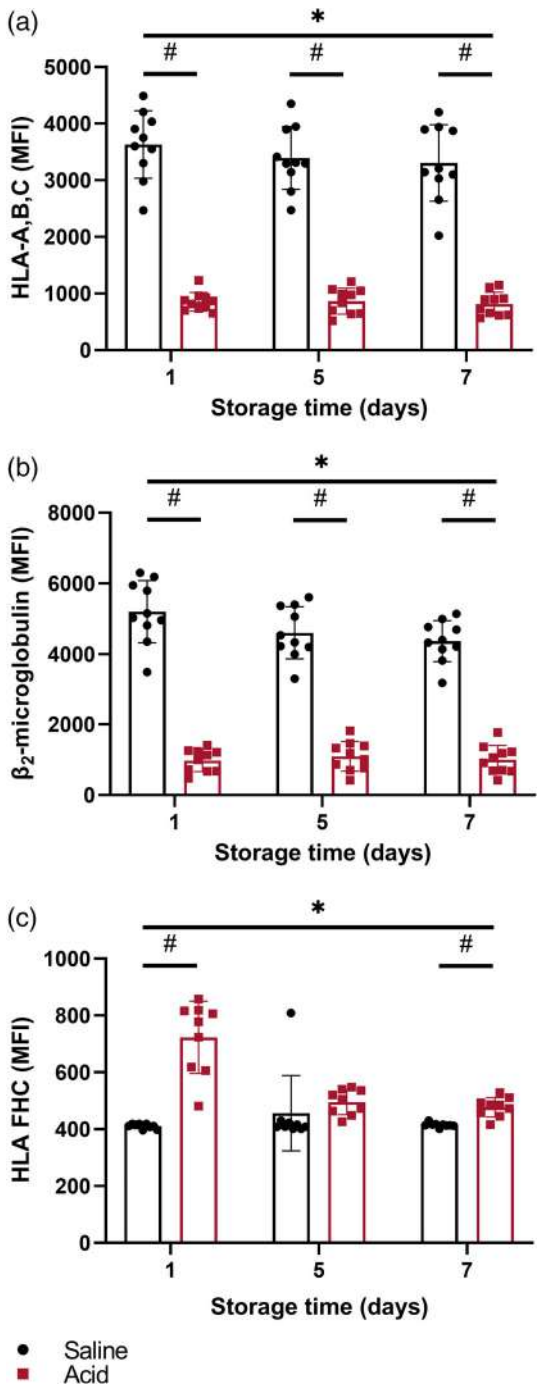

FIGURE 1 Acid treatment disrupted human leucocyte antigen (HLA) Class-I on the platelet surface. Saline- and acid-treated platelets were stained with antibodies directed against (a) HLA-A, B, C, (b) β_2-microglobulin, or (c) HLA Class-I free heavy chain (FHC) and measured using flow cytometry. Data were analysed using two-way repeated measures analysis of variance (ANOVA) with post hoc Bonferroni correction and *t*-tests. Data represent mean ± standard deviation ($n = 10$); $^{*}p < 0.05$ for ANOVA and $^{\#}p < 0.05$ for *t*-tests. MFI, median fluorescent intensity.

Prism version 9.4.1. Data from acid- and saline-treated platelets across the storage period were compared using two-way repeated measures analysis of variance with Bonferroni correction. Where applicable, saline and acid treatments were compared at single time points using independent paired *t*-tests. In all instances, $p < 0.05$ was considered as significant.

TABLE 1 Reactivity of patient anti-sera containing HLA Class-I and HPA antibodies with acid-treated platelets.

Platelet immunofluorescence test (PIFT)			
	PIFT IgG ratio		
Serum tested	Saline	Acid	Significance
HLA Class-I serum 1#	18.39 ± 7.46	5.62 ± 1.96	$p = 0.0004$*
HLA Class-I serum 2#	5.79 ± 2.14	3.37 ± 0.97	$p = 0.004$*
HPA-1a serum§	12.01 ± 6.79	7.04 ± 2.27	$p = 0.025$*
HPA-5a serum§	7.06 ± 4.27	4.89 ± 2.29	$p = 0.022$*
HPA-5b serum§	3.88 ± 1.79	3.10 ± 1.15	$p = 0.043$*
Monoclonal antibody immobilization assay (MAIPA)			
	MAIPA IgG ratio		
Serum tested	Saline	Acid	Significance
HLA Class-I serum 1#	60.21 ± 15.09	17.9 ± 9.34	$p = 0.0001$*
HLA Class-I serum 2#	17.36 ± 15.18	7.55 ± 6.07	$p = 0.048$*
HPA-1a serum§	80.26 ± 31.31	58.34 ± 12.33	$p = 0.054$
HPA-5a serum§	17.99 ± 10.46	15.07 ± 9.72	$p = 0.326$
HPA-5b serum§	5.92 ± 11.25	4.21 ± 7.36	$p = 0.260$

Note: Saline- and acid-treated platelets were incubated with anti-sera containing only anti-HLA class-I antibodies (#) or anti-sera containing an anti-HPA antibody and anti-HLA class-I antibodies (§) and tested by PIFT and MAIPA. PIFT and MAIPA ratios were calculated by dividing the test result by the negative control result as measured in MFI or OD, respectively (PIFT ratio: positive >2.0; MAIPA ratio: positive >3.0). Data represent mean ± standard deviation ($n = 8$).
$^{*}p < 0.05$ from paired *t*-tests.

RESULTS

HLA Class-I expression post acid treatment

Acid treatment reduced antibody binding to HLA Class-I by an average of 75% (Figure 1a; $p < 0.0001$) compared to controls on Day 1. The reduction was maintained throughout platelet storage. There was concomitant reduction in HLA Class-I native conformation, as determined by dissociation of the β_2M subunit following acid treatment, with an ~81% reduction compared to the control on Day 1 (Figure 1b; $p < 0.0001$). β_2M levels remained constant during storage. Exposure of the free heavy chain increased almost two-fold in acid-treated platelets (Figure 1c; $p < 0.0001$), confirming the loss of β_2M from the HLA Class-I structure [26]. Free heavy chain was highest on Day 1 of storage (day of acid treatment) and decreased thereafter.

TABLE 2 Platelet specifications and metabolic parameters for apheresis platelets post HLA disruption over 7 days of storage.

Measure	Storage day	Saline	Acid	Significance
Platelet content ($\times 10^9$ platelets/L)	1	295.24 ± 30.00	138.40 ± 50.63	$p < 0.0001$*
	5	277.65 ± 35.98	153.15 ± 47.63	
	7	223.82 ± 39.94	129.17 ± 40.05	
Recovery (%)	1	99.27 ± 12.97	46.47 ± 20.53	$p < 0.0001$*
Viable cells (%)	1	96.48 ± 2.55	88.17 ± 4.61	$p < 0.0001$*
	5	97.10 ± 0.93	91.07 ± 4.02	
	7	96.60 ± 1.45	91.99 ± 3.06	
Nonviable cells (%)	1	2.22 ± 0.93	10.51 ± 3.32	$p < 0.0001$*
	5	2.39 ± 0.72	8.42 ± 3.99	
	7	2.75 ± 1.06	7.28 ± 2.77	
MPV (fL)	1	5.03 ± 0.42	6.24 ± 0.47	$p = 0.001$*
	5	4.80 ± 0.47	5.31 ± 0.36	
	7	4.75 ± 0.43	5.32 ± 0.40	
pH	1	7.19 ± 0.13	7.16 ± 0.14	$p = 0.254$
	5	7.32 ± 0.17	7.25 ± 0.16	
	7	7.38 ± 0.12	7.26 ± 0.14	
Glucose consumption (mM/platelet/day)	1–5	0.52 ± 0.08	1.74 ± 0.71	$p = <0.0001$*
	5–7	0.47 ± 0.21	1.13 ± 0.48	
Lactate production (mM/platelet/day)	1–5	0.76 ± 0.10	1.41 ± 0.47	$p = 0.01$*
	5–7	0.90 ± 0.68	1.64 ± 0.73	
MMP (MFI)	1	498.33 ± 142.31	289.56 ± 56.17	$p = 0.001$*
	5	794.40 ± 257.51	466.30 ± 122.74	
	7	770.00 ± 269.34	528.60 ± 135.72	

Note: Data were analysed using two-way repeated measures analysis of variance applicable with post hoc Bonferroni correction. Data represent mean ± standard deviation ($n = 10$).
*$p < 0.05$ significance.

Reactivity to patient sera

Binding of patient antibodies directed against HLA Class-I and HPA on platelets was measured using PIFT and MAIPA (Table 1). Acid treatment significantly reduced binding of HLA Class-I IgG antibodies using PIFT. Reactivity to HPA-1a, HPA-5a and HPA-5b in PIFT was also significantly reduced in acid- versus saline-treated platelets. However, there were no significant differences in the binding of any of the three HPAs using MAIPA.

Platelet viability, metabolism and component specifications

Acid treatment resulted in significant platelet loss (Table 2). The mean platelet recovery post acid treatment was 47% compared to 99% for the controls (Table 2). Over 80% of acid-treated platelets remained viable during storage, as indicated by calcein-AM staining. Similarly, staining of non-viable platelets with FM 4-64® showed a significantly higher proportion of dead platelets in acid-treated components (Table 2). The MPV was significantly higher in acid-treated platelets, indicating that they were larger and potentially more activated (Table 2).

Platelet metabolism during storage was accelerated by acid treatment, with significantly higher glucose consumption and lactate production (Table 2). This was not associated with a decrease in pH (Table 2). Mitochondrial function (matrix metalloproteinase, MMP) was significantly decreased in acid-treated platelets (Table 2).

Platelet markers and activation

Surface abundance of CD61 was not affected by HLA disruption (Figure 2a; $p = 0.314$), although CD41a was slightly reduced (Figure 2b; $p = 0.007$). Both glycoproteins remained stable during storage. CD42b was also significantly reduced by acid treatment (Figure 2c; $p = 0.002$), but remained stable during storage. Glycoprotein VI (GPVI) was the most affected platelet glycoprotein examined (Figure 2d; $p < 0.0001$) and was significantly reduced immediately post acid treatment, increasing with subsequent storage.

The effect of HLA disruption on platelet glycans was investigated using lectin binding. Acid-treated platelets had significantly less sialic

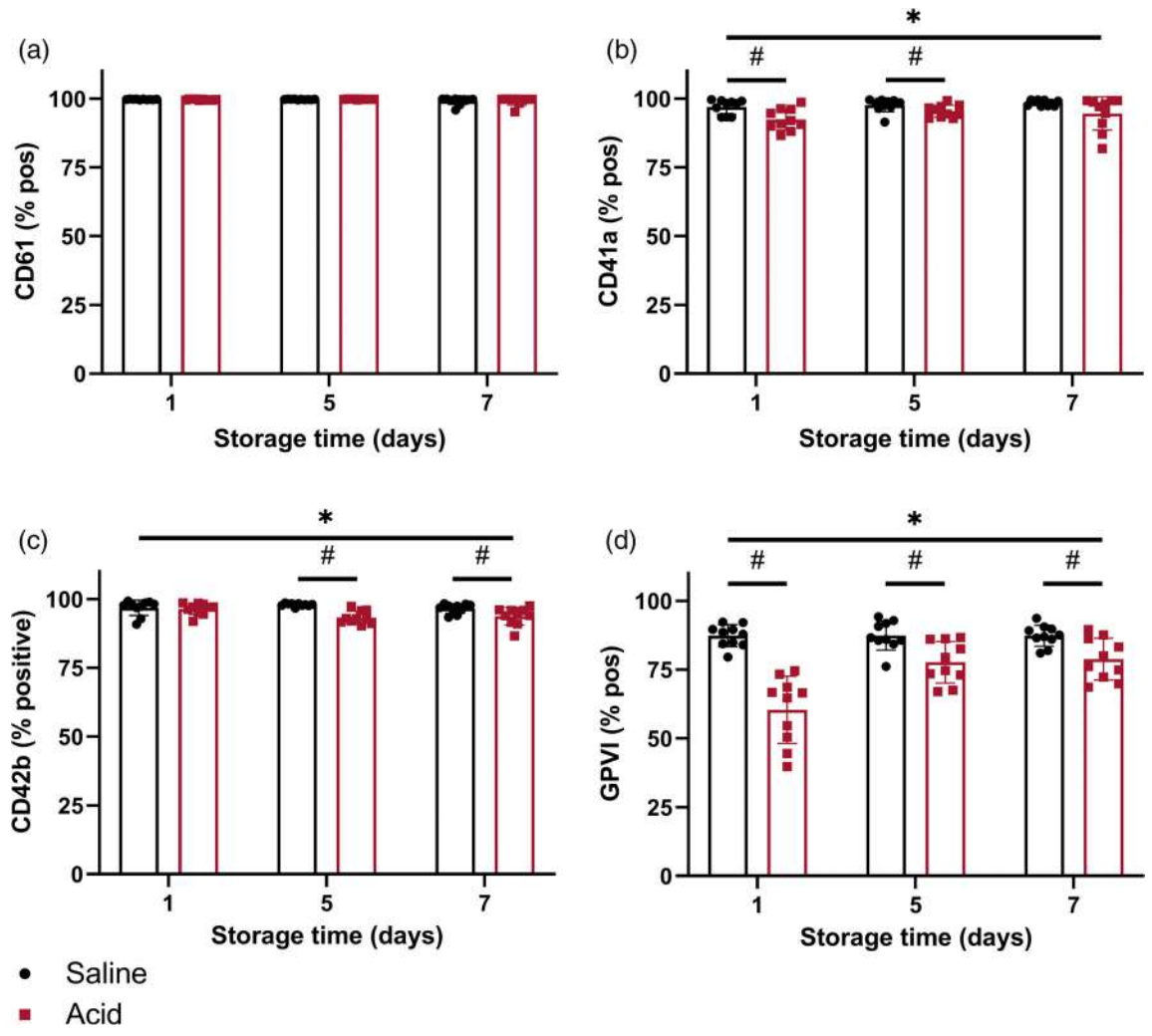

FIGURE 2 Effect of human leucocyte antigen disruption on platelet markers. Platelets were stained and measured by flow cytometry. Platelet glycoprotein expression was tested in unstimulated platelets. (a) CD61, (b) CD41a, (c) CD42b, (d) GPVI. Data were analysed using two-way analysis of variance (ANOVA) with post hoc Bonferroni correction and *t*-tests. Data represent mean ± standard deviation ($n = 10$); $^{*}p < 0.05$ for ANOVA and $^{\#}p < 0.05$ for *t*-tests.

acid and galactose on their surface as measured by SNA (Figure 3a; $p = 0.009$) and RCA-1 binding, respectively (Figure 3b; $p = 0.029$). The differences were most marked on Day 1 of storage, becoming less so on Days 5 and 7.

Basal surface levels of CD62P were significantly increased in acid-treated platelets compared to controls (Figure 4a; $p < 0.0001$), indicating degranulation and platelet activation. Following TRAP-6 stimulation, maximum CD62P was blunted by HLA disruption compared to controls, although the difference was small (Figure 4a; $p = 0.0013$). HLA depletion did not affect basal activation of the GPIIb/IIIa complex in unstimulated platelets (Figure 4b; $p = 0.564$). However, activation following ADP stimulation was significantly lower in acid-treated platelets ($p < 0.0001$). AN51 binding was also significantly lower following HLA disruption (Figure 4c; $p = 0.045$), indicating increased CD42b activation, most notably on Day 1 immediately after acid treatment ($p = 0.0061$). The proportion of platelets with externalized PS was significantly higher in acid-treated platelets (Figure 4d; $p < 0.0001$), which decreased during storage.

Platelet aggregation and coagulation

Platelet aggregation in response to collagen was significantly enhanced in acid-treated platelets compared to controls throughout storage (Figure 5a; $p = 0.0008$). The time to initiation of clot formation (R-time) as measured by TEG was significantly shorter following HLA disruption (Figure 5b; $p = 0.0004$), particularly on Day 1 of storage. Importantly, clot strength (MA) was retained in the acid-treated platelets throughout storage, and it was no different to control platelets (Figure 5c; $p = 0.152$). There were no differences in other clot formation parameters (clot strengthening, maximum clot strength, K-time; data not shown).

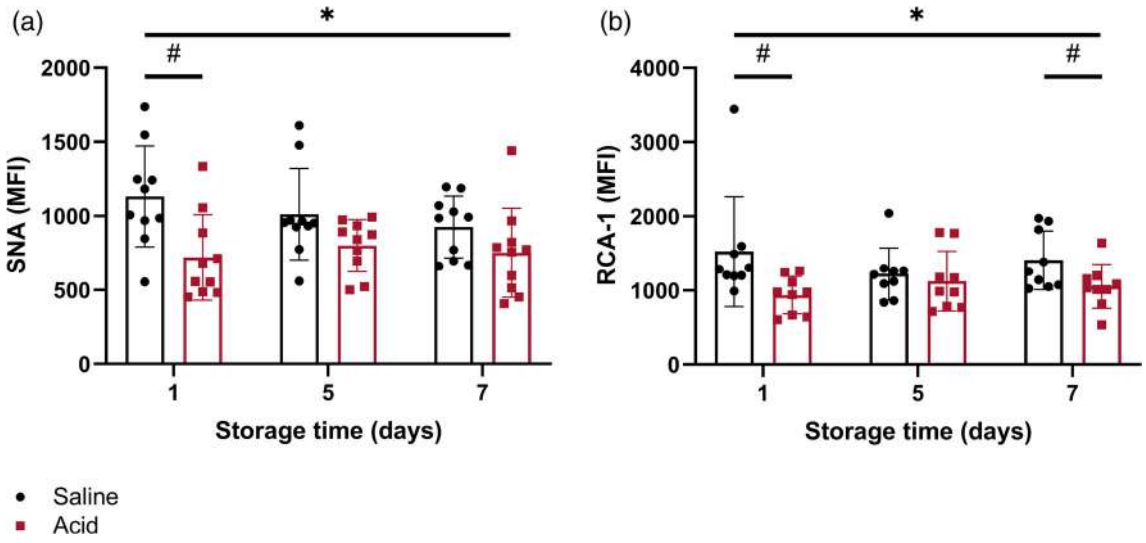

FIGURE 3 Glycosylation status of platelets post human leucocyte antigen disruption. The binding of the lectins (a) SNA and (b) RCA-1 was measured by flow cytometry. Data were analysed using two-way analysis of variance (ANOVA) with post hoc Bonferroni correction and *t*-tests. Data represent mean ± standard deviation ($n = 10$); $^{*}p < 0.05$ for ANOVA and $^{\#}p < 0.05$ for *t*-tests. MFI, median fluorescent intensity.

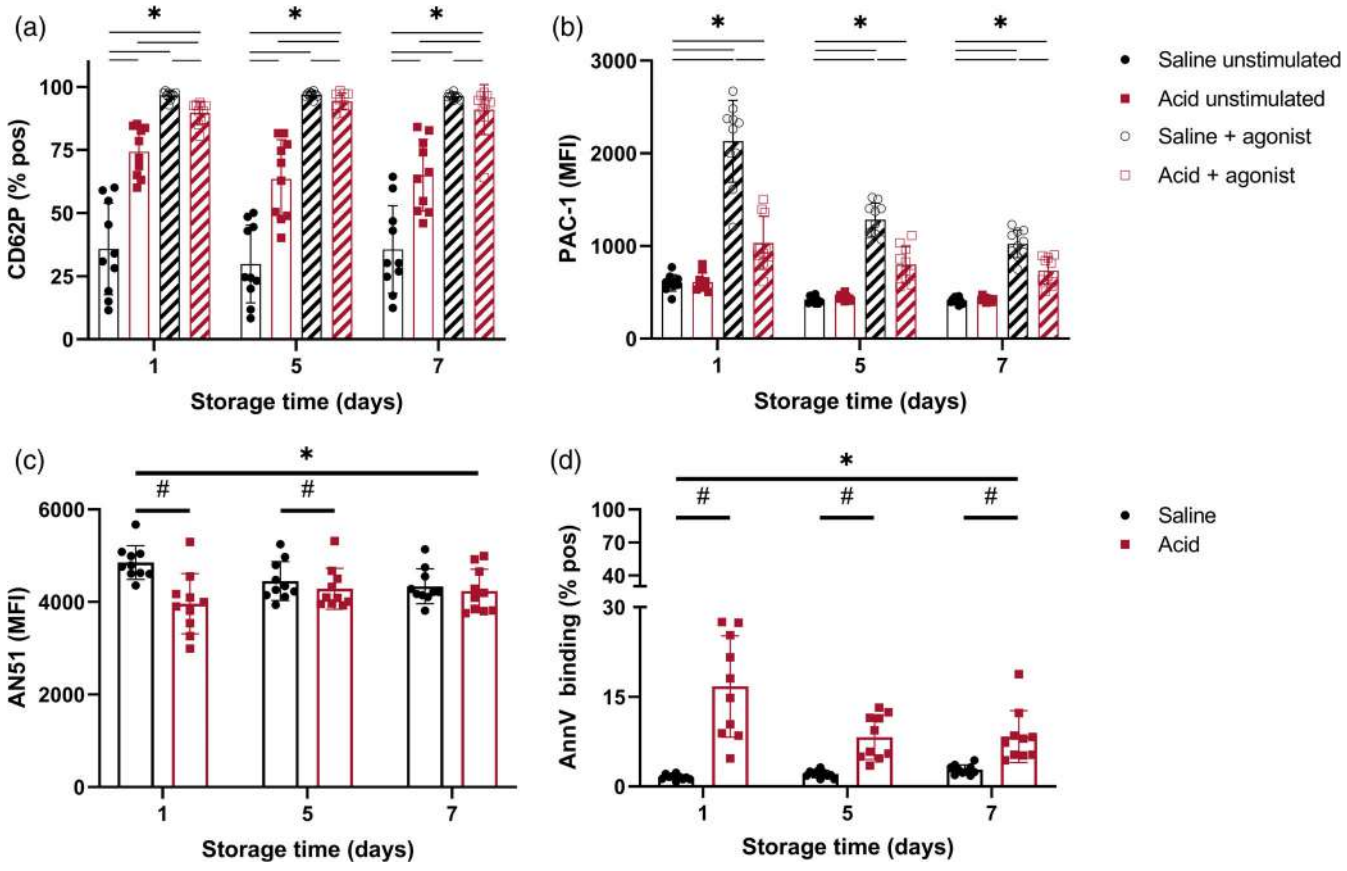

FIGURE 4 Effect of human leucocyte antigen disruption on platelet receptor activation. Unstimulated and stimulated platelets were stained and measured by flow cytometry. Platelet activation was assessed using (a) CD62P ± TRAP-6 stimulation and (b) GPIIb/IIIa activation as measured by PAC-1 ± ADP stimulation. Unstimulated platelets only were measured by flow cytometry. (c) CD42b activation as measured using AN51 and (d) phosphatidyl serine exposure as measured by AnnV binding. Data were analysed using two-way analysis of variance (ANOVA) with post hoc Bonferroni correction and *t*-tests. Data represent mean ± standard deviation ($n = 10$); $^{*}p < 0.05$ for ANOVA and $^{\#}p < 0.05$ for *t*-tests. MFI, median fluorescent intensity.

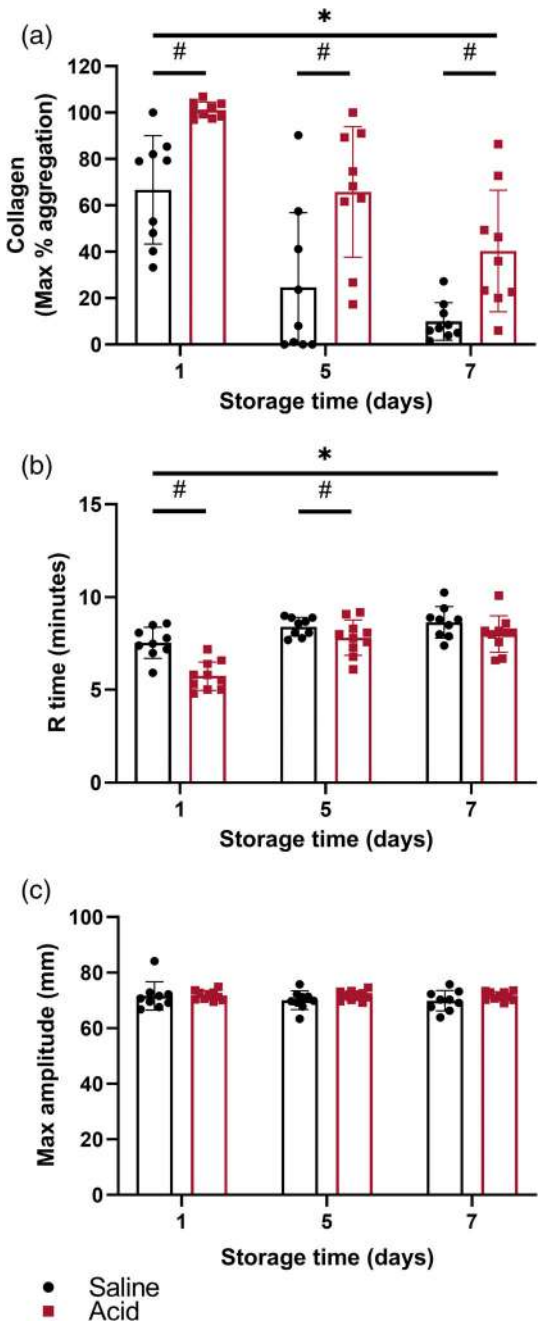

FIGURE 5 Platelet aggregation and coagulation. (a) Platelet aggregation in response to exposure to collagen was measured by light transmission aggregometry. (b) Time to clot formation (R time; R) and (c) maximum amplitude (MA) as measured by thromboelastography. Data were analysed using two-way analysis of variance (ANOVA) with post hoc Bonferroni correction and *t*-tests. Data represent mean ± standard deviation ($n = 10$); $^{*}p < 0.05$ for ANOVA and $^{\#}p < 0.05$ for *t*-tests.

Platelet releasates

PF4 and sCD62P release were significantly higher after HLA disruption and subsequent storage ($p = 0.016$ and 0.0003, respectively; Figure 6a,b). While RANTES and sCD40L were also slightly higher, the differences were not significant ($p = 0.239$ and 0.104, respectively; Figure 6c,d).

DISCUSSION

Citric acid treatment effectively disrupted HLA Class-I antigens on the platelet surface. Although disruption was accompanied by platelet loss and increased platelet metabolism and activation, acid-treated platelets retained their clotting and aggregation functions.

Acid treatment was effective in disrupting HLA Class-I levels on apheresis platelets. The extent of reduction achieved in this study was within the range reported in other studies [13, 19, 26–28], as was the concomitant dissociation of β_2M [14, 19, 28], The reduction was maintained over storage, as observed in previous studies [29, 30], indicating β_2M did not reassociate with the free heavy chain of HLA; nor was there de novo synthesis or further externalization of HLA molecules.

The disruption of HLA Class-I on the platelet surface was associated with significant decreases in HLA-specific reactivity to patient anti-sera in both PIFT and MAIPA, although a complete loss of antigenicity was not observed. PIFT was performed because it is a whole-cell assay and therefore indicates how patient anti-sera reacts to intact acid-treated platelets. However, PIFT detects binding of serum antibodies to platelets and cannot discriminate between HLA and HPA antibody binding. Therefore, MAIPA was also performed, wherein antibody–antigen complexes were released from lysed platelets and detected with monoclonal antibodies specific for anti-HLA Class-I or platelet glycoproteins, to determine specific antigenicity testing. HPA-1a, -5a and -5b binding were significantly reduced when measured using PIFT but not MAIPA, confirming that acid treatment selectively reduced HLA Class-I, but not HPA antigenicity.

HLA disruption led to loss of platelets in the components, reducing platelet viability and increasing platelet metabolism and mitochondrial depolarization. The pH was not significantly changed by acid treatment because of the buffering effect of PAS-E. The degree of platelet loss and viability in this study was greater than that reported in other studies, potentially due to difference in storage solutions [18, 26, 29, 31]. However, the same viability was retained throughout storage, which has not been previously demonstrated.

In this study, platelets were stored in 60% SSP+ 40% plasma. Previous studies have indicated that platelet quality was superior when acid-treated components were resuspended in 100% plasma compared to PAS-E [32].

Overall, HLA disruption had minimal effect on platelet surface receptors. GPVI, a labile glycoprotein [33], was significantly reduced by acid treatment over storage. HLA disruption activated platelets, as evidenced by increased surface CD62P and PS and release of soluble factors PF4 and sCD62P. Activation of CD42b, determined by AN51 binding, was observed in HLA-disrupted platelets immediately after treatment but appeared to return to its resting conformation following subsequent storage.

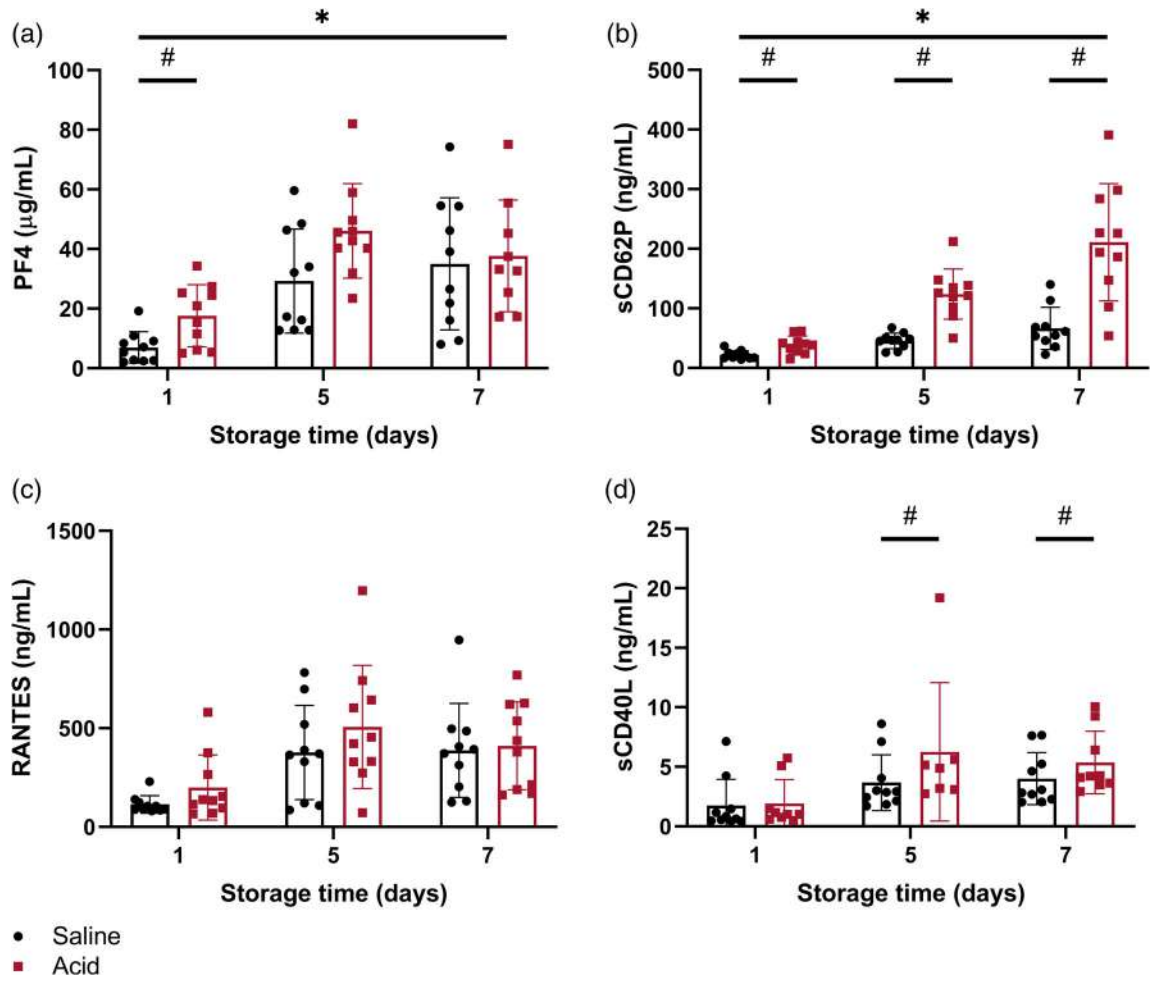

FIGURE 6 Platelet releasates. Supernatants were prepared from platelet samples and ELISAs were used to measure: (a) PF4; (b) sCD62P; (c) RANTES; (d) sCD40L. Data were analysed using two-way analysis of variance (ANOVA) with post hoc Bonferroni correction and *t*-tests. Data represent mean ± standard deviation ($n = 10$); $^{*}p < 0.05$ for ANOVA and $^{\#}p < 0.05$ for *t*-tests.

Acid treatment significantly blunted the ability of platelets to respond to both a strong (TRAP-6) and a weak agonist (ADP), as evidenced by reduced CD62P and PAC-1 binding post stimulation. Aggregation responses to collagen were significantly stronger in HLA-disrupted platelets, which has been previously observed [26]. The enhanced response may be due to a conformational change in $\alpha_2\beta_1$, conferring a higher affinity for collagen [26], again indicating that HLA-disrupted platelets were more activated. This is further supported by TEG measurements, whereby acid treatment reduced the time to initial clot formation.

Acid treatment increased desialylation and degalactosylation of platelet glycoproteins. Desialylation leads to platelet clearance in vivo via Ashwell–Morell receptors on hepatocytes [34]. Similarly, deglycosylation can increase phagocytosis by macrophages, as demonstrated with cold-stored platelets [35]. Furthermore, PS externalization acts as an 'eat me' signal to professional phagocytes and endothelial cells [36]. Together, this suggests that acid-treated platelets may be more readily cleared from the circulation following transfusion. However, previous studies have indicated that HLA-disrupted platelets escape complement-mediated destruction and antibody-mediated phagocytosis [19, 26]. Additionally, a small volume of autologous acid-treated platelets transfused into two female volunteers survived 6.25 days post transfusion in comparison to 7.95 days for untreated platelets, suggesting they have comparable in vivo survival [31]. Transfusion success has been reported in a limited number of HLA-refractory patients who received acid-treated platelet components, with a platelet increment or cessation of bleeding observed in 5/7 patients who received acid-treated components [14, 31, 37, 38]. The likelihood of a successful transfusion with acid-treated platelets would depend not only on the degree of HLA disruption but also on the anti-HLA antibody type, specificity, avidity, titre and ability to activate complement in the transfusion recipient [39]. An important consideration is the level of platelet activation observed in this study. Pro-coagulant platelets have been characterized by increased PS externalization, increased CD62P, decreased CD42b and GPVI and mitochondrial depolarization [40]. The increase in PS externalization and CD62P, together with reduced GPVI, suggests that HLA-disrupted platelets have an activated and potentially more pro-coagulant phenotype. Many haematology/oncology patients with thrombocytopaenia experience clinically significant bleeding [41].

If HLA-disrupted platelets can maintain platelet increment, this suggests they may be beneficial when administered prophylactically.

It should be noted that while HLA-disrupted platelets are unlikely to become the standard of care for refractory patients, they could potentially be used as a bespoke product manufactured on demand when compatible components are unavailable for transfusion and patient support is urgently required. The costs and labour associated with HLA disruption are greater than standard platelets components, and clinical trials are required to demonstrate their utility.

There are some limitations to this study. This study had a saline (treatment) control, but no untreated control was included because of low availability of triple-dose platelets. Furthermore, only in vitro measures of platelet quality and function were assessed in this study. HLA sensitivity to serum could be tested only after Day 5 of storage rather than immediately after treatment because of the inter-state location of the testing laboratory.

In this study, acid treatment significantly decreased HLA antigenicity of platelet components. While platelet loss and decreased viability were observed, acid-treated platelets remained functional, retaining their ability to aggregate and form clots. Further development of this product to align HLA-disrupted platelet components with routine blood component manufacturing procedures may enable the interim provision of platelet components to support refractory patients when HLA compatible units cannot be sourced.

ACKNOWLEDGEMENTS

Australian Governments fund Australian Red Cross Lifeblood for the provision of blood, blood products and services to the Australian community. We wish to thank Dr Lacey Johnson who provided invaluable assistance and advice, and Htet Htet Aung for performing biochemical measurements.

A.M.D., J.D. and D.C.M. designed the study; A.M.D., R.R. and G.P. performed the research; A.M.D. and D.C.M. analysed the data and drafted the manuscript; all authors critically reviewed the 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 from the corresponding author upon reasonable request.

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How to cite this article: Davis AM, Rawson R, Pahn G, Daly J, Marks DC. Platelets retain function and can be stored following disruption of human leucocyte antigens. Vox Sang. 2024;119:675–85.

Received: 5 December 2023 | Revised: 19 March 2024 | Accepted: 19 March 2024

DOI: 10.1111/vox.13637

ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Aggregates in apheresis-derived platelet concentrates: A 5-year single-centre experience

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Funding information
The authors received no specific funding for this work.

Abstract

Background and Objectives: The phenomenon of aggregates in apheresis-derived platelet concentrates (APCs) has not yet been fully elucidated. Initially, visible aggregates (IVA) usually dissolve within 24 h after collection, but some persist till the end of the shelf life (persistent aggregates, PA). A study conducted at the Croatian Institute of Transfusion Medicine aimed to identify factors that influence the aggregate occurrence in APCs.

Materials and Methods: We conducted a cross-sectional study for the 2018–2022 period and collected data on APCs with IVA. We analysed APCs discarded due to PA separately for two apheresis technologies and compared them to the control group.

Results: Significantly more donations were discarded in the IVA group compared with the control group and total number of discarded APCs. A total of 205 APCs were discarded due to PA (14.7% of IVA APCs and 1.27% of all APCs collected). Amicus APCs with PA had a significantly lower platelet count and mean platelet volume. They were obtained by procedures with less anticoagulant used. In contrast to Amicus APCs, Haemonetics APCs with PA had a significantly higher platelet count. None of the donor-related factors examined was predictive of PA.

Conclusion: APCs with IVA are more often discarded, not only due to aggregates, but also for impairment of other quality control parameters. Type of apheresis technology, being one of the most common risk factors for IVA, was not confirmed as the main risk factor for PA. There seem to be some donor-related causal factors.

Keywords

blood donation, plateletpheresis, quality control

Highlights

- Apheresis-derived platelet products with aggregates are frequently discarded, not only because of aggregates but also due to impairment of other quality control parameters.
- Type of apheresis technology is identified as the main risk factor for initially visible aggregates but not for persistent aggregates.
- Donor factors, still not identified, could be a risk factor for persistent aggregates in platelet concentrates.

INTRODUCTION

Aggregates in apheresis-derived platelet concentrates (APCs) are a well-known phenomenon. The international forum focused on aggregates in platelet concentrates conducted among 21 blood establishments showed a wide difference in experiences among blood banks [1]. One of the possible reasons is the lack of a mechanism for objective measurement of aggregates. Instead, they are assessed by visual inspection and can be descriptively graduated by size and number.

The mechanisms of cell separation and shear stress to which platelets are exposed during the apheresis procedure differ depending on the machine used. It is known that some types of apheresis devices cause more activation, thereby contributing to more frequent formation of aggregates in APCs but not in donor bloodstream [2, 3]. Procedure-related aggregates are considered 'friendly' because they dissolve in most cases, and such APCs are issued for clinical use [4, 5]. There is a lack of studies on the effect of APCs with aggregates on the platelet corrected count increment in patients because APCs with persistent aggregates (PA) are in most cases routinely discarded in blood banks [1].

Some researchers found a strong relationship of PA in APCs with donor history of aggregates [6–8]. This could imply that there are one or more donor-related factors responsible for the occurrence of aggregates in APCs. These factors have not been identified so far, although haematocrit and higher platelet count in donors are mentioned as a potential donor-related risk factor [9]. One study identified female gender as a risk factor [10]. There are suggestions to conduct studies related to donor factors that could influence or predict the formation of aggregates because apheresis technology is not the only factor influencing their formation [2, 5].

Croatian Institute of Transfusion Medicine (CITM) is the largest blood establishment in Croatia with ~100,000 whole blood (WB) donations and ~3000 apheresis donations per year. During apheresis procedures, both platelets and plasma are collected. APCs account for about 17% of total platelet production, and we tend to increase this number. In our blood establishment, aggregates are present only in APCs but not in WB-derived platelet concentrates (both resuspended in the mixture of plasma and platelet additive solution). This study aimed to assess donor-, procedure- and product-related factors in APCs with aggregates and compare them with the control group.

MATERIALS AND METHODS

We conducted a cross-sectional study during a 5-year period (2018–2022) and collected available data on donations and donors with aggregates in APCs. During the observed period, APCs were collected as a single platelet product using two apheresis technologies, Amicus Separator Fresenius Kabi (AM) and MCS+ 9000, Haemonetics (HAE), both resuspended in the platelet additive solution (PAS III for AM APCs and PAS III M for HAE APCs). WB to anticoagulant (AC) ratio was set to 9:1. Collection of APCs and their further processing took place in adjacent buildings, so transport between them was very short (1–2 min). They were transported in insulated boxes, so that outside temperature did not influence the temperature of the products. APCs were collected from Monday to Saturday. To examine the possible impact of apheresis procedure timing on the occurrence of aggregates, we divided the collections in three time slots based on when the procedure started: first 07:10–09:10 AM, second 9:40–11:05 AM and third 11:20 AM–14:20 PM. Also, to examine the occurrence of aggregates during different seasons, donations were divided as follows: winter (December to February), spring (March to May), summer (June to August) and autumn (September to November).

After being delivered to the blood processing department, each donation was left to rest for 1–2 h at room temperature. Visual assessment for swirling and aggregates was performed at four time points, that is, immediately after collection, after the rest period and before being inserted in an agitator, at the time of taking samples for quality control (shortly after the rest period) and at issue time. Each donation with visible aggregates after the rest period on the collection day was recorded (initially visible aggregates, IVA). We collected the available data on these donations and donors for the period from 1 January 2018 to 31 December 2022 and compared them with the control group.

Control group was formed randomly for donations in the 1 January 2018–31 December 2020 period from donors who did not have aggregates in APCs donated during the 2018–2022 period. There were 185 donations from 183 donors in the control group. Platelet products were selected to be equally distributed through each month over the years included.

We performed an analysis of subgroup of APCs discarded due to PA and compared them with the control group. PA are defined as aggregates that do not dissolve until the expiry date (5 days for APCs without bacterial screening and 7 days for APCs with bacterial screening). Due to a low share of female donors in Croatia, we were not able to analyse variables between men and women. To avoid a bias due to different technologies of apheresis devices and donor platelet (PLT) count criteria (for HAE, donors with a higher number of PLT are chosen), we separately analysed data for each technology.

Samples for complete blood count, platelet count and mean platelet volume (MPV) analysis in APCs were collected in K2EDTA tubes and CELL-DYN Ruby (Abbott) haematology analyser was used. Total blood volume (TBV) was calculated using the Nadler equation: for men: $TBV = (0.3669 \times height^3) + (0.03219 \times weight) + 0.6041$; and for women: $TBV = (0.3561 \times height^3) + (0.03308 \times weight) + 0.1833$. Body mass index (BMI) was classified into four categories (<18.5 underweight, 18.5–24.9 normal weight, 25–29.9 overweight and >30 obesity) [11].

MedCalc® Statistical Software version 22.006 (MedCalc Software Ltd., Ostend, Belgium) was used for statistical analysis. Since data were not normally distributed, non-parametric tests were used. Numerical data were expressed as median and interquartile range. The χ^2-test was used to examine the association between two categorical variables. The Mann–Whitney *U* test was used to examine the

difference in numerical variables between the two groups. The level of statistical significance was set at $p < 0.05$. A backward stepwise logistic regression was used to identify the possible predictors of PA among the donor-related variables. At each step, variables were added if p value <0.05.

RESULTS

A total of 16,085 APCs were collected at CITM during the 2018–2022 period (55% AM and 45% HAE), and 1575 (9.8%) of them were non-conforming. Two hundred and five (1.27%) APCs were discarded due to PA, which was the third most common reason for discarding, after procedural issues (658 APCs, 4.1%) and out-of-specification quality control results (248 APCs, 1.5%). Distribution of the total number of APCs, IVA and PA according to the type of apheresis device is shown in Figure 1.

Out of 16,085 APCs, there were 1396 (8.7%) APCs with IVA, and the number of such APCs showed an increasing trend (Figure 2).

In products with IVA, most of the aggregates dissolved and APCs were issued. However, 325 (23.3%) APCs with IVA were discarded for various reasons, PA being the reason in most of the products ($n = 205$, 14.7%), followed by quality control failures ($n = 53$, 3.8%). The total number of discarded APCs was significantly higher in the IVA group ($p < 0.001$, $\chi^2 = 37.26$) compared with the control group, where only 7 (3.8%) APCs were discarded.

APCs with IVA (1396 donations) were collected from 456 donors. APCs with PA (205 donations) were collected from 139 donors. About 25% of donors in the PA group had repetitive aggregate occurrence (Figure 3).

Most of the donors with single occurrence of PA donated more than once, and their remaining donations resulted in either IVA or no aggregates at all. Donors did not necessarily donate on the same type of device. The percentage of APCs with PA per donor during the observed period is shown in Figure 4.

There was no difference in ABO blood groups between donors in both IVA and PA groups compared with controls. No difference was observed between non-O and O blood groups of donors in the IVA and PA groups. In donors with three and more aggregate occurrences, there was no difference in ABO blood groups compared with the control group either.

Most of the donations in all the groups were collected from overweight donors, BMI: 25–29.9 (53% of IVA, 58% of PA and 59% of

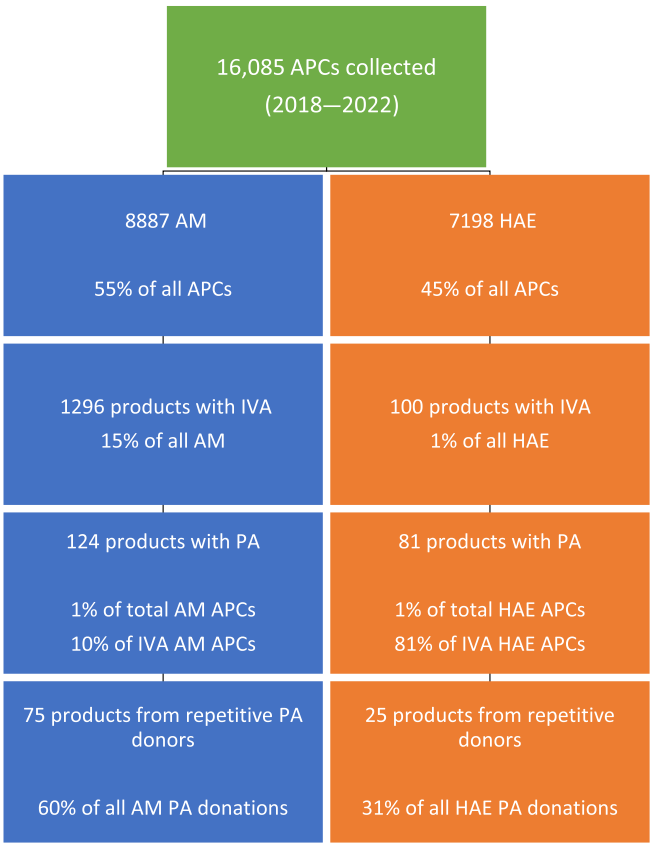

FIGURE 1 Total number of apheresis-derived platelet concentrates (APCs) collected, initially visible aggregates (IVA) and persistent aggregates (PA), according to type of apheresis device (AM = Amicus Separator, Fresenius Kabi; HAE = MCS+ 9000, Haemonetics).

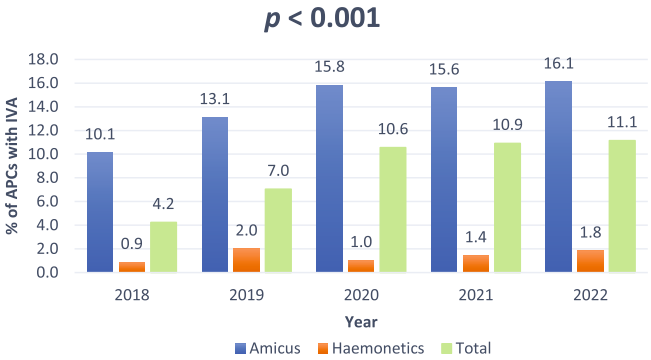

FIGURE 2 Percentage of apheresis-derived platelet concentrates (APCs) with initially visible aggregates (IVA) according to apheresis devices; comparison of proportions.

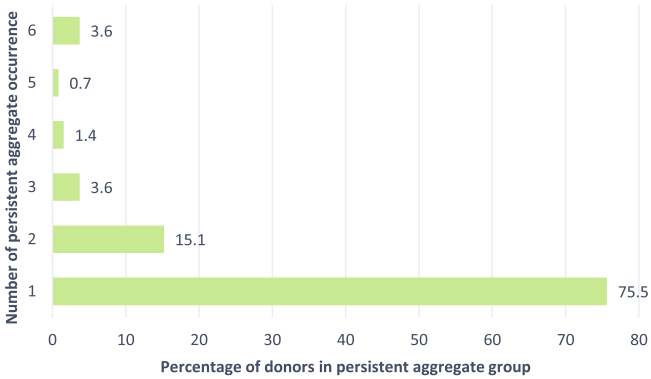

FIGURE 3 Percentage of donors by number of aggregate occurrences in apheresis-derived platelet concentrates with persistent aggregates (2018–2022).

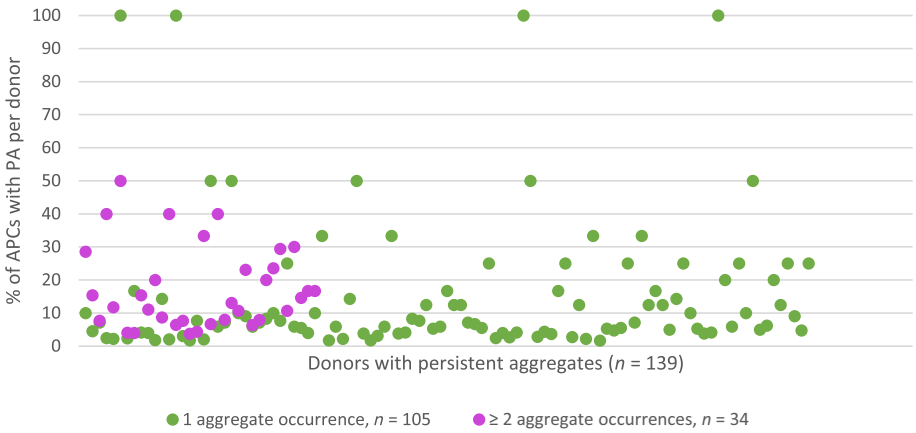

FIGURE 4 Percentage of apheresis-derived platelet concentrates (APCs) with persistent aggregates (PA) in total number of donated APCs per each donor with PA during the observed period (2018–2022).

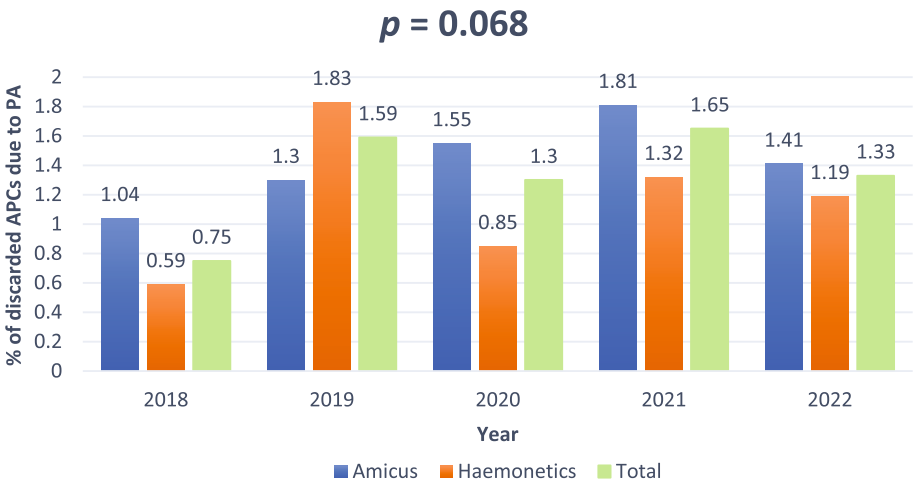

FIGURE 5 Discarded apheresis-derived platelet concentrates (APCs) due to persistent aggregates (PA) throughout the observed period according to apheresis devices; comparison of proportions.

controls). In IVA and PA donations, the second most represented group was obesity, BMI > 30 (33% of IVA and 28% of PA), in contrast to the control group (12%). The rest of donations were from normal weight donors, BMI: 18.5–24.9.

There was no significant difference in time slots or seasons of performing apheresis procedures in the IVA and PA groups compared with the control group.

In the IVA group, 1296 (93%) procedures were performed on AM and 100 (7%) on HAE. In the control group, 94 (51%) procedures were performed on AM and 91 (49%) on HAE ($p < 0.001$). In the PA group, 124 (61%) procedures were performed on AM and 81 (39%) on HAE, with no statistically significant difference compared with the control group ($p = 0.055$). Distribution of APCs with PA according to apheresis devices is shown in Figure 5. In contrast to the analysis of all PA cases according to the device type, where no statistically significant difference between devices was found, the analysis of donations ($n = 100$) donated by 34 donors with repetitive PA occurrence (≥2 in the observed period) showed that more procedures were performed on AM ($n = 75$) than on HAE ($n = 25$), $p < 0.001$ (comparison of proportions).

Donor, procedure and product characteristics of PA APCs were analysed and compared with the control group, separately for each technology. Results are shown in Table 1.

Backward logistic regression was done for donor-related variables for both apheresis technologies. In the final model, BMI was retained for AM, whereas the number of previous apheresis donations, red blood cell and white blood cell count were retained for HAE. The receiver operating characteristic curve analysis resulted in area under the curve (AUC) <0.7 for all of the variables.

Comparison of the final whole blood to AC ratio (WB/AC) resulted in a variation of WB/AC between 6:1 to 12:1 in AM procedures (mostly being 8:1–9:1) and 6:1–9:1 in HAE procedures (mostly being 7:1). Significantly more anticoagulant was used in the control group compared with both IVA and PA groups in AM procedures. As for HAE procedures, there was no significant difference between the groups in the final volume of AC used.

TABLE 1 Apheresis-derived platelet concentrates discarded for persistent aggregates compared with the control group.

	Amicus		Haemonetics	
	PA	Control	PA	Control
Characteristics	***n* = 124**	***n* = 94**	***n* = 81**	***n* = 91**
Donor age (years)	43 (35–47)	41 (32–46)	44* (35–51)	40 (29–48)
Donor body mass index	28* (26–30)	27 (25–29)	28 (25–30)	27 (25–29)
Total blood volume (L)	5.7* (5.4–6.3)	5.5 (5.3–5.9)	5.6 (5.2–6)	5.7 (5.4–6.1)
N of previous donations (WB + apheresis)	46 (28–69)	47 (25–71)	54** (34–76)	37 (20–65)
N of previous donations (only apheresis)	16 (5–31)	16 (4–34)	27*** (9–43)	10 (3–27)
n of donations in 1-year period before actual donation	5 (3–8)	5 (3–8)	7* (4–10)	5 (3–8)
Donor RBC ($\times 10^{12}$/L)	5.27 (4.96–5.53)	5.18 (4.92–5.44)	5** (4.68–5.29)	5.2 (4.95–5.43)
Donor haematocrit	0.47 (0.45–0.49)	0.47 (0.45–0.49)	0.45** (0.43–0.47)	0.47 (0.45–0.49)
Donor WBC ($\times 10^{9}$/L)	6.3 (5.7–7)	6.3 (5.4–7.2)	6.3 (5.6–7.1)	6.5 (5.8–7.2)
Donor PLT count ($\times 10^{9}$/L)	241 (217–265)	230 (215–254)	276 (250–314)	271 (241–296)
Donor MPV (fL)	7.37 (6.74–8.27)	7.78 (7.33–8.46)	7.04 (6.44–7.63)	7.15 (6.76–7.98)
Apheresis procedure duration (min)	60 (55–70)	63 (55–68)	75 (70–81)	75 (70–85)
Anticoagulant volume infused (mL)	338* (307–375)	359 (340–387)	387 (350–424)	398 (357–441)
Total blood volume processed (mL)	2900 (2619–3183)	2939 (2684–3158)	2712 (2362–2961)	2750 (2402–3044)
APC volume (mL)	317 (315–321)	317 (313–320)	297* (295–300)	296 (293–299)
APC PLT count ($\times 10^{9}$/L)	865*** (804–968)	1159 (1107–1234)	1304** (1220–1369)	1240 (1156–1309)
APC MPV (fL)	3.98*** (3.74–4.29)	5.24 (4.74–5.73)	5.34 (4.91–5.86)	5.34 (4.93–5.68)

Abbreviations: APC, apheresis-derived platelet concentrate; MPV, mean platelet volume; *n*, number; *N*, total number; PA, persistent aggregates; PLT, platelets; RBC, red blood cell; WB, whole blood; WBC, white blood cell.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; Mann–Whitney test.

DISCUSSION

Aggregates in platelet concentrates are of great interest to blood bank experts owing to the multitude of unanswered questions about the reasons for their appearance and clinical significance. IVA, which usually are procedure-related [2], are not considered to be a major problem because most of the aggregates dissolve within the first few days [1], although a question of clinical relevance remains unanswered. However, in some APCs with IVA, aggregates remain permanent until the expiry date and as such are not eligible for issuing.

Our study indicated that apheresis technology was the main contributing factor to IVA occurrence (93% of APCs with IVA were collected using AM and 7% using HAE). APCs with aggregates collected on AM tended to dissolve in most of the cases.

Situation was more complicated in the PA group. In the total number of PA donations, there was no statistically significant difference ($p = 0.055$) between the two apheresis technologies, but significant difference was reached in the subgroup of donors with repeated occurrence of PA (75% collected using AM and 25% using HAE, $p < 0.001$). Due to the low number of donors with repetitive PA ($n = 34$), it is not conclusive whether this finding was related to donor factor(s), apheresis factor(s) or both (donor factor that may become manifested in visible aggregates with one type of the apheresis technology), especially because four donors donated on both device types, and the donations ended up as APCs with PA. This was an important observation to be further investigated.

In this study, we demonstrated the correlation of IVA with a higher risk of APC rejections due to the types of nonconformity other than aggregates, particularly deviations in quality control results. Low platelet count was identified as the leading cause of quality deviation. The increased rejection of APCs in the IVA group was statistically significant, suggesting a lower quality of APCs with IVA. In addition to APCs rejected due to PA, such nonconformities increase economic costs and decrease platelet supply. Several published studies did not find impaired in vitro quality of APCs with aggregates [8, 9].

Feys et al. investigated several factors in APCs with PA and compared them with controls. They showed that APCs with PA had accelerated storage lesion due to increased degranulation (measured by P-selectin) and increased binding of von Willebrand factor [12]. One study reported a lower platelet count in products with aggregates, although these were whole blood-derived platelet concentrates. They showed that pre-storage temperature was the reason for aggregate formation and, consequently, lower PLT count in the final product [13].

To the best of our knowledge, our study is the first study assessing variables for PA for two technologies. To avoid bias associated with the procedure, the main analysis for all donor-, procedure- and product-related variables was performed separately for both the technologies used. The results differed for each technology.

Platelet count in the product was higher in HAE APCs with PA, which is in concordance with a previously published study [14]. However, in AM APCs with PA, both PLT count and MPV were lower. This

could, to some extent, be explained by a higher level of platelet activation in this type of device [2]. Larger platelets tend to have a greater degree of platelet activation [15, 16] and are, therefore, accumulated, so the cell counter cannot count them as individual cells (a mechanism similar to the one observed in pseudothrombocytopenia) [17].

The volume of anticoagulant used was significantly lower in AM APCs with both IVA and PA compared to AM control group. This, along with disproportions of the WB/AC ratio in AM, might be one of the key contributors to the formation of aggregates (mainly IVA and not necessarily PA) in AM APCs. Lowering the WB/AC ratio has been suggested for the prevention of aggregates [5, 18], and at CITM, it is set to 9:1 for both AM and HAE. However, the final volume of anticoagulant used, as well as the WB/AC ratio, varied significantly in AM in our study.

Donor-related factors that may contribute to the appearance of aggregates cannot be overseen. Previously published studies found that only some donors were more often associated with the appearance of aggregates in APCs than others [1, 6, 8]. Our study confirmed this observation and suggested the possible additional donor-related factors for PA (donor age, BMI, number of previous donations).

In our study, AM APCs with PA originated from donors with a higher BMI. The rationale behind this is not quite clear. Elevated platelet activation and aggregation are usually associated with obesity [19], but it should be borne in mind that most of the platelet activation studies related to obesity were performed in patients with already-known cardiovascular diseases. Several studies conducted in non-patient population showed no difference in platelet activation between non-obese and obese individuals [20, 21]. Recently, it has been reported that there are platelet phosphorylation changes related to obesity, resulting in dysregulation of the main signalling pathways, including activation of platelets [22].

Earlier studies suggested that donors of APCs with aggregates had higher PLT counts in their blood [9, 14]. In our study, donors with PA had higher PLT counts, but these differences did not show statistical significance.

Donors of HAE APCs with PA were significantly older, but donor age was not predictive of PA. Literature data suggest that older age is usually related not only to lower platelet counts but also to increased platelet activity [23, 24]. Also, for HAE APCs with PA, a higher number of previous donations was recorded (both apheresis and whole blood) compared with HAE control group, which was statistically significant. In regression analysis, only previous number of apheresis donations was retained in the final model but resulted in AUC <0.7. At this moment, we cannot establish a causal relationship of this finding with PA occurrence, and it will be further monitored.

Low WBC count in donors was described as the only independent donor-related factor for aggregate occurrence during peripheral blood haematopoietic stem cell collection using the Spectra Optia cell separator [25]. A study on the safety of plateletpheresis carried out on Amicus and MCS+ devices showed a significant decline in lymphocyte and monocyte counts, but not in neutrophil count on both types of devices, with greater difference for the AM device [26]. We did not study a WBC differential, but rather total WBC count in donors, from pre-donation samples. There was no statistically significant difference in WBC count in studied groups, and logistic regression did not prove WBC count to be a predictive factor for PA.

Platelet ABO (H) type modulates platelet function [27]. However, it seems that ABO type does not influence the occurrence of aggregates in APCs; in our study, no difference between blood types was found in IVA and PA donor groups or in donors with repetitive PA occurrence compared with controls.

A recently published study on variation in platelet activation state depending on the time of day showed a slightly increased platelet activation in donors during morning hours (samples collected between 8 and 10 AM) [28]. Theoretically, this could implicate more aggregates in APCs collected earlier during the day, but our study did not show that more IVA or PA procedures were performed during the morning. A study examining the difference in PLT counts in the same donors between morning and afternoon found a minor increase in PLT counts during the day, but this difference was not statistically significant [29].

Our study is a contribution to the identification of risk factors for the occurrence of aggregates in APCs. It also shows that APCs with IVA are more susceptible to rejection due to a higher incidence of nonconformities, not only because of the presence of PA but also because of other quality-related nonconformities.

Our study implicates that IVA occurrence is most likely caused by apheresis device technology, but other factors contribute to the formation of PA, which should be further investigated. The donor-related factors examined could not predict PA, but there was a limited number of variables available for this cross-section study. Blood group and time of the day of performing apheresis procedures do not influence the occurrence of aggregates in APCs. We plan to further investigate donor-related factors to see whether there is a practical way to diminish the occurrence of aggregates in APCs and, consequently, reduce the number of discarded platelet products.

ACKNOWLEDGEMENTS

M.V. designed the research study, collected and analysed data and wrote the first draft of the manuscript; A.H. and I.J. supervised the research and reviewed and edited the manuscript; T.V. designed the research study, supervised the research and reviewed and edited the manuscript.

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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How to cite this article: Vinković M, Hećimović A, Jukić I, Vuk T. Aggregates in apheresis-derived platelet concentrates: A 5-year single-centre experience. Vox Sang. 2024;119: 686–92.

Received: 2 February 2024 | Revised: 3 April 2024 | Accepted: 4 April 2024

DOI: 10.1111/vox.13640

ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Proliferation of psychrotrophic bacteria in cold-stored platelet concentrates

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Abstract

Background and Objectives: Platelet concentrates (PC) are stored at 20–24°C to maintain platelet functionality, which may promote growth of contaminant bacteria. Alternatively, cold storage of PC limits bacterial growth; however, data related to proliferation of psychotrophic species in cold-stored PC (CSP) are scarce, which is addressed in this study.

Materials and Methods: Eight laboratories participated in this study with a pool/split approach. Two split PC units were spiked with ~25 colony forming units (CFU)/PC of *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Serratia liquefaciens*, *Pseudomonas fluorescens* and *Listeria monocytogenes*. One unit was stored under agitation at 20–24°C/7 days while the second was stored at 1–6°C/no agitation for 21 days. PC

Carl McDonald was retired from NHS Blood and Transplant.

were sampled periodically to determine bacterial loads. Five laboratories repeated the study with PC inoculated with lyophilized inocula (~30 CFU/mL) of *S. aureus* and *K. pneumoniae*.

Results: All species proliferated in PC stored at 20–24°C, reaching concentrations of ≤10^9 CFU/mL by day 7. Psychrotrophic *P. fluorescens* and *S. liquefaciens* proliferated in CSP to ~10^6 CFU/mL and ~10^5 CFU/mL on days 10 and 17 of storage, respectively, followed by *L. monocytogenes*, which reached ~10^2 CFU/mL on day 21. *S. aureus* and *K. pneumoniae* did not grow in CSP.

Conclusion: Psychrotrophic bacteria, which are relatively rare contaminants in PC, proliferated in CSP, with *P. fluorescens* reaching clinically significant levels (≥10^5 CFU/mL) before day 14 of storage. Cold storage reduces bacterial risk of PC to levels comparable with RBC units. Safety of CSP could be further improved by implementing bacterial detection systems or pathogen reduction technologies if storage is beyond 10 days.

Keywords

bacterial contamination of platelet concentrates, bacterial growth in platelet concentrates, cold-stored platelet concentrates, platelet concentrates

Highlights

- Psychrotrophic bacteria, which are rare contaminants in platelet concentrates (PC), can proliferate in cold-stored PC at levels comparable with red blood cell concentrates.
- Fast-growing psychrotrophic bacteria, such as *Pseudomonas fluorescens*, can reach clinically significant levels (≥10^5 CFU/mL) in PC before day 14 of cold storage.
- Common mesophilic PC contaminants such as *Klebsiella pneumoniae* and *Staphylococcus aureus* do not survive or grow in cold-stored PC.

INTRODUCTION

Platelet concentrates (PC) are mainly used to prophylactically treat thrombocytopenic patients; however, PC are also used to manage active bleeding patients. PC are manufactured from whole blood or collected via apheresis and are stored in either plasma or a mix of plasma and platelet additive solution (PAS), and can be subjected to pathogen reduction, irradiation or volume reduction. Each of these manufacturing methods and post-production treatments influences the biochemical and physiological profiles of PC. Biological changes in PC that are developed during storage are known as the platelet storage lesion. PC manufacturing methods and storage conditions have a direct impact on platelet functionality [1].

Many studies have been performed to determine optimal PC storage conditions. Reports of studies conducted in the early 1970s showed that cold storage of PC (1–6°C) resulted in improved platelet aggregation response and reduced bleeding times. However, cold-stored PC (CSP) had reduced platelet circulation time due to rapid clearance compared with PC stored at room temperature (RT, 20–24°C). PC storage at RT was thus preferred as it reduced patient exposure to multiple PC transfusions and avoided the challenge of maintaining a dual PC inventory [2].

Cold storage of PC has regained attention in recent years to treat patients with active bleeding due to the superior haemostatic function of this blood product [2]. Importantly, new studies have shown that PC storage in plasma or PAS does not significantly affect the in vitro quality of CSP [3]. In September 2019, the US Food and Drug Administration (FDA) granted a variance for PC storage, allowing PC to be stored at 1–6°C for up to 14 days without agitation. This variance was requested by the US Army Blood Program for use during military operations to treat actively bleeding patients. More recently, in June 2023, the FDA granted permission for the cold storage of apheresis PC suspended in plasma or PAS with a shelf life of up to 14 days if transferred to the cold within 4 h of collection or pathogen reduction treatment [4].

One of the major issues encountered with RT storage of PC is the enhanced risk of bacterial growth; however, very few studies have addressed this concern with CSP as it is assumed that refrigeration limits bacterial proliferation. An interesting study was conducted by Kahn and Syring in 1975 [5]. Whole blood was intentionally inoculated with four bacterial species: *Escherichia coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. Inoculated whole blood was then centrifuged and platelet-rich plasma (PRP, 24–32 mL) was extracted. PRP was stored for 3 days at either RT with agitation or statically at 4°C. Results of the study showed that

P. aeruginosa did not survive in RT-stored PRP but *E. coli*, *E. cloacae* and *S. epidermidis* all proliferated in PRP stored at RT. In contrast, all organisms remained viable at very low concentrations but did not proliferate when PRP was stored at 4°C [5]. More recently, a study was conducted to challenge bacterial growth in CSP compared with RT-stored PC. Ten-mL apheresis PC units stored in minibags (pH SAFE, Blood Cell Storage, Inc.) were inoculated with 1000 colony forming units (CFU)/mL of *Acinetobacter baumannii*, *E. coli*, *P. aeruginosa*, *Staphylococcus aureus* and *S. epidermidis*. PC minibags were stored for 5 days at 20–22°C under agitation or at 4°C with no agitation. High levels of bacteria were reached for all species in PC stored at 20–22°C, while bacterial loads remained constant (*E. coli*, *P. aeruginosa* and *S. aureus*) or decreased (*A. baumannii* and *S. epidermidis*) in CSP [6]. More recently, Brown et al. (2022) spiked double apheresis PC units at initial concentrations ranging from 442 to 733 CFU/mL of *E. coli*, *S. aureus*, *S. epidermidis*, *Serratia marcescens*, *A. baumannii*, *Pseudomonas fluorescens*, *Bacillus cereus* and *Streptococcus pyogenes* [7]. Spiked PC were split into two equal units: one unit was held under agitation at RT for 2 h and then placed in cold storage while the second split was stored in refrigeration 8 h post-collection. No significant impact on bacterial growth was observed between the two groups.

The multinational study discussed herein aimed at addressing the gaps of information existing in the literature regarding the potential safety risk posed by CSP units inoculated with real-life concentrations of transfusion relevant bacterial strains, including psychrotrophic species.

MATERIALS AND METHODS

Platelet concentrates

Eight sites participated in this study which are listed in Table 1. Products included single and double apheresis platelet units, and buffy coat platelet pools collected or manufactured in 100% plasma or PAS, as described in Table 1. Ethical approval and/or donor consent for the research use of PC units was obtained at those centres where approval was required.

Platelet unit spiking

The study had a pool and split approach. The protocol summarized in Figure 1 was followed by eight participant sites worldwide. Five transfusion relevant bacterial reference strains were used for PC spiking experiments and included *Klebsiella pneumoniae* PEI-B-P-08 and *S. aureus* PEI-B-P-63, which have previously been shown to proliferate well in RT-stored PC, and three psychrotrophic species—*Serratia liquefaciens* PEI-A-184 (fast grower in red blood cell concentrates [RBCC]), *P. fluorescens* PEI-B-P-77 (fast grower in RBCC) and *Listeria monocytogenes* PEI-A-199 (slow grower in RBCC) [8, 9].

Enumerated frozen stocks were prepared for all bacteria, which were thawed and diluted on the spiking day. Serial dilutions were prepared to inoculate each split PC unit at a target concentration of

TABLE 1 Study participants and platelet components.

Study site	Species tested	Platelet unit type	Plasma or platelet additive solution (PAS)
Austrian Red Cross, Blood Centre Linz, Austria[a]	All species	Buffy coat pools	PAS: SSP+
Canadian Blood Services, Ottawa, Ontario, Canada[a]	All species	Combination of single apheresis units and buffy coat pools	Plasma
Cerus Corporation, Concord, California, USA	All species	Double apheresis units	PAS: Intersol
US Army Institute of Surgical Research, Texas, USA	All species	Double apheresis units	Plasma
German Red Cross Blood Service NSTOB, Springe, Germany[a]	All species	Buffy coat pools	PAS: SSP+
Héma-Québec, Québec City, Québec, Canada[a]	All species	Double apheresis units	Plasma
Institut für Laboratoriums- und Transfusionsmedizin, Herz- und Diabetesze ntrum, Germany	All species	Double apheresis units	PAS: SSP+
South African National Blood Service, South Africa[a]	*K. pneumoniae*	Buffy coat pools	PAS: SSP+
	S. aureus	Combination of single apheresis units and buffy coat pools	Plasma
	P. fluorescens	Apheresis	Plasma
	S. liquefaciens	Buffy coat pools (two replicates)	PAS: SSP+
		Apheresis (one replicate)	Plasma
	L. monocytogenes	Buffy coat pools (one replicate)	PAS: SSP+
		Apheresis (two replicates)	Plasma

[a]Participant sites also conducted studies with lyophilized bacterial inocula (BIOBALLS).

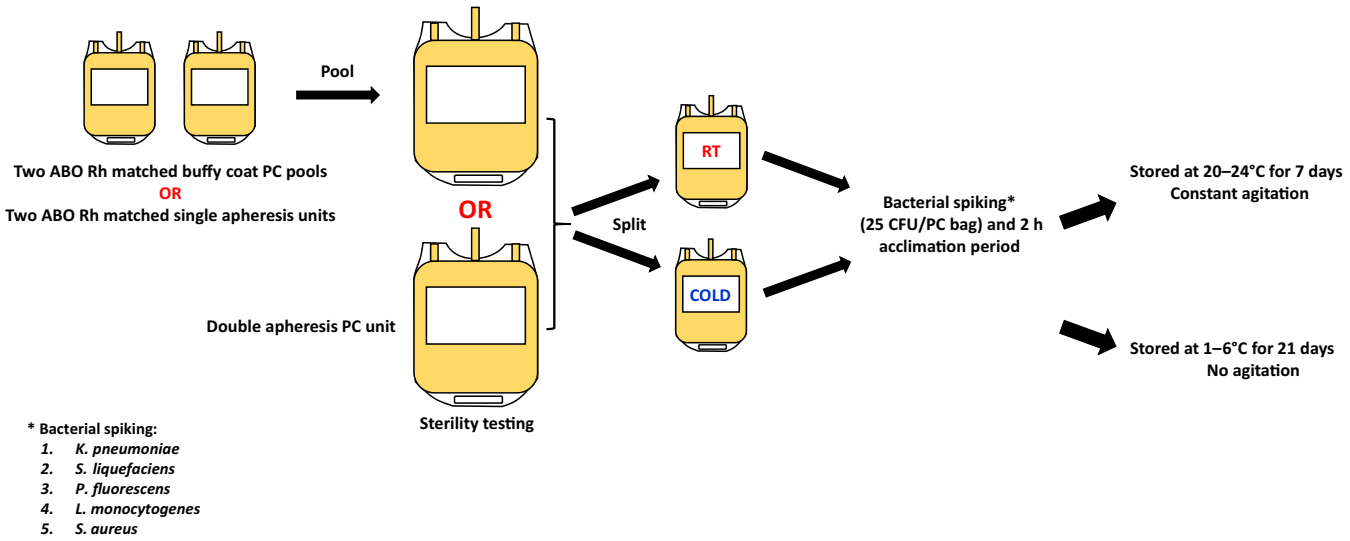

FIGURE 1 Experimental design describing process for platelet concentrates (PC) spiking and two arms of the study with PC stored under standard conditions (room temperature: 20–24°C/agitation) or in the cold with no agitation. CFU, colony forming units.

25 colony forming units (CFU)/PC bag. Samples from the serial dilutions were plated to estimate the initial bacterial load per millilitre based on the volume of the PC units. PC were inoculated with bacteria and then stored for 2 h under standard PC storage conditions (20–24°C, agitation). After the 2-h acclimation period, one split PC unit remained under standard storage for 7 days, while the other split PC unit was stored under refrigeration (1–6°C) with no agitation for 21 days. PC stored at 20–24°C were sampled on days 1, 2, 3, 4 and 7 of storage, and CSP were sampled on days 7, 10, 14, 17 and 21 of storage, for determination of bacterial loads by serial dilutions and plating on appropriate agar media. The protocol was repeated in triplicate for each bacterial species at each participant site.

Additional spiking studies were conducted by five of the eight participant laboratories (Table 1). A similar protocol as the one described above was followed with PC spiked with lyophilized inocula (BIOBALLS, bioMérieux) of two species, *K. pneumoniae* PEI-B-P-08 and *S. aureus* PEI-B-P-6.

Statistical analysis

T-test was used to compare log10 of bacterial concentration between species in each of the two PC storage conditions, 20–24°C under agitation and 1–6°C static, and between PC units suspended in plasma versus SSP+ PAS. Comparison between PC suspended in plasma and Intersol PAS (PAS C) was not possible as only one participant site used Intersol. *T*-test was also used to compare the growth of *S. aureus* and *K. pneumoniae* in PC spiked with either dilutions from frozen bacterial stocks or BIOBALLS. A *p*-value <0.05 was considered statistically significant different.

RESULTS

Comparable initial bacterial loads in PC units

Estimated initial bacterial load in the PC units inoculated with diluted enumerated stocks on day 0 varied from approximately 10^{-2} to 10^{1} CFU/mL ($p > 0.05$, Figure 2).

Bacterial growth under PC standard storage conditions varies between testing sites

As shown in Figure 3, all species tested grew in PC units stored at 20–24°C under agitation over 7 days. *K. pneumoniae* and *P. fluorescens* displayed fast growth reaching concentrations greater than 10^{5} by day 2, proliferating to approximately 10^{9} CFU/mL by day 7 of storage. *S. liquefaciens* grew well in six participant laboratories reaching loads of 10^{2}–10^{7} by day 2 of PC storage, which increased to 10^{6}–10^{8} by day 7 of PC incubation. Interestingly, this species did not survive in PC pools suspended in PAS SSP+ in one of the participant sites. Growth of *S. aureus* was not consistent between sites, it varied from 10^{1} to 10^{7} CFU/mL on day 2 of PC incubation in seven sites but reached loads of 10^{7}–10^{8} CFU/mL by day 7. Surprisingly, in one site, which used apheresis and buffy coat PC in plasma, this bacterium grew to 10^{7} CFU/mL by day 1 and maintained this load until the end of PC storage. The growth of *L. monocytogenes* also had great variability in bacterial loads on day 2 of storage (10^{1}–10^{7} CFU/mL) within sites, but it reached concentrations of 10^{6}–10^{7} CFU/mL by day 7.

Growth of *K. pneumoniae* was significantly faster ($p < 0.05$) than that of other species at all testing days. *P. fluorescens* grew significantly faster than *S. aureus* and *L. monocytogenes* on all testing days

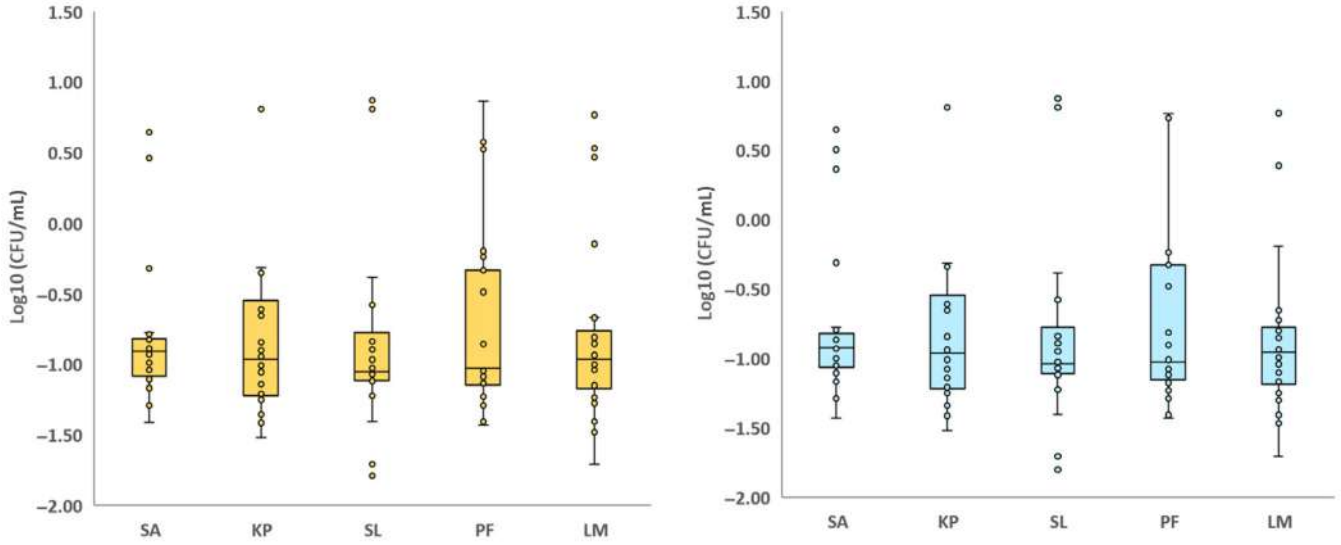

FIGURE 2 Initial bacterial loads in platelet concentrates (PC) inoculated with diluted enumerated stocks. (a) Graph showing log10 of initial concentration of bacteria in PC units stored under standard conditions (20–24°C/agitation). (b) Graph showing log10 of initial concentration of bacteria in cold-stored PC units with no agitation. CFU, colony forming units; KP, *K. pneumoniae*; LM, *L. monocytogenes*; PF, *P. fluorescens*; SA, *S. aureus*; SL, *S. liquefaciens*.

($p < 0.05$) except day 7 for *S. aureus* ($p = 0.11$). Comparison of bacterial loads for all other species on the different testing days did not yield significant differences ($p > 0.05$).

Psychrotrophic bacteria proliferate in CSP units

Bacterial proliferation in CSP is shown in Figure 3. Mesophilic bacteria tested in this study included *K. pneumoniae* and *S. aureus*. *K. pneumoniae* did not survive in any of the PC units at all testing laboratories, demonstrating that this strain is sensitive to cold storage. Similarly, *S. aureus* did not grow in CSP in seven of the eight testing sites; however, in one site, it surprisingly grew to 10^5 CFU/mL on day 21, despite not being detectable on any of the prior testing days. Microbial identification confirmed the identity of the organism as *S. aureus*.

The three psychrotrophic bacteria tested herein displayed different growth dynamics in CSP. *S. liquefaciens* did not survive in two of the eight sites that used pooled PC prepared in PAS SSP+. In another site, which used apheresis PC suspended in SSP+, this species was only detected on day 21 ($\sim 10^1$ CFU/mL). In the site where apheresis PC were prepared with Intersol, *S. liquefaciens* grew very slowly reaching a concentration of 10^5 CFU/mL on day 21 of PC storage while in the remaining five sites, this bacterium grew to $\sim 10^5$ CFU/mL on days 10 or day 14 of PC storage, and up to $\sim 10^8$ CFU/mL by day 21. *P. fluorescens* did not survive in one site but grew consistently fast in the remaining seven sites, reaching loads of 10^5 CFU/mL in some cases by day 7 of PC storage and 10^9 CFU/mL by day 21. Again, this bacterium grew slowly in apheresis PC units prepared with Intersol, reaching a load of 10^5 CFU/mL on day 14. In one site, which used apheresis PC suspended in plasma, this bacterium only had detectable colonies on day 21 ($\sim 10^1$ CFU/mL). *L. monocytogenes* consistently grew slower than the other two psychrotrophic species. It did not grow in two sites in all three repetitions, and in one site, in two of the three replicates. In the five laboratories where it proliferated, the bacterial load at the end of PC storage (day 21) varied from 10^2 to 10^4 CFU/mL.

Effect of PAS on bacterial growth is strain-dependent

Growth of *K. pneumoniae*, *S. aureus*, *S. liquefaciens* and *P. fluorescens* in PC stored at 20–24°C was not different between PC suspended in plasma and SSP+ ($p = 0.86$, $p = 0.99$, $p = 0.50$ and $p = 0.94$, respectively). Similarly, no difference in growth of *S. liquefaciens* and *P. fluorescens* was observed CSP between units suspended in plasma and SSP+ ($p = 0.99$, $p = 0.96$, respectively). Interestingly, a slower growth rate was observed for *K. pneumoniae*, *S. liquefaciens* and *P. fluorescens* in units suspended in Intersol compared to plasma and SSP+. In contrast, *L. monocytogenes* displayed a higher growth rate in PC suspended in SSP+ compared to plasma although statistical significance was only reached on days 17 and 21 in units stored at 1–6°C ($p = 0.40$).

Lyophilized (BIOBALLS) inoculum does not impact proliferation of *K. pneumoniae* and *S. aureus* in PC

Each BIOBALL contained approximately 30 CFU of bacteria. The growth patterns for *K. pneumoniae* and *S. aureus* in PC units inoculated with BIOBALLS and incubated under PC standard storage conditions were similar to those of PC inoculated with diluted enumerated stocks ($p > 0.05$, Figure 4). As observed for PC units inoculated with diluted enumerated stocks, these two bacteria did not grow in CSP inoculated with BIOBALLS (data not shown).

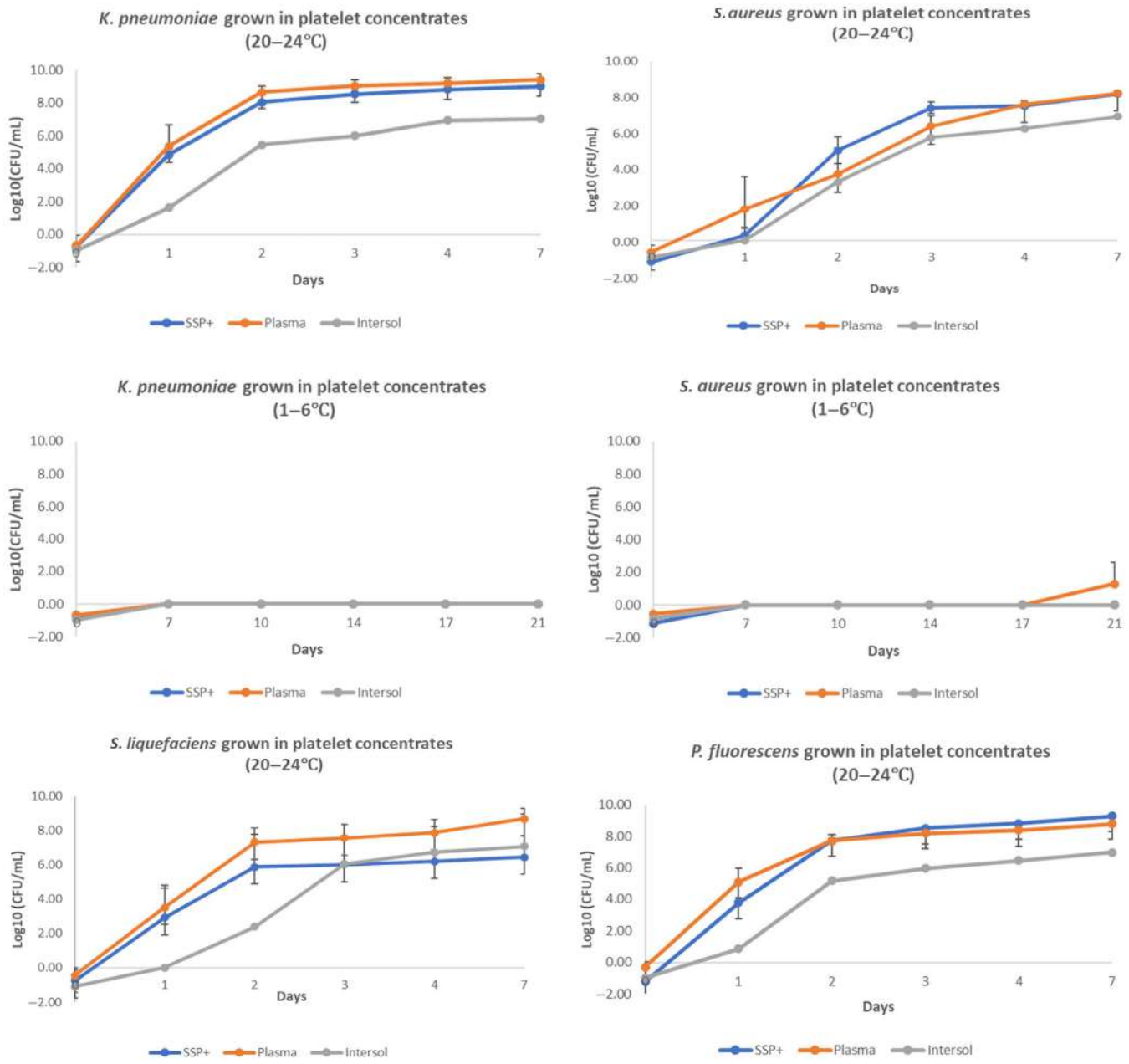

FIGURE 3 Bacterial proliferation in platelet concentrates (PC) stored at room temperature (RT) versus cold-stored PC. Graphs show bacterial growth in PC inoculated with diluted enumerated stocks over 7 days of storage under standard conditions (RT: 20–24°C/agitation) or in PC stored at 1–6°C without agitation over 21 days. Aggregated data from technical triplicates performed at each of the eight participant sites are shown for each bacterial species ± standard error. CFU, colony forming units.

DISCUSSION

The risk of transfusing PC contaminated with bacteria has been greatly reduced in high-income countries in the last three decades as a result of the implementation of successful mitigation strategies, including improved donor disinfection, diversion of the first aliquot of collected blood, PC screening with culture or rapid methods, or PC treatment with pathogen reduction technologies. There however remains a residual safety risk as evidenced by reports of septic transfusion events involving PC which have been screened with culture methods, rapid tests or subjected to pathogen reduction, or contaminated post-pathogen reduction treatment [10, 11].

Cold storage of PC has regained attention as a means to treat actively bleeding patients, and it has been proposed that the storage of PC units at 1–6°C limits bacterial growth. Studies conducted by Ketter [6] and more recently by Brown [7] have confirmed that cold storage of PC restricts growth of mesophilic bacteria, and as shown in our study with *K. pneumoniae*, it can even have a bactericidal effect. However, we have also demonstrated that bacterial strains able to proliferate in RBCC [9], have the ability to grow in CSP. Slow growing

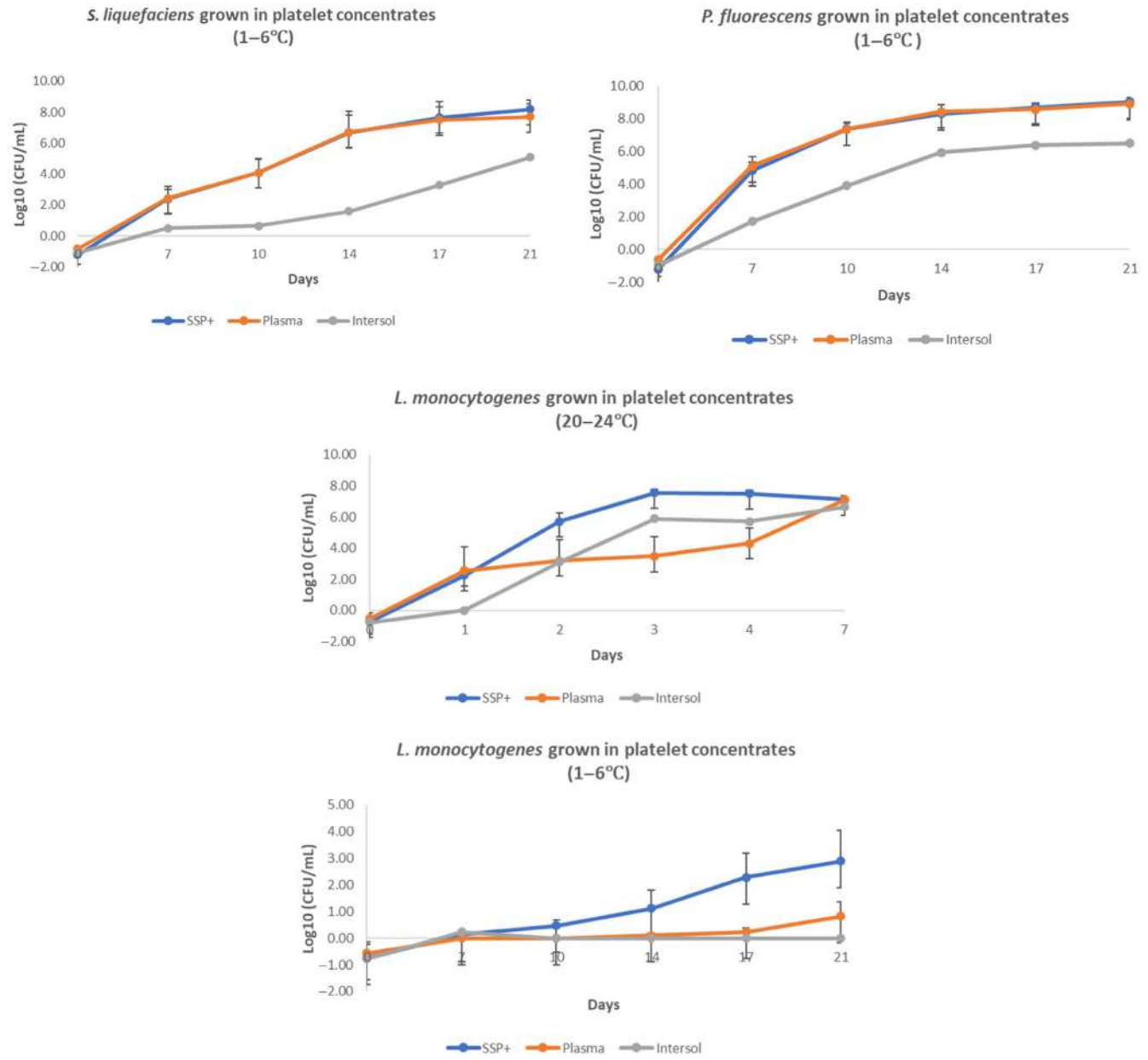

FIGURE 3 (Continued)

species such as *L. monocytogenes* pose a minimal safety risk since clinically relevant bacterial loads (i.e., 10^5 CFU/mL) [12] are not reached within 21 days of cold storage. However, other species which display fast growth in CSP, such as *S. liquefaciens* and *P. fluorescens*, can pose a safety risk to transfusion patients in units stored for more than 10 days, though these species are relatively uncommon PC contaminants.

Notably, variation in proliferation of psychrotrophic strains in CSP was observed within sites, with some reporting loss of viability. Interestingly, the type of PAS influenced bacterial proliferation. Our data showed that while Intersol delayed proliferation of *K. pneumoniae*, *S. liquefaciens* and *P. fluorescens*, SSP+ promoted growth of *L. monocytogenes* in CSP. The differential growth observed in PC suspended in the two types of PAS was unexpected as previous studies have demonstrated faster bacterial growth in PC suspended in both, Intersol and SSP+ [13, 14]. Furthermore, Kou et al. [15] have recently shown comparable bacterial proliferation in apheresis PC suspended in plasma and SSP+ PAS. It is therefore plausible that the slow growth observed in our studies in PC suspended in Intersol is species- and/or strain-dependent and should be verified in further studies performed at different sites to allow for statistical comparisons.

It would be interesting to complement the data obtained in this multinational study with a delayed cold storage approach. Studies have been conducted to maximize PC availability by delayed cold storage of PC with the aim of avoiding dual inventories. Wood et al. conducted a pool and split study with two arms of buffy coat PC; one group of PC was stored at 2–6°C immediately after pool PC preparation while the second group was stored at RT for 4 days and then

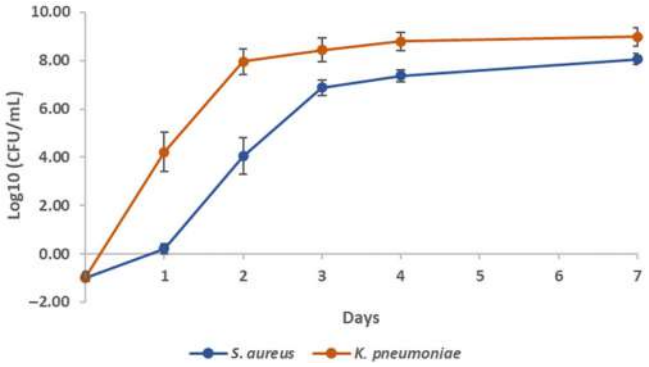

FIGURE 4 Graph shows bacterial growth in platelet concentrates inoculated with BIOBALLS over 7 days of storage under standard conditions (room temperature: 20–24°C/agitation). Aggregated data from technical triplicates performed at each of five participant sites (Table 1) are shown for each bacterial species ± standard error.

moved to refrigeration for a total cold storage of 21 days. The authors concluded that the metabolic and platelet activation profiles between the two groups of PC were similar [16]. Braathen et al. conducted a study comparing cold storage and delayed cold storage on day 7 of apheresis PC prepared in PAS-IIIM (T-PAS+, Terumo BCT) for up to 21 days [17]. In this study, the authors reported poorer in vitro quality of delayed CSP compared with regular cold storage, likely due to faster platelet metabolism during PC storage at RT during the first 7 days. It is unknown if any differences in metabolic changes observed in delayed CSP will influence bacterial proliferation. Bacteria could either continue growing once PC are moved to refrigeration, remain at a constant load or lose viability, which is a subject of current investigation.

Our study was comprehensive as it included PC components prepared in plasma or PAS with different approaches at eight sites located on different continents. It is acknowledged that only one strain of each species was tested, and therefore, other species and strains could complement our results. We however have provided novel data regarding transfusion relevant psychrotrophic bacteria, which, though relatively uncommon blood contaminants, are able to proliferate in CSP just as in RBCC and could pose a safety risk to transfusion patients if clinically significant loads are reached. The safety risk posed to patients could be limited by restricting the storage of cold PC as much as operationally feasible. Alternatively, CSP which have remained in storage for longer than 10 days could be screened with culture or rapid methods. Safety of CSP could also be improved if they are treated with pathogen reduction technologies prior to storage. Overall, our results demonstrate that cold storage of PC results in bacterial safety comparable with RBCC, which is a significant improvement over RT storage.

ACKNOWLEDGEMENTS

The authors thank volunteer blood donors and Production staff at each participant facility. International Society of Blood Transfusion provided funding for this study.

S.R.-A. and C.M. conceived and coordinated the study. D.K. performed experimental work at Canadian Blood Services and collected and analysed data from all participant sites. A.C., K.M.C., M.C., J.F., U.G., P.K., P.L., T.L., T.N., J.P., C.R., A.S., B.S., S.S., T.V. and S.Z. conducted and/or coordinated experimental work and data collection in their respective sites. S.R.-A. wrote the manuscript, which was reviewed by all co-authors.

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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How to cite this article: Ramirez-Arcos S, Kumaran D, Cap A, Cardenas KM, Cloutier M, Ferdin J, et al. Proliferation of psychrotrophic bacteria in cold-stored platelet concentrates. Vox Sang. 2024;119:693–701.

Received: 17 January 2024 | Revised: 31 March 2024 | Accepted: 7 April 2024

DOI: 10.1111/vox.13642

ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Characterization of a novel mouse platelet transfusion model

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Funding information
Columbia University Department of Pathology and Cell Biology; Herbert Irving Comprehensive Cancer Center at Columbia University; NIH/NCI Cancer Center, Grant/Award Number: P30CA013696

Abstract

Background and Objectives: Platelet transfusions are increasing with medical advances. Based on FDA criteria, platelet units are assessed by in vitro measures; however, it is not known how platelet processing and storage duration affect function in vivo. Our study's aim was to develop a novel platelet transfusion model stored in mouse plasma that meets FDA criteria adapted to mice, and transfused fresh and stored platelets are detectable in clots in vivo.

Study Design and Methods: Platelet units stored in mouse plasma were prepared using a modified platelet-rich plasma (PRP) collection protocol. Characteristics of fresh and stored units, including pH, cell count, in vitro measures of activity, including activation and aggregation, and post-transfusion recovery (PTR), were determined. Lastly, a tail transection assay was conducted using mice transfused with fresh or stored units, and transfused platelets were identified by confocal imaging.

Results: Platelet units had acceptable platelet and white cell counts and were negative for bacterial contamination. Fresh and 1-day stored units had acceptable pH; the platelets were activatable by thrombin and adenosine diphosphate, agreeable with thrombin, had acceptable PTR, and were present in vivo in clots of recipients after tail transection. In contrast, 2-day stored units had clinically unacceptable quality.

Conclusion: We developed mouse platelets for transfusion analogous to human platelet units using a modified PRP collection protocol with maximum storage of 1 day for an 'old' unit. This provides a powerful tool to test how process modifications and storage conditions affect transfused platelet function in vivo.

Keywords

mouse model, platelet, platelet activation, platelet aggregation, platelet storage, platelet transfusion

Highlights

- Here, we describe a novel mouse platelet model stored in mouse plasma in platelet transfusion bags.
- Our mouse platelet model demonstrates acceptable in vitro measures of quality and excellent post-transfusion recovery, and can participate in clotting in vivo when stored for 24 h.
- We anticipate that our mouse platelet model will have broad applications for studying platelet transfusion.

INTRODUCTION

Platelets are anuclear discoid cell fragments derived from megakaryocytes, are required for haemostasis, and play crucial roles in inflammation [1–4]. Platelet transfusions are provided for bleeding prophylaxis in profoundly thrombocytopenic patients and promote clot stabilization in bleeding patients. Approximately 2.5 million platelet units were distributed in 2021, representing a 0.8% increase over 2019 [5, 6]. Notably, 2021 was marked by severe platelet shortages due to the COVID-19 pandemic; advances in chemotherapy, cardiac surgery techniques and critical care will also likely increase demand for platelet transfusions. There are several FDA-approved platelet preparation and storage methods, along with processing modifications, such as irradiation and pathogen reduction technologies [7]; these may affect platelet function in vivo. Given platelet functional plasticity and multiple clinical contexts entailing their use, elucidating how these parameters modulate platelet function is an unmet clinical need.

FDA regulations define human platelet unit parameters as: pH ≥ 6.2 at the end of storage, platelet number ≥3.0×10^{11} (apheresis) or 5.5×10^{10} (whole blood [WB]), residual white blood cell (WBC) number ≤5.0×10^{6}, and 85% of platelets retained after leukoreduction [8]. Because most platelet units in the United States are stored in plasma at room temperature, they also need to be free of bacterial contamination. Although the gold standard for assessing platelet viability is to measure post-transfusion recovery (PTR) after autologous transfusion of radiolabeled platelet units [9], detecting circulating platelets after transfusion does not measure their functional capacity. Nonetheless, platelet activity in vitro can be assessed by functional assays (e.g., light transmission aggregometry, platelet function analyser studies, thromboelastography) or by measuring platelet activation using flow cytometry. Indeed, several studies characterizing the function in vitro of cold-stored platelets in WB [10, 11] and of freeze-dried platelets [12] were published recently; furthermore, an elegant study also demonstrated increased thrombin generation in human autologous transfusions of cold-stored versus room temperature platelets [13]. Additionally, using these approaches, numerous in vitro studies suggest that platelet function deteriorates during storage [14–16].

Although multiple studies examined various aspects of platelet function and transfusion using mouse models and multiple groups collect blood in citrate [17–19], in most of these studies, platelet concentrates were stored in a modified Tyrode's solution, with or without other additives (e.g., albumin, apyrase, prostacyclin), to prevent platelet activation during storage [20–25]. However, in the United States, platelet concentrates prepared from WB may not be stored in platelet additive solutions, and most apheresis platelet units are also not stored in platelet additive solution [6, 26]. As such, to develop a tractable model to study how platelet processing methods and storage affect platelet function, we developed a reproducible mouse model for transfusing platelets stored in plasma, which satisfies the (adjusted) human FDA platelet transfusion criteria. Our model, which uses a modified pooled platelet-rich plasma (PRP) collection technique, provides platelet units with acceptable pH, WBC counts per unit and PTR. We also developed a novel flow cytometry approach to assess platelet aggregation in vitro and showed, using confocal microscopy, that these transfused platelets participate in clotting in vivo.

MATERIALS AND METHODS

Mice

CD-1 (CD-1; strain no. 022) outbred mice were purchased from Charles River Laboratories. C57BL/6J-TG(UBC-GFP)30Sach/J (green fluorescent protein [GFP] mice; strain no. 004353) and B6.Cg-Tg (CAG-DsRed*MST)1Nagy/J (red fluorescent protein [RFP] mice; strain no. 006051) mice were purchased from The Jackson Laboratory. All mice were maintained on a 12:12 light/dark cycle and a standard chow diet (ad libitum). All mouse experiments were approved by Columbia University's Institutional Animal Care and Use Committee (IACUC).

Platelet unit preparation

The protocol was modified from the PRP collection protocols used for human platelets [26]. *Fresh frozen plasma (FFP) preparation*: To prepare mouse FFP, WB was collected aseptically from GFP mice via cardiac puncture into 15-mL conical tubes with 12.3% CPDA-1 and stored undisturbed at room temperature for 30 min. The WB was then centrifuged at 3000×*g* for 30 min at 20°C; all centrifugation steps used the Thermo Scientific Legend XTR centrifuge with an acceleration of 9 and deceleration of 7. The plasma layer was carefully collected, leaving ~1 mL plasma above the buffy coat (BC) layer so as not to disturb the BC. Plasma was frozen at −20°C in 1 mL aliquots in sterile microcentrifuge tubes. Immediately before use, FFP aliquots were thawed at 37°C and passed through a 0.2-μm filter (Pall, 4612). *Platelet unit preparation*: On the day of blood collection, WB was collected aseptically from GFP mice via cardiac puncture in 12.3% CPDA-1 [27, 28], and the WB sat undisturbed for 30 min at room temperature. GFP WB was diluted with sterile phosphate buffered saline (PBS) at a ratio of 1:2 of WB:PBS, separated into ~4–6 mL aliquots in 15-mL conical tubes, and then centrifuged at 120×*g* for 10 min at 20°C. The PRP and BC with some contaminating red blood cells (RBCs) were placed into new 15-mL conical tubes (hereafter referred to as PRP + BC). PRP + BC was centrifuged at 80×*g* for 20 min at 20°C, and the resulting PRP was collected carefully without disturbing the BC and RBC pellet. PRP platelets were quantified by flow cytometry (see below), pelleted at 1942×*g* for 10 min at 20°C and then re-suspended in FFP to a final concentration of ~$2.5–3.0 \times 10^{5}$ platelets/μL. Platelet units were stored in mini platelet aliquot bags (BCSI, 6044) with shaking at room temperature.

Platelet unit analysis and flow cytometry

Platelet units were analysed for cellular composition (by flow cytometry) and pH and assessed for bacterial contamination. *Flow cytometry*: Platelet units were stained with antibodies against CD41a (1:100, eBioMWReg30, PE-Cy7, ThermoFisher), Ter119 (1:1000, APC-efluor 780, ThermoFisher) and CD45 (1:400, 30-F11, Brilliant Violet 605, Fisher) to detect platelets, RBCs and WBCs, respectively. Data were collected with an Attune NxT flow cytometer with the No-Wash No-Lyse Filter kit (ThermoFisher Scientific; United States) and analysed with FlowJo software. WBCs were quantified using the Leucocount Kit (BD, 340523) and analysed with FlowJo software. *Platelet unit pH* was measured daily by adding 25 µL of the platelet unit to pH strips with a pH range of 5–9 (Fisher, 13-640-519). *Bacterial contamination assessment*: Platelet units were cultured at the time of unit preparation, and after 24 and 48 h of storage, by inoculating paediatric blood culture bottles (BD BACTEC Peds Plus/F, 442020) and incubating them for 1–5 days at 37°C. The culture medium was sampled by adding 1–3 drops to microscope slides followed by Gram stain (BD BBL™ Gram Stain Kits; B12539) and imaging.

Platelet activation and aggregation

Platelet activation was assessed by incubating 1 µL of the platelet unit with 0.5 U/mL thrombin (Sigma-Aldrich, 604980) diluted in fluorescence-activated cell sorting (FACS) buffer (PBS + 0.2 mg/mL bovine serum albumin + 0.9 mg/mL ethylenediaminetetraacetic acid) [29] at room

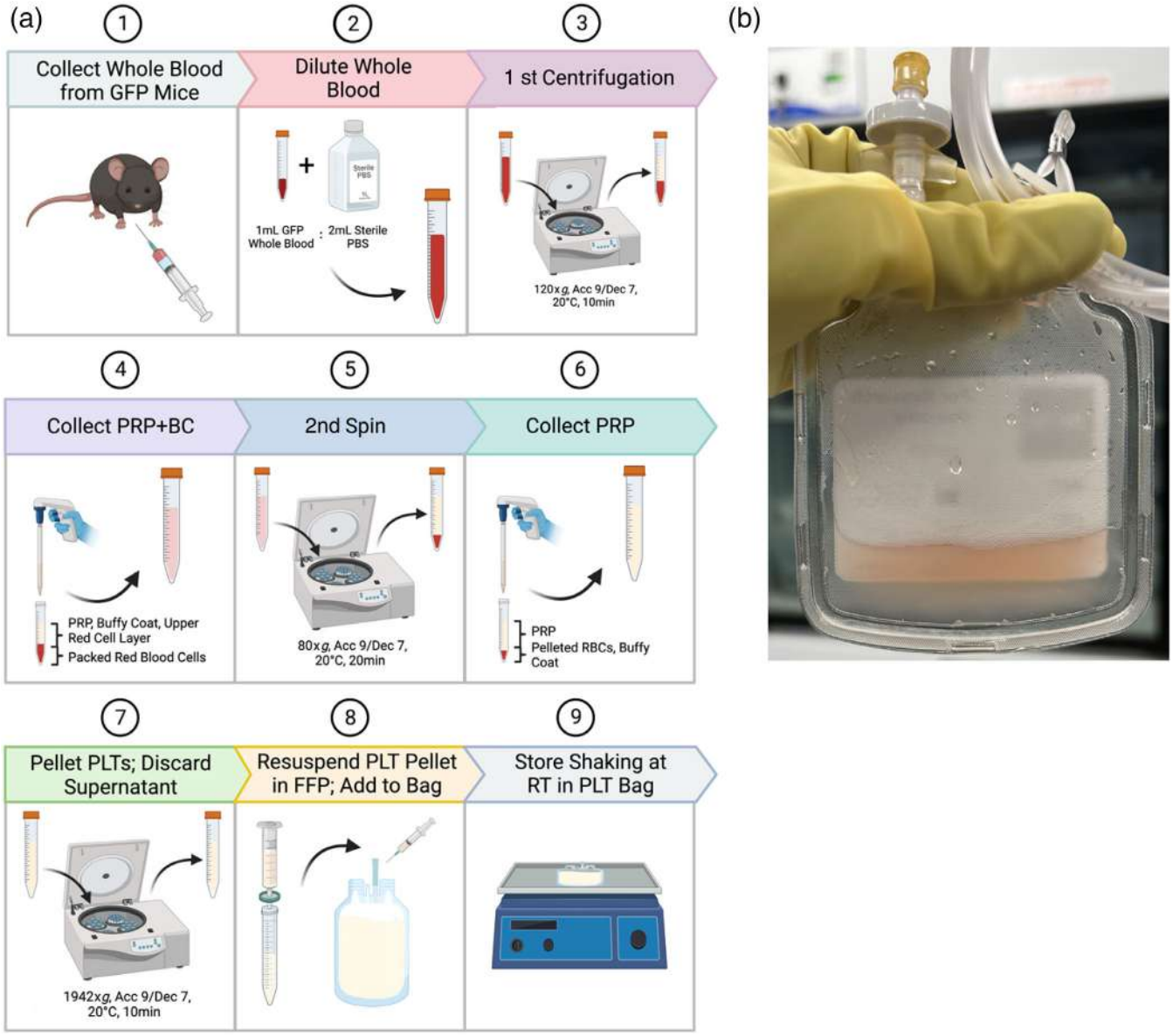

FIGURE 1 Mouse platelet unit method. (a) Sequential steps to make the mouse platelet unit (Created with BioRender.com). (b) Platelet unit in mini platelet aliquot bag (BCSI, 6044); unit volume is ~6 mL. FFP, fresh frozen plasma; GFP, green fluorescent protein; PLT, platelets; PRP, platelet-rich plasma; RT, room temperature.

temperature for 2 min or with 10 mM adenosine diphosphate (ADP) (Fisher, 22-515-225) at 37°C for 5 min. Platelets were washed with 1-mL FACS buffer, and then stained with t antibodies against CD41a, Ter119, CD45 and CD62P (P-selectin; 1:100, Psel.KO2.3, APC, ThermoFisher). Ter119+ RBCs and CD45+ WBCs were excluded and CD62P expression quantified on CD41a + platelets. *Platelet aggregation* was measured by incubating 1 µL of the transfusate (as prepared for platelet PTR; see below) in a total volume of 200 µL with 0.5 U/mL thrombin diluted in FACS buffer at room temperature for 2 min or with 10 µL 20 mM ADP at 37°C for 5 min. Platelets were washed and stained using the platelet activation protocol. GFP and RFP platelets were evaluated by flow cytometry; platelets doubly expressing GFP and RFP were considered to be aggregated.

Post-transfusion recovery

To determine PTR, a fresh WB unit was prepared from RFP mice. Briefly, WB was collected from 1 RFP mouse by cardiac puncture into CPDA-1 (as above). RFP WB sat for 30 min and then was mixed in a 2:1 ratio with a GFP+ platelet unit (fresh, 1- or 2-day stored) to create the transfusate; of note, RBCs in RFP WB do not express RFP (data not shown). Anaesthetized CD-1 recipient mice received 150 µL of the transfusate by retro-orbital transfusion. WB was then collected from transfusion recipients by tail prick at 5 min, 1, 24, 48 and 72 h post-transfusion. The ratio of circulating GFP-positive platelets to RFP-positive platelets, relative to this ratio in the transfusate, measured using flow cytometry, was used to determine PTR at each time point.

Scanning confocal microscopy

Transfusates were prepared by mixing GFP platelet units with freshly collected RFP WB in a 3:1 ratio. Anaesthetized CD-1 mice were transfused retro-orbitally with 200 µL of fresh, 1-day stored or 2-day stored transfusate. After 1 h, a 3-mm section of the tail tip was transected and left to clot. Once clotted, animals were euthanized and 1 cm of the tail tip was transected and sliced longitudinally. Tail tip sections were mounted on microscope slides and imaged by scanning confocal microscopy (Leica TCS SP8, Leica LASX software). The number and percentage of GFP- and RFP-fluorescent platelets were counted and compared with the transfusate composition for each time point. Confocal microscopy images were analysed using ImageJ software [30].

Statistical analysis

For comparison of three or more groups, a one-way or repeated measures two-way analysis of variance (ANOVA) with Tukey's multiple comparisons post-test was utilized; $p \leq 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism.

RESULTS

Platelet unit composition and pH change during storage

Per microliter, an average 300-mL human platelet unit contains at least 1×10^6 platelets, an average of 1.7×10^3 RBCs [31] and fewer than 16.7 WBCs ($<5 \times 10^6$ WBCs/unit). Although there is no standard for mouse platelet dose for a platelet transfusion, other groups have published platelet counts ranging from 1.0×10^5 platelets/µL to 4×10^6 platelets/µL [22, 32–35]. Although the final platelet concentration depends on the total volume of FFP into which the PRP is ultimately re-suspended, to minimize the mice required for the unit and

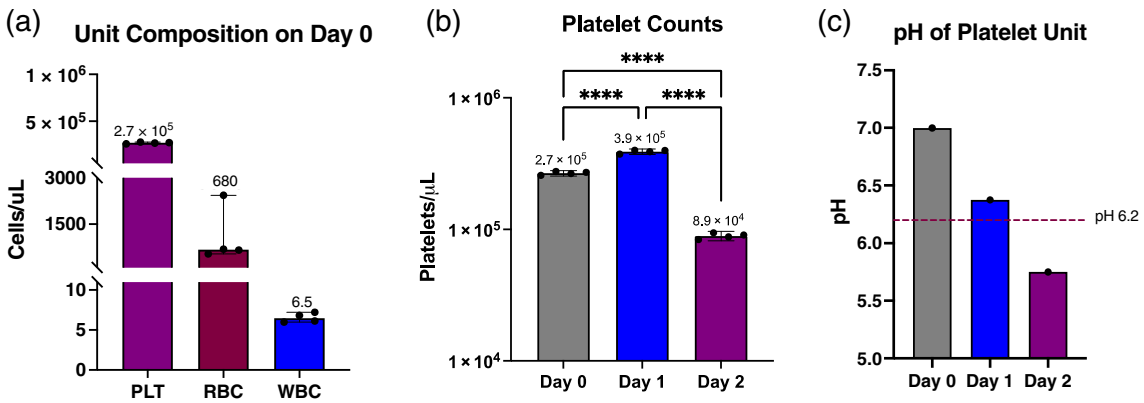

FIGURE 2 Composition and pH of platelet units change during storage. Aliquots of platelet units were stained with antibodies to detect platelets (PLT), red blood cells (RBCs) and white blood cell (WBCs). (a) Freshly manufactured platelet unit contained, on average, 2.7×10^5 platelets/µL, 680 RBCs/µL and 6.5 WBCs/µL. (b) Platelet counts of each unit were calculated throughout storage; platelet count increases to 3.9×10^5/µL after 1 day of storage and decreased to 8.9×10^4/µL after 2 days. (c) Platelet unit pH was tested with pH strips and decreased throughout storage. Dashed line indicates pH 6.2, which is the lower FDA established threshold for human platelet units. Data are representative of one of two experiments and technical replicates are shown in (a) and (b), with data representing mean ± SD. Data were analysed with a one-way ANOVA with Tukey's multiple comparisons post-test and ****$p < 0.0001$.

to allow for sufficient volume for bacterial culturing, in vitro assays and PTR experiments, we use a minimum platelet count of 1.0×10^5 platelets/μL for the final platelet counts of fresh units.

Mouse platelet units were prepared from GFP mice as described in the *Materials and Methods* section (platelet unit method, Figure 1). The unit's platelet count per microliter varied depending on the volume of mouse plasma into which the platelets were ultimately re-suspended, but trends between experimental replicates were consistent. For one experimental replicate reported here, fresh platelet units contained the following per microliter: 2.7×10^5 platelets, 680 RBCs and 6.5 WBCs (Figure 2a). Because platelets have a short half-life, we quantified platelet count as a function of storage age. Intriguingly, platelet counts increased to 3.9×10^5 platelets/μL after 1 day of storage, but significantly decreased below baseline to 8.9×10^4 platelet/μL after 2 days of storage (Figure 2b). Per FDA guidelines, human platelet units require pH ≥ 6.2 [36, 37]. Across all experiments, mouse platelet unit pH ranged from 7.0 to 7.5 on the day of preparation, 6.4–6.75 on Day 1 of storage and 5.75–6.25 on Day 2 of storage (experimental replicate shown in Figure 2c). Platelet unit 'swirl', an indirect measure of platelet discoid shape and suitable pH [38], was observed with fresh and 1-day stored platelets (Video S1); platelet swirling was significantly reduced in 2-day stored units. To ensure that platelet units were free from bacterial contamination, samples were incubated in paediatric blood culture bottles. All units tested negative for bacterial contamination during

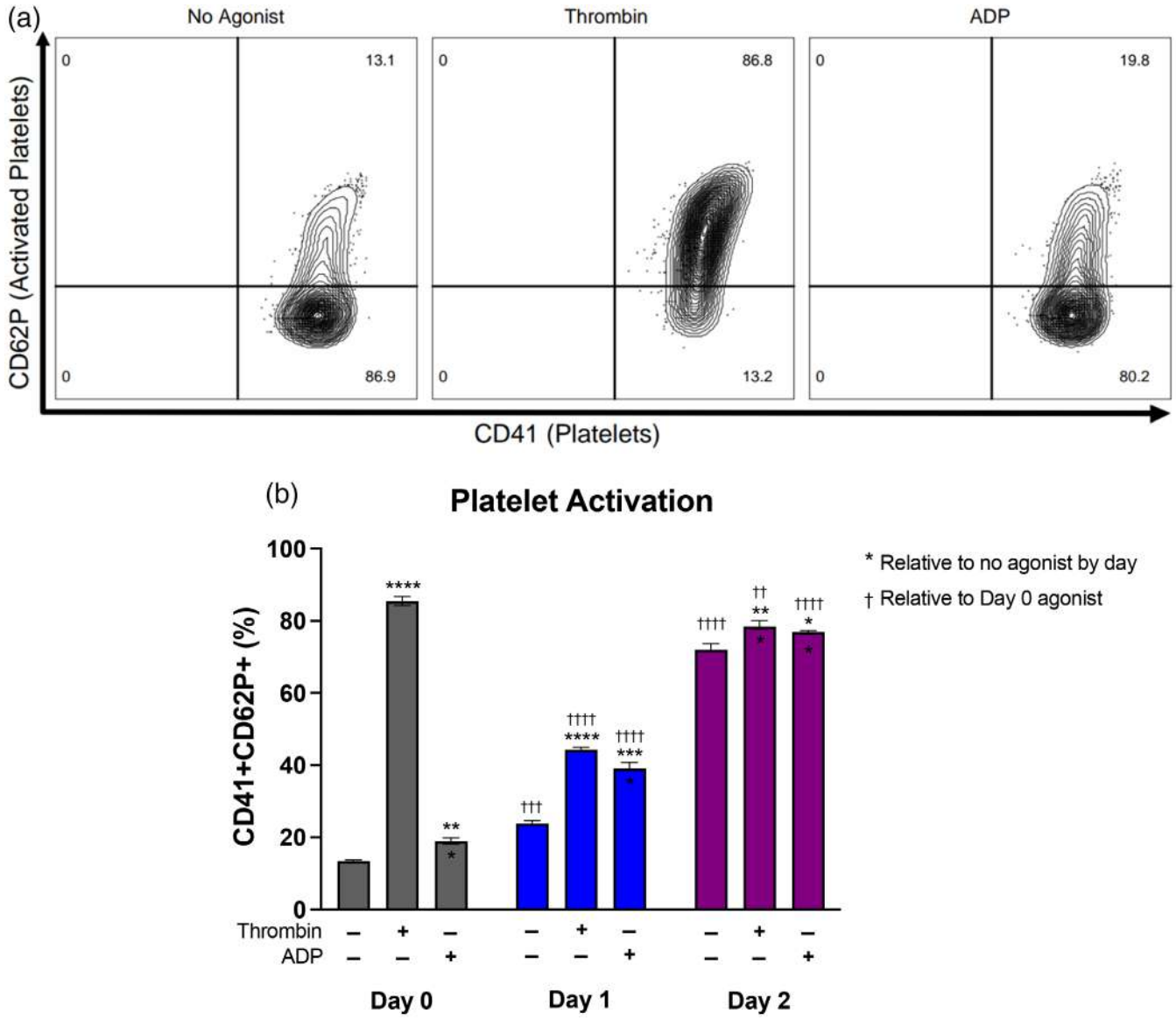

FIGURE 3 Platelet unit storage modulates CD62P expression and agonist responses. Platelet units were prepared from green fluorescent protein mice and aliquots from fresh and stored units (1 and 2 days) were stained to detect platelets (PLT), red blood cells (RBCs) and white blood cell (WBCs). Ter119+ RBCs and CD45+ WBCs were excluded from the analysis. CD41+ platelets were assessed for CD62P expression. (a) Representative flow plots and (b) frequency of CD41+ platelets in response to no agonist, thrombin (0.5 U/mL) and adenosine diphosphate (ADP) (10 mM). Data are representative of one of two experiments and data in (b) are mean ± SD. Data were analysed by two-way ANOVA with Tukey's multiple comparisons post-test; "*" compares baseline to an agonist by day, whereas "†" compares the agonist condition from Day 1 or Day 2 to Day 0; ** or †† is $p < 0.01$, *** or ††† is $p < 0.001$, **** or †††† is $p < 0.0001$.

storage (data not shown). These results suggest that this method of preparing mouse platelet units falls in line with modified FDA criteria for human platelets. These mouse platelet units contain sufficient numbers of platelets, are lower than the established thresholds for RBCs and WBCs, and maintain a neutral pH for 1 day of storage. As observed with human platelet units, prolonged storage leads to more acidic pH and reduced viability. Based on these observations, mouse platelet units prepared on the day of collection are considered 'fresh' and after 1 day of storage are considered 'old'.

Storage of platelet units modulates CD62P expression and agonist response

As platelets are often transfused to improve haemostasis and restore clotting capacity, it is essential to test whether platelets become activated upon agonist exposure. To test whether these platelet units could be activated throughout storage, the percentage of platelets expressing CD62P (P-selectin) was quantified at baseline and after incubation with platelet agonists (i.e., thrombin and ADP; Figure 3). Freshly prepared platelet units had low CD62P+ platelet frequencies (mean 13.4%, Figure 3b), which significantly increased throughout storage, demonstrating that platelets were becoming activated during storage. Fresh platelets responded to thrombin and ADP, as measured by significant increases in CD62P+ platelet frequencies (6.3 and 1.4-fold over fresh baseline, respectively). Platelet units stored for 1 day retained functional responses to agonists, as CD62P+ platelet frequencies increased in response to thrombin and ADP (1.85- and 1.6-fold over 1-day baseline, respectively), but responses were less dramatic than seen with freshly prepared platelets. Lastly, 2-day stored platelets had diminished functional responses to thrombin and ADP (1.08- and 1.06-fold over 2-day stored baseline, respectively).

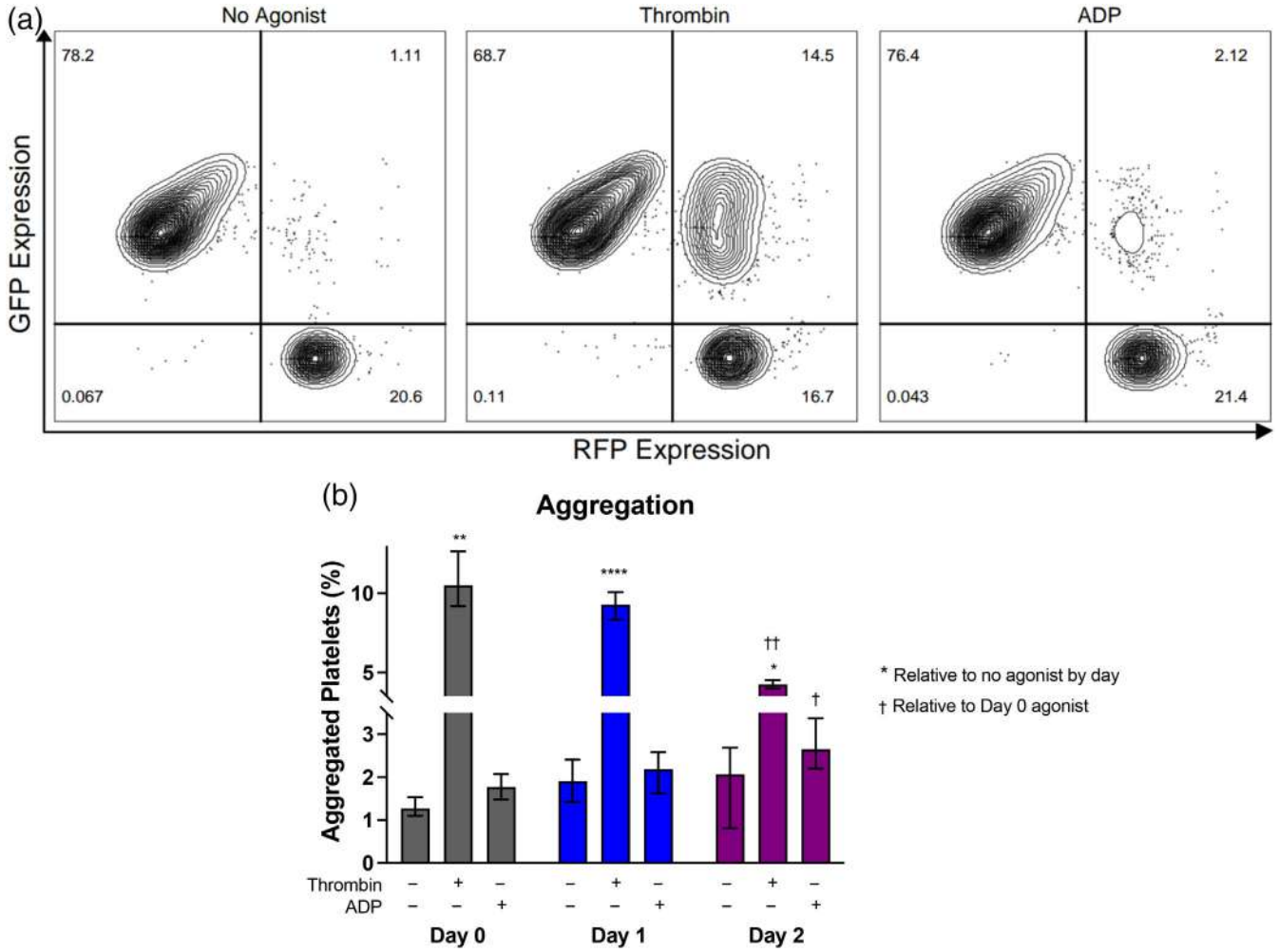

FIGURE 4 Platelet aggregation diminishes with prolonged storage. Green fluorescent protein (GFP) platelet units after different times in storage were mixed with fresh red fluorescent protein (RFP) whole blood (WB), incubated in FACS buffer with thrombin (0.5 U/mL) or adenosine diphosphate (ADP) (10 mM), and then analysed by flow cytometry for the percentage of GFP+RFP+ (i.e., double positive) platelet 'clumps', signifying platelet aggregation. (a) Representative flow plots of fresh GFP platelet units and fresh RFP WB exposed to vehicle, thrombin and ADP. (b) Percentage of GFP+RFP+ platelet aggregates formed from GFP units (fresh, 1-day or 2-day stored) mixed with fresh RFP WB and unstimulated or activated with thrombin or ADP. Data shown are representative of one of two experiments and data represent mean ± SD. Data were analysed with by two-way ANOVA with Tukey's multiple comparisons post-test and "*" compares baseline to an agonist by day, whereas "†" compares the agonist condition from Day 0 to Day 1 or Day 2 (* or † is $p < 0.05$; ** or †† is $p < 0.01$; **** is $p < 0.0001$).

Taken together, these data demonstrate that otherwise unmanipulated platelet units become activated throughout the storage duration and lose functional capacity to respond to agonists.

Platelet aggregation diminishes with prolonged storage

Another measure of platelet function is the ability to aggregate and form clots. As conventional aggregometry assays require large volumes of blood (i.e., 800 μL), we adapted a recently reported assay [39], to develop a new method using flow cytometry that requires smaller volumes. To that end, we avoided ex vivo platelet labelling using platelets collected from two strains of transgenic mice with platelets expressing either GFP or RFP, thereby enabling visualization of platelet aggregation in vitro by flow cytometry. As such, individual platelets express GFP or RFP and platelet aggregates are positive for both GFP and RFP (Figure 4a, representative flow plots).

To test how our platelet preparation affected aggregation, GFP platelet units (fresh or stored) were mixed with fresh WB from RFP animals (which have RFP platelets) followed by agonist exposure. Fresh GFP platelets mixed with RFP WB had few detectable aggregates (Figure 4a, left and Figure 4b), suggesting that these platelets are not activated. To induce activation and promote aggregation, fresh platelets were treated with thrombin (Figure 4a, middle) or ADP (Figure 4a, right); thrombin-treated, but not ADP-treated, fresh platelets displayed significant increases in GFP+RFP+ frequencies, as compared with the 'no agonist' control (Figure 4b). Similar to fresh GFP units, thrombin treatment, but not ADP, led to significant increases in aggregation of 1-day stored GFP units, as compared with the 'no agonist' control. A similar trend was observed with 2-day stored GFP platelet units, although aggregation frequencies were diminished. Taken together, these data show low levels of platelet aggregation in otherwise unmanipulated platelet units throughout a 2-day storage period. Additionally, thrombin exposure consistently induced platelet aggregation, but its effect diminished throughout storage. Lastly, platelet exposure to ADP did not promote significant aggregation. In sum, these data, along with those in Figure 3, suggest that this platelet processing procedure and storage conditions yield a functional platelet product by in vitro metrics.

PTR of fresh and stored platelet units

To test whether platelets from these units circulated in vivo, GFP platelets (fresh or stored) were mixed with RFP WB at a ratio of 2:1 and transfused into wild-type mice (Figure 5a). Peripheral blood was collected at defined time points and the GFP:RFP ratio was normalized to the percentage of GFP+ platelets in the transfusate to determine PTR. Fresh and 1-day stored platelets had similar PTRs over the first 24 h post-transfusion; diversion of PTRs was evident at 72 h post-transfusion but both remained above 60% (Figure 5b). In contrast, 2-day stored platelets had a PTR under 60% at the first time point (i.e., 5 min). Taken together, these data demonstrate that fresh and 1-day stored platelet units had excellent PTR, even at 72 h post-transfusion, but 2-day stored platelets do not circulate well post-transfusion.

Transfused GFP platelets from fresh and 1-day stored units participate in clotting

To determine whether transfused platelets contribute to clot formation in vivo, recipient mice were transfused with a 3:1 ratio of GFP

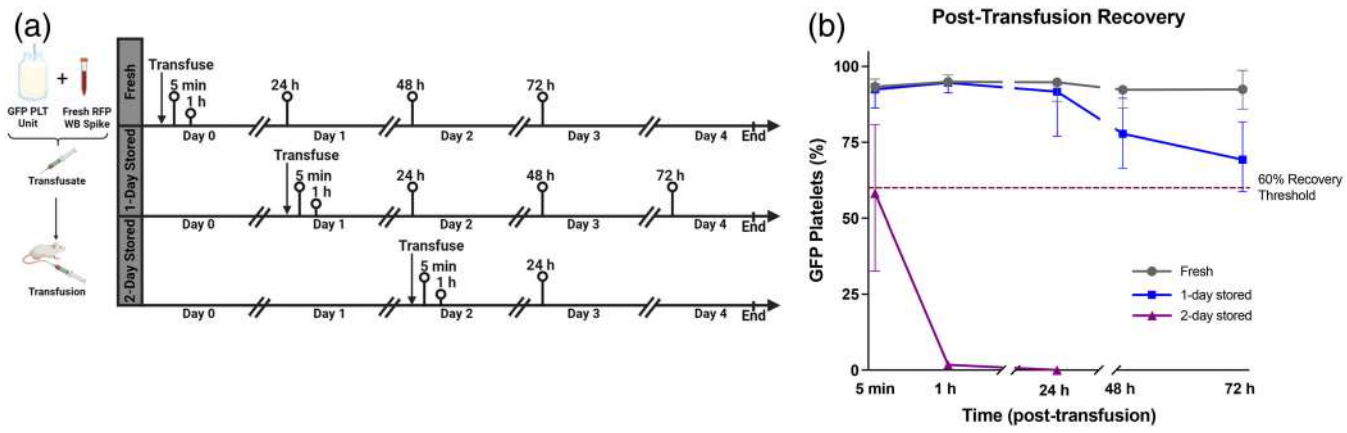

FIGURE 5 Post-transfusion recovery (PTR) of fresh and stored platelet units. (a) Schematic of PTR protocol. Green fluorescent protein (GFP) platelet units stored for different durations (fresh, 1-day stored, 2-day stored) were mixed 2:1 with fresh red fluorescent protein whole blood and 150 μL were transfused retro-orbitally into recipient CD-1 mice ($n = 5$ per group). Post-transfusion recovery was quantified at different times post-transfusion by flow cytometry. Created with BioRender.com. (b) Graph of PTR for fresh, 1-day stored and 2-day stored GFP platelet units. The percentages of GFP platelets were normalized to the composition of the transfusate from each day transfused. Fresh and 1-day stored platelet units had a PTR of >60% even 72 h after transfusion, whereas 2-day stored units had very poor PTR, with only 2% GFP platelets circulating at 1 h, and no GFP platelets circulating by 24 h.

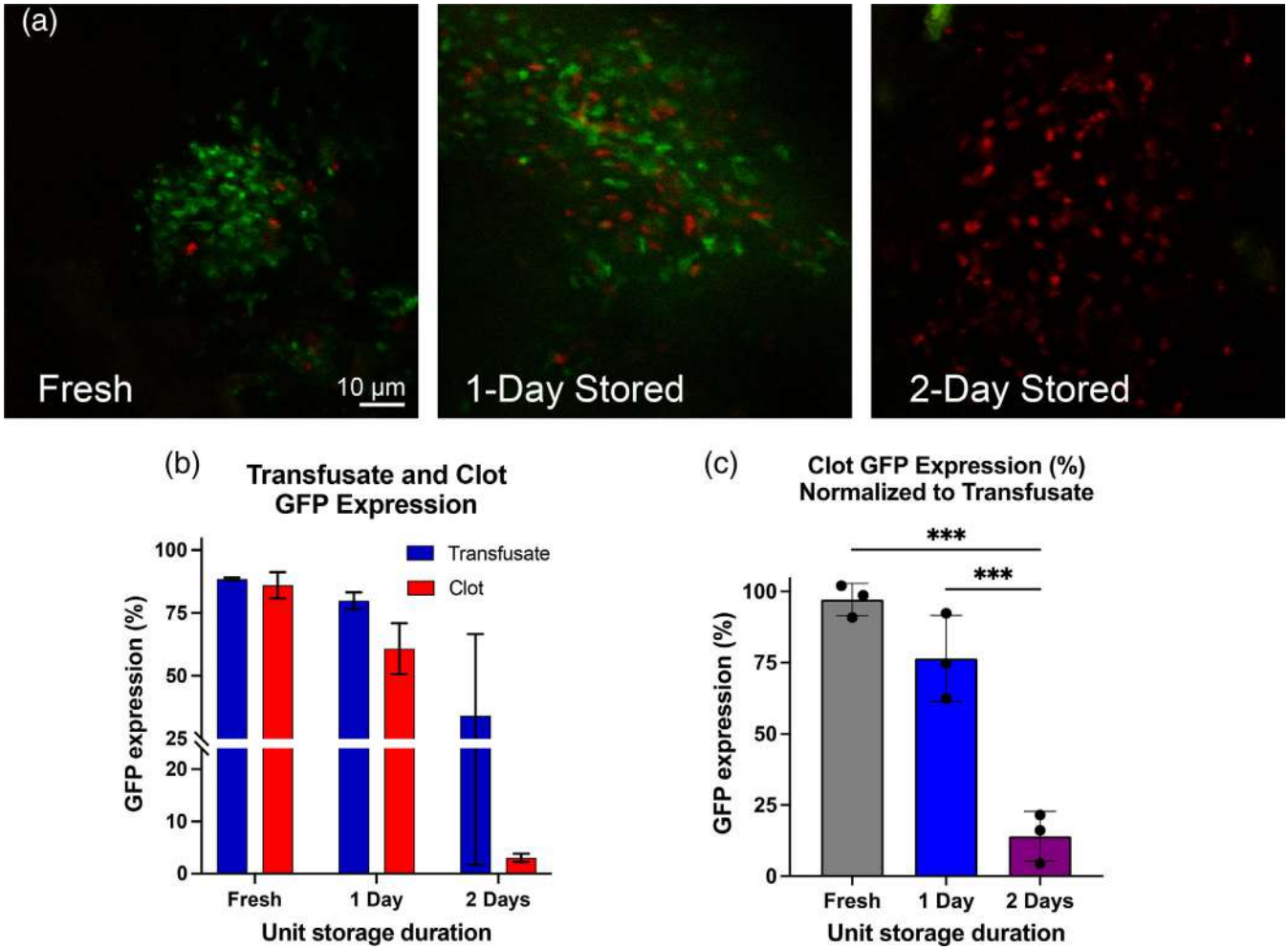

FIGURE 6 Transfused green fluorescent protein (GFP) platelets are identified in clots using fresh and 1-day stored platelet units but not 2-day stored platelet units. Recipient mice were transfused with a 3:1 transfusate of fresh or stored GFP units mixed with fresh red fluorescent protein (RFP) whole blood, followed by tail transection and mounting of tail tips for multiphoton microscopy. (a) GFP and RFP platelet contributions to the clots from fresh, 1-day stored and 2-day stored platelet units. Bar, 10 µm. (b) Graph comparing levels of GFP-expressing platelets as a percentage of total fluorescence (GFP + RFP) in the transfusate (blue bars) and clot (red bars); the percentage of GFP in the transfusate compared with clots for each day of storage was not significant. (c) When normalized to the percentage of GFP-expressing platelets in the transfusate by day of storage, the percent of GFP expression in the clot was significantly reduced for platelet units stored for 2 days (***$p < 0.001$). Data shown are cumulative from three independent experiments, with $n = 2$ recipients per group. Data were analysed with a one-way ANOVA with Tukey's multiple comparisons post-test and ***$p < 0.001$.

platelet units (fresh or stored) and fresh RFP WB, which served as a loading control. Post-transfusion, a 3-mm section of the tail tip was transected, and tails permitted to clot. Following euthanasia, tail tips were imaged by scanning confocal microscopy (Figure 6a). The number of GFP and RFP platelets in each clot from each recipient was counted and the frequency of GFP platelets in the clot calculated; this frequency was compared with the GFP frequency in the transfusate (Figure 6b). Of note, significantly fewer GFP platelets from 2-day stored units were detected in clots, as compared with fresh and 1-day stored platelets. However, to assess whether a disproportionate frequency of GFP platelets was actually detected in clots, as compared with the transfusate, GFP frequencies were normalized (Figure 6c); GFP platelets from fresh and 1-day stored units were detected in clots at similar frequencies, but 2-day stored platelets were significantly reduced. Taken together, these data show that transfused fresh and 1-day stored GFP platelet units participate in clotting in this tail transection model, whereas transfused 2-day stored GFP platelets are not found in these clots.

DISCUSSION

We present a model for mouse platelet transfusion involving storing mouse platelet concentrates in plasma with sufficient platelet counts and acceptably low numbers of RBCs and WBCs. Fresh and 1-day stored platelets in these units activate in vitro, aggregate in vitro and in vivo, exhibit excellent PTR at 72 h, and participate in clotting. Additionally, this mouse model is highly reproducible between experimental replicates.

There are several similarities and differences between this mouse model and human platelet units. In our hands, we found that diluting WB in PBS before centrifugation allows for better separation of PRP, which was previously published [25, 33]. We then pellet and resuspend the mouse platelet units in FFP, instead of fresh donor plasma, to reduce the time required for platelet unit manufacturing. Surprisingly, performing the serial centrifugation steps without leukoreduction resulted in fewer WBCs per microliter in the final product, as compared with leukoreduction using a neonatal leukoreduction filter (data not shown). Although this deviates from the protocol for manufacturing human PRP, this centrifugation without leukoreduction led to less manipulation of the murine unit, which others have also used for PRP preparations [40]. Additionally, the platelet counts in the mouse units increased between fresh and 1 day of storage; this is also seen with human platelet units manufactured from WB, presumably either because small platelet clumps disaggregate during storage with agitation [41] or because these stored platelets are still dividing, as was observed by others [42–44]. Lastly, in the mouse model, platelet units that have been stored for 2 days have low pH, and the resulting platelets do not circulate or participate in clot formation. Thus, in this model, when mouse platelet units are stored at room temperature with agitation, the limit of storage is 1 day. We recognize that a storage limit of 1 day is short, as compared with the 5- to 7-day storage duration of human platelets. However, mouse platelets have a circulatory lifespan of 5 days, as compared with 10 days for human platelets [45]. Of note, although human RBCs can be stored for 42 days, the analogous mouse model equivalent is 6–14 days for an 'old RBC unit' [46–48]; thus, perhaps it is not surprising that the platelet storage lesion is also accelerated for murine platelets, with only 1 day of storage corresponding to the 'end of storage'.

In summary, this mouse model for manufacturing platelet concentrates stored in plasma takes advantage of GFP and RFP transgenic lines. Using this model, the effects of various storage modifications, including irradiation, pathogen reduction technologies, platelet additive solutions and cold storage, can be studied using relevant in vivo and in vitro outcome measures. This model will also provide a powerful tool to interrogate important clinical scenarios, including platelet transfusion efficacy during intracerebral haemorrhage and mechanisms influencing platelet alloimmunization.

ACKNOWLEDGEMENTS

The authors acknowledge that these studies used the Confocal and Specialized Microscopy Shared Resource of the Herbert Irving Comprehensive Cancer Center at Columbia University, funded in part through the NIH/NCI Cancer Center Support Grant P30CA013696. The authors appreciate Dr. Steven Spitalnik for his suggestions, support and critical review of our manuscript. This work was supported by Departmental funding from the Department of Pathology and Cell Biology, Columbia University Irving Medical Center, New York, NY, USA.

T.A.T., K.E.H. and E.F.S. designed the studies and the experiments. D.G. collected and analysed data from mouse experiments. T.S. assisted with microscopy and G.J.B. assisted with microbiology. All the authors were involved in the interpretation of data. K.E.H. and E.F.S. wrote the manuscript. All the authors contributed to the manuscript and approved the submitted version.

CONFLICT OF INTEREST STATEMENT

Although not related to this manuscript, Krystalyn E. Hudson has a sponsored research agreement with Alpine Immune Sciences. All other authors have no conflicts of interest to disclose.

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.

How to cite this article: Gordy D, Swayne T, Berry GJ, Thomas TA, Hudson KE, Stone EF. Characterization of a novel mouse platelet transfusion model. Vox Sang. 2024;119:702–11.

Received: 17 January 2024 | Revised: 15 March 2024 | Accepted: 24 March 2024

DOI: 10.1111/vox.13631

ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Analysis of maternal Fc gamma receptor IIIb isoantibodies using immunomagnetic negative selected neutrophils

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Funding information
Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

Abstract

Background and Objectives: The isolation of neutrophils and subsequent detection of anti-human neutrophil antigens (HNA) antibodies are crucial in clinical medicine for the diagnosis of autoimmune neutropenia, neonatal alloimmune neutropenia (NAIN) and transfusion-related acute lung injury (TRALI). This study reports two cases of maternal anti-Fc-gamma-receptor-IIIb (FcγRIIIb) isoimmunization without NAIN symptoms and compares the efficiency of immunomagnetic negative selection (IMNS) with traditional dextran/Ficoll for neutrophil isolation in HNA serological assays.

Materials and Methods: Investigating two cases of maternal anti-FcγRIIIb isoimmunization, neutrophils from three donors were isolated from 8 mL of whole blood using IMNS and dextran/Ficoll. Serological assays included the granulocyte agglutination and immunofluorescence test, monoclonal antibody immobilization of granulocyte antigens and the LABScreen Multi (One Lambda). IMNS and dextran/Ficoll were compared in terms of cell yield, viability, time, cost and purity.

Results: Maternal anti-FcγRIIIb isoantibodies with *FCGR3B* gene deletion were detected in both cases. Newborns and fathers exhibited specific gene combinations: *FCGR3B*02/FCGR3B*02* (Case 1) and *FCGR3B*02/FCGR3B*03* (Case 2). IMNS outperformed dextran/Ficoll, yielding four times more neutrophils (average neutrophil counts: $18.5 \times 10^3/\mu L$ vs. $4.5 \times 10^3/\mu L$), efficiently removing non-neutrophil cells and reducing processing time (30–40 min vs. 70–90 min), although it incurred a higher cost (2.7 times).

Conclusion: Two cases of maternal anti-FcγRIIIb isoantibodies, unrelated to NAIN, were identified. Although neutropenia has not been described in these cases, we emphasize the importance of identifying asymptomatic cases with the potential for severe neutropenia. Additionally, IMNS is introduced as a rapid, high-yield,

Juliana Oliveira Martins and Elyse Moritz contributed equally to the manuscript.

high-purity neutrophil isolation technique, beneficial for serological assays detecting anti-HNA antibodies.

Keywords

dextran/Ficoll, Fc-gamma-receptor-IIIb, human neutrophil antigen-1, immunomagnetic negative selection of neutrophils, neonatal alloimmune neutropenia, neutrophil isolation

Highlights

- Two cases of maternal anti-Fc-gamma-receptor-IIIb isoantibodies, unrelated to neonatal alloimmune neutropenia, have been described. It is important to document asymptomatic cases when an antibody with the potential to induce severe neutropenia is present.
- Immunomagnetic negative selection (IMNS) outperformed dextran/Ficoll, yielding four times more neutrophils, efficiently removing non-neutrophil cells and eliminating red blood cells without the lysis step. IMNS also reduced processing time, although it incurred a higher cost.
- IMNS-isolated neutrophils exhibit high purity and reduced activation or apoptosis, resulting in a good alternative for neutrophil isolation in serological assays aimed at detecting anti-human neutrophil antigen antibodies.

INTRODUCTION

The Fc-gamma-receptor-IIIb (FcγRIIIb; CD16b) is an immunogenic and polymorphic neutrophil glycoprotein encoded by the *FCGR3B* gene. In the first domain of the glycoprotein, the human neutrophil antigen-1 (HNA-1) system is expressed, comprising four antigenic epitopes: HNA-1a, HNA-1b, HNA-1c and HNA-1d, encoded by three alleles (*FCGR3B*01*, *FCGR3B*02* and *FCGR3B*03*) [1–4].

The HNA-1 system participates in alloimmune and autoimmune events, serving as a target for specific antibodies involved in transfusion-related acute lung injury (TRALI), neonatal alloimmune neutropenia (NAIN) and autoimmune neutropenia [5–8]. Individuals with the deletion of the *FCGR3B* gene (*FCGR3B*null*) are observed in 0.1% of Caucasians, with a higher prevalence in Africans (1% in South Africa, 2.3% in Uganda and 7% in Ghana) and Italians (1.9%) [9–11]. Although rare, FcγRIIIb-null (or HNA-1-null) individuals may develop anti-FcγRIIIb isoantibodies after allogeneic exposure (by pregnancy or transfusion), which can lead to severe cases of NAIN [12, 13].

NAIN is a rare and often serious condition caused by transplacental passage of maternal immunoglobulin G (IgG) antibodies against HNA expressed in the foetus and inherited from the father, resulting in the destruction of foetal neutrophils [8]. Serious infections, mainly of skin and umbilical cord, occur in one out of five patients with NAIN, and the duration of neutropenia is, on average, 1–4 months [14]. The absolute neutrophil count (ANC) for the definition of neutropenia during the first days of life is <6.0–7.0 (6–24 h), <3.0 (24–72 h), <2.5 (72–240 h) $\times$ 10^9/L for term infants and <3.0 (24 h), <1.0 (72 h) $\times$ 10^9/L for preterm infants. From 14 days to 1 year, the ANC cut-off is <1.0 $\times$ 10^9/L [15].

The diagnosis of NAIN includes the detection of HNA antibodies in the mother's serum, combined with the presence of the corresponding antigen in the neutropenic neonate. The serological investigation protocol usually includes the screening tests: granulocyte immunofluorescence test (GIFT) and granulocyte agglutination test (GAT) for an HNA-incompatible mother and child. The identification of antibody specificity can be performed by the monoclonal antibody immobilization of granulocyte antigens (MAIGA) and/or the bead-based techniques. The combination of GIFT, GAT and MAIGA assays is considered the 'gold standard' for HNA testing and demands the isolation of freshly collected neutrophils from genotyped donors as a source of target antigens [16].

For neutrophil isolation, most laboratories use dextran sedimentation followed by Ficoll density gradient centrifugation (dextran/Ficoll). This is a very simple and low-cost procedure; however, it is time-consuming and involves a series of cell manipulations, with consequences for the neutrophil activation status [17]. Currently, cell isolation by immunomagnetic negative selection (IMNS) is widely used in transplant laboratories to isolate lymphocytes for cross-matching tests [18]. However, in neutrophil laboratories, the use of IMNS is not widely propagated due to a lack of knowledge or comparative studies.

Here, we report two cases of maternal isoimmunization against the FcγRIIIb glycoprotein, with the newborns presenting no clinical or laboratory signs of NAIN. We detected anti-FcγRIIIb isoantibodies using neutrophils isolated by two different techniques: IMNS and dextran/Ficoll, which were applied to GIFT, GAT and MAIGA. This is the first study to compare the two methods in terms of time, cost, purity, neutrophil yield and viability.

MATERIALS AND METHODS

Study population

The two women with anti-FcγRIIIb isoantibodies were identified in a previous study where we investigated the prevalence of HNA

alloantibodies in 147 non-transfused pregnant women presenting red blood cell (RBC) alloantibodies [19]. Patients were recruited from a public hospital in Sao Paulo (SP, Brazil), and blood collections were performed after obtaining informed consent, during the postpartum period.

Considering the presence of the anti-FcγRIIIb antibody, the families were summoned 4 months later to receive clinical guidance, and blood collection from the mother, father and child was performed after obtaining informed consent.

This study was approved by the Ethical Committee of the Universidade Federal de São Paulo, with the number 0846/2015.

Case 1

A 24-year-old pregnant Brazilian woman with no history of previous pregnancies, abortions or blood transfusions presented to our service for delivery at 40 weeks of gestation. She was included in the study of Martins et al. [19] because of the presence of the anti-Lewis a alloantibody. No abnormalities were observed during the pregnancy. The child was born weighing 3422 g and experienced no complications. Because of favourable health conditions, no laboratory tests were requested, and both the mother and child were discharged 2 days after delivery.

Case 2

A 37-year-old woman from Ghana, Africa, presented to the hospital for management of her current pregnancy due to hypertension and haemolytic disease of the foetus and newborn (HDFN) caused by anti-D and anti-C RBC alloantibodies. The patient's obstetrical status was G4P3A0 with a history of recurrent blood transfusions during all previous deliveries. She had a history of haemolysis, elevated liver enzymes and low platelets syndrome in the last pregnancy and HDFN in the last two pregnancies.

The infant was delivered by caesarean section at 33 weeks of gestation, with a birth weight of 3540 g, presenting severe HDFN requiring an exchange transfusion on the second day and intravenous immunoglobulin (IVIg) on the third day of life. The newborn developed sepsis on the eleventh day and was treated with broad-spectrum antibiotics. Discharge occurred after 20 days of hospitalization with no development of neutropenia.

Serological techniques

Neutrophil isolation from donors with known HNA phenotype

Blood samples from three donors were collected in EDTA tubes. Neutrophils were isolated from 8 mL of whole blood using two different techniques: (1) the dextran/Ficoll (Sigma-Aldrich, MO, USA), as described before [20] and (2) the IMNS using the EasySep™ Direct Human Neutrophil Isolation Kit (StemCell Technologies, BC, Canada). The negative selection of neutrophils uses monoclonal antibodies bound to magnetic beads that recognize specific cell surface markers expressed in non-neutrophils cells, promoting cell separation through a magnetic column. The IMNS was performed with 1 mL of buffy coat obtained from 8 mL of whole blood, following the supplier's protocol.

After isolation, cells were resuspended to a final volume of 1 mL, and differential blood cell counts for each isolation method were determined using an automated haematology analyser (CELL-DYN Ruby, Abbott Diagnostics, USA). Neutrophil concentrations were adjusted according to the serological tests: GAT and MAIGA, 5.0×10^3 cells/μL; GIFT, 1.0×10^5 cells/μL.

Granulocyte agglutination test

The isolated neutrophils (2 μL, 5.0×10^3 cells/μL) were incubated with patient's serum or control serum (6 μL) on a Terasaki plate for 2 h at 37°C. The test was performed in duplicate, and the results were considered positive in the presence of agglutination, which was evaluated using an inverted microscope.

Granulocyte immunofluorescence test

GIFT was performed by incubating the neutrophil suspension (40 μL, 1.0×10^5 cells/μL) with the patient's serum (40 μL) for 30 min at 37°C, followed by two washing steps and incubation with fluorescein isothiocyanate-labelled rabbit F(ab′)2 anti-human IgG (Dako, Hamburg, Germany) for 20 min at room temperature. After washing, cell-bound fluorescence-labelled antibodies were detected by flow cytometry. The results were considered positive when the mean fluorescence intensity reached a value two times higher than that of the negative control serum [20]. The GIFT can be performed with paraformaldehyde-fixed or unfixed neutrophils. We used unfixed cells since the flow cytometric acquisition and analysis were performed immediately after completing the labelling protocol.

Bead-based assay

The test was performed using the LABScreen Multi (LSM) kit (One Lambda Inc., CA, USA), capable of identifying alloantibodies against all HNA specificities with the use of microspheres coated with the specific purified antigens. Tests were conducted following the manufacturer's instructions using the LABScan™ 100 platform (Luminex, USA), and the results were analysed by the Fusion 4.6™ software (One Lambda, Inc, USA). Results were considered positive for the HNA-1 system when the mean fluorescence intensity (MFI) ≥1000, and the normalized background ratio was ≥10.

TABLE 1 Immunohaematological results of mothers, fathers and infants in two cases of maternal anti-FcγRIIIb isoimmunization.

			Genotyping (epitopes)					Leucocyte antibodies	
Families	**Blood type**	**RBC alloantibody**	**HNA-1**	**HNA-2 (%)[a]**	**HNA-3**	**HNA-4**	**HNA-5**	**HLA**	**HNA**
Case 1									
Mother	A+	Anti-Lewis a	Null	81	3a	4a	5a	Class I	Anti-1abc
Father	O+	NT	1bd	58	3a	4a	5a	NT	NT
Son	A+	NT	1bd	85	3a	4a	5a	NT	NT
Case 2									
Mother	O−	Anti-D + C	Null	78	3a	4a	5ab	Class I, II	Anti-1abc
Father	A+	NT	1bcd	88	3a	4a	5a	NT	NT
Son	A+	NT	1bcd	85	3a	4a	5ab	NT	NT

Abbreviations: FcγRIIIb, Fc-gamma-receptor-IIIb; HLA, human leukocyte antigen; HNA, human neutrophil antigen; NT, not tested; RBC, red blood cell.
[a]Phenotyping of CD177 by flow cytometry. Values represent the percentage of positive neutrophils.

The anti-HNA-1 positive serum samples were also evaluated by GIFT, GAT and MAIGA in the granulocyte laboratory of the Institute for Clinical Immunology, Transfusion Medicine, and Haemostasis, Justus Liebig University, Giessen, Germany.

HNA-1 genotyping

Genotyping of the *FCGR3B* coding region responsible for the HNA-1 alleles expression (HNA-1a, HNA-1b, HNA-1c and HNA-1d) was performed by sequence-specific polymerase chain reaction (PCR), as previously described [20]. The human growth hormone gene was used as an internal positive PCR control (439-bp).

Cell viability

A membrane integrity-based cell viability assay was conducted using NucGreen Dead 488 reagent (Life Technologies, CA, USA) which was added, in the concentration of 2.5%, to the neutrophil suspension (5 μL, 1.0×10^5 cells/μL), followed by a 3-min incubation at room temperature and subsequent flow cytometric analysis. Cell viability was calculated using total number of cells as 100% compared with the number of NucGreen-positive cells.

RESULTS

The maternal anti-FcγRIIIb isoantibodies against the incompatible children were not associated with NAIN

Case 1

Screening tests for HNA antibodies in mother's serum revealed an antibody reactive to all epitopes of the HNA-1 system (HNA-1a, HNA-1b, HNA-1c and HNA-1d), characterized as a pan-reactive anti-FcγRIIIb. The blood sample collected from the child 4 months after delivery showed no alterations in the ANC (3.19×10^9/L).

The father and child were genotyped as *FCGR3B*02/FCGR3B*02*, coding for the epitopes HNA-1b1d; however, no *FCGR3B* gene was present in genomic DNA from the mother, confirming the incompatibility (Table 1).

Case 2

Screening tests in the mother's serum revealed an anti-FcγRIIIb; however, neutropenia was not detected in the child during hospitalization. As expected for a preterm neonate, there was a considerable reduction in the number of neutrophils on the third day of life, although still within the expected values. A normal ANC was also observed on the 40th day of life (ANC = 6.3×10^9/L) (Figure 1).

The *FCGR3B* gene was deleted in the mother; however, the newborn and the father were genotyped as *FCGR3B*02/FCGR3B*03* (coding for the epitopes HNA-1b1c1d), confirming the incompatibility with the mother (Table 1).

Comparison of neutrophil isolation techniques: IMNS as a faster technique with higher cell yield

The neutrophil isolation techniques, dextran/Ficoll and IMNS, were performed on three HNA-typed donors, and isolated neutrophils were used as an antigen source for the serological tests GAT and GIFT. The IMNS presented a cell yield approximately four times higher when compared with dextran/Ficoll (average neutrophil counts: 18.5×10^3/μL vs. 4.5×10^3/μL), allowing the processing of a four-times greater number of samples in the GAT or GIFT. Furthermore, in comparison to dextran/Ficoll, this technique managed to remove a greater number of lymphocytes, monocytes, eosinophils and basophiles and was able to completely remove RBC without the lysis step (Table 2).

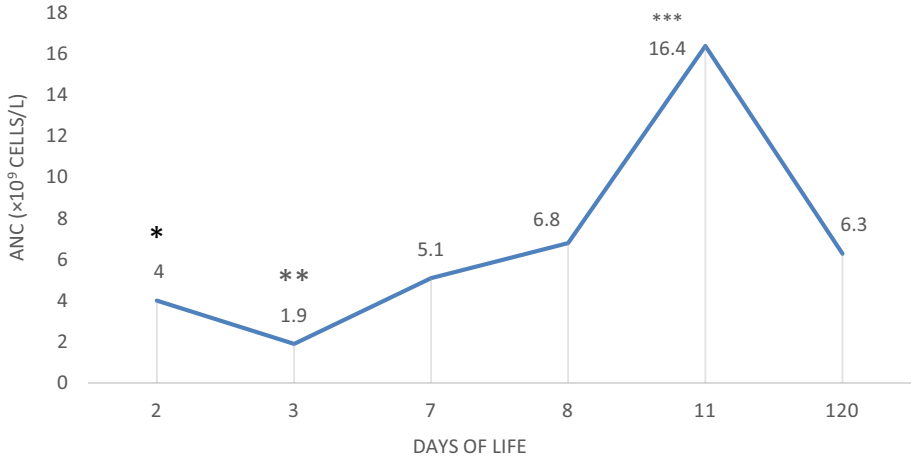

FIGURE 1 Absolute neutrophil count (ANC) representation during the first 120 days of life of the child reported as Case 2. *ANC before exchange transfusion; **ANC during the use of intravenous immunoglobulin; ***ANC during the use of large spectrum antibiotics.

TABLE 2 Characteristics of the neutrophil isolation techniques used to study anti-FcγRIIIb isoantibodies against the cells of three previously genotyped healthy subjects.

	Donor 1		Donor 2		Donor 3	
	Dextran/Ficoll	IMNS	Dextran/Ficoll	IMNS	Dextran/Ficoll	IMNS
Cell type [$\times 10^9$/L (%)]						
WBCs	5.9	23.4	4.2	18.4	4.6	15.8
Neutrophils	5.4 (91.2)	22.7 (97.1)	3.9 (92.4)	17.7 (96.2)	4.3 (95.4)	15.3 (96.8)
Lymphocytes	0.3 (4.8)	0.3 (1.2)	0.2 (3.6)	0.3 (1.8)	0.1 (2.6)	0.2 (1.4)
Monocytes	0.1 (2.2)	0.3 (1.1)	0.1 (1.5)	0.2 (1.1)	0.05 (1.1)	0.1 (0.9)
Eosinophils	0.1 (1.2)	0.0 (0.0)	0.1 (1.4)	0.02 (0.1)	0.04 (0.9)	0.2 (0.9)
Basophils	0.04 (0.7)	0.1 (0.4)	0.04 (1.0)	0.1 (0.8)	0.01 (0.1)	0.0 (0.0)
RBCs	0.00	0.00	0.00	0.00	0.00	0.00
Platelets	0.00	0.00	0.00	0.00	0.00	0.00
Viability	0.87	0.93	0.89	0.91	0.82	0.89
Number of tests						
GAT (2 µL/test)	1074	4680	840	3680	910	3160
GIFT (40 µL/test)	26.7	117	21	92	22.8	79
Cost	**Dextran/Ficoll**		**IMNS**			
Catalogue number	31392-50G/17-1440-02		19666			
Company	Sigma/GE healthcare		Stemcell			
Price per sample (USD)	0.60/1.03 (1.63)		4.44			
Time spent (min)	70–90		30–40			

Note: Neutrophils were obtained from 8 mL of whole blood, with results based on a final volume of 1 mL.
Abbreviation: FcγRIIIb, Fc-gamma-receptor-IIIb; GAT, granulocyte agglutination test; GIFT, granulocyte immunofluorescence test; IMNS, Immunomagnetic negative selection; RBCs, red blood cells; WBCs, white blood cells.

The cell viability was analysed through NucGreen, which is a cell-impermeant stain that emits a green fluorescence when bound to DNA. The viability of both methods was adequate (≥80%); however, in the three donors tested, the percentage of viable cells was slightly higher in the IMNS when compared with dextran/Ficoll (average of 91% vs. 86%) (Table 2).

The time spent performing the dextran/Ficoll technique was, on average, 70–90 min, whereas the IMNS technique took 30–40 min

TABLE 3 Results of the serological techniques applied to detect anti-HNA antibodies in maternal sera using a cell panel obtained from three previously genotyped donors.

	Neutrophil donors	GAT		GIFT[a] (median)		LSM[b]			MAIGA	
Cases	Genotype	D/F	IMNS	D/F	IMNS	Beads	MFI	NBG	3G8	LNK16
1	*FCGR3B*01/*01*	3+/4+	3+/4+	2.7	3	HNA-1a	2415	11	Neg	Neg
	*FCGR3B*02/*02*	3+/4+	3+/4+			HNA-1b	2273	24		
	*FCGR3B*01/*03*	Neg	Neg	Neg	Neg	HNA-1c	1513	25		
2	*FCGR3B*01/*01*	4+/4+	4+/4+	13.3	14.4	HNA-1a	11,546	75	Pos	Pos
	*FCGR3B*02/*02*	4+/4+	4+/4+			HNA-1b	9601	173		
	*FCGR3B*01/*03*	3+/4+	3+/4+			HNA-1c	13,268	264		

Note: 3G8 and LNK16 denote anti-CD16 monoclonal antibodies.
Abbreviations: D/F, dextran/Ficoll protocol for neutrophil isolation; GAT, granulocyte agglutination test; GIFT, granulocyte immunofluorescence test; HNA, human neutrophil antigen; IMNS, immunomagnetic negative selection; LSM, LABScreen Multi; MAIGA, monoclonal antibody immobilization of granulocyte antigen; MFI, mean fluorescence intensity; NBG, normalized background ratio; Neg, negative; Pos, positive.
[a]Ratio between the MFI values obtained in the test and control serum. Results >2 were considered positive.
[b]Cut-off HNA-1a, HNA-1b and HNA-1c values: MFI ≥1000 and NBG ≥10.

for complete cell separation. The cost of the techniques may vary, but considering the products described in Table 2, IMNS had a cost 2.7 times higher than dextran/Ficoll.

Identification of anti-FcγRIIIb isoantibodies

All serological screening techniques performed were able to detect the presence of anti-FcγRIIIb isoantibodies in the serum of both women. GAT and GIFT tested positive in all homozygous cells, and the LSM showed results above the established cut-off in both cases. MAIGA was negative in the first case, probably due to the low antibody titre, as evidenced by the low fluorescence intensity in LSM; and positive in the second case with two anti-CD16 monoclonal antibodies (3G8, LNK16) (Table 3).

The GAT presented strong agglutination with cells isolated by both techniques, and no visible difference in agglutination pattern was observed. However, during the microscopic analysis, we observed that IMNS preparation had fewer impurities, which facilitated the identification of weaker reactions.

The GIFT also presented positive results (two times higher the negative control) in both cell isolation techniques, with the IMNS presenting higher MFI results when compared to dextran/Ficoll, but no statistical difference was found (Table 3).

The validation of the IMNS technique for neutrophil isolation was also performed with four control sera from the workshop of granulocyte immunobiology of the International Society of Blood Transfusion-containing the antibodies anti-HNA-1a, anti-HNA-1b, anti-HNA-2 and anti-HNA-3a.

DISCUSSION

The present study describes two cases of maternal isoimmunization against FcγRIIIb not associated with NAIN and examines the value of the implementation of IMNS by comparing it with the traditional dextran/Ficoll method for neutrophil isolation.

The two FcγRIIIb-null mothers with anti-FcγRIIIb isoantibodies represent 1.4% (2/147) of the original and selected cohort composed of mothers with RBC alloantibodies induced by pregnancy and 22.2% (2/9) of mothers with HNA antibodies in the same cohort. RBC alloimmunization has been associated with a fivefold increased risk of developing anti-HNA alloantibodies, reflecting the individual's efficient immune response to allogeneic antigens [19].

The first case reported in this study confirms the previous findings that HNA antibodies can be found in the first pregnancy [5, 6]. Unfortunately, we could only access the child's ANC on the 40th day of life for the following reasons: (1) blood counts in term newborns are not routinely performed in the health service; (2) the infant was healthy, with no signs of infection; (3) the identification of an anti-FcγRIIIb isoantibody in the mother's serum occurred after hospital discharge. Based on these data, for this case, we cannot confirm the absence of neutropenia in the child at the time of the birth; however, no clinical symptoms of neutropenia were present. It is known that about 32% of infants with NAIN are asymptomatic, and only 19% of the cases show evidence of infections at the presentation [5]. In most cases, neutropenia is detected incidentally when ANC is performed due to the presence of other clinical conditions, as observed by van den Tooren-de Groot et al. [6], in which 14/35 (40%) cases of NAIN were detected only because cell counting was performed for other unrelated disorders.

The newborn in the second case was diagnosed with a severe HDFN due to the presence of anti-D and anti-C RBC alloantibodies, in addition to the presence of maternal anti-FcγRIIIb isoantibody. He presented a considerable drop in the number of neutrophils on the third day of life, as expected for a moderate preterm; however, an exchange transfusion and IVIg administration in the second and third day, respectively, prevented proper evaluation of the ANC (Figure 1). Due to the absence of neutropenia within the 120 days of life, the diagnosis of NAIN was ruled out in this case.

Although neutropenia was not identified in the cases described, either due to a lack of neutrophil counts or because a transfusion was a confounding factor, we believe it is important to report asymptomatic cases in the presence of an antibody with the potential to cause severe neutropenia, with clinical manifestations like omphalitis, skin infections, fever, meningitis, pneumonia and sepsis.

As also observed by Zupańska et al. [14], the incidence of HNA-1 immunization seems to be higher than the incidence of NAIN, which, moreover, manifests itself at various grades of severity. The rate of cell destruction depends on many factors, including the antigen density on the neutrophil membrane. It is known that gene copy number variation seems to be a special feature of *FCGR3B*, with the occurrence of gene duplication combined with recombination. An allele drop out on one chromosome could possibly lead to reduced HNA-1 expression in the child, justifying the absence of NAIN [4].

Although the cases described here of maternal isoimmunization without foetal compromise were discovered by chance, the isolation of neutrophils and subsequent detection and identification of anti-HNA antibodies are very important in clinical medicine for the correct diagnosis of potentially serious conditions, such as autoimmune and alloimmune neutropenia, and TRALI [5, 7]. Furthermore, the identification of an anti-HNA antibody, especially in cases of autoimmune neutropenia, enables a reduction in patients' exposure to invasive exams to establish a diagnosis.

According to the International Working Party on Granulocyte Immunology, the combination of the GAT and GIFT with typed donor cells is considered the gold standard in the detection of antibodies against neutrophils [16]. For decades, the neutrophil isolation technique for detection of anti-HNA antibodies used by the granulocyte laboratories has been dextran sedimentation followed by Ficoll density gradient centrifugation. However, the lengthy procedure with multiple washing steps and the presence of monocytes may lead to neutrophil activation, affecting the results of serological tests such as GAT in which neutrophils are actively involved [17]. Additionally, neutrophil isolation by dextran/Ficoll is time-consuming and requires a series of steps, such as preparation of reagents, incubation, long centrifugations and lysis of RBC, resulting in considerable cell loss [21]. To obtain a sufficient amount of cells after the isolation, a high volume of whole blood from donors is needed.

Our results show that the IMNS method for neutrophil isolation is considerably faster than dextran/Ficoll, requires a smaller amount of blood and achieves a higher yield and quality of isolated cells. The neutrophils are intact and not activated, improving performance in assays such as GAT and GIFT. Furthermore, the IMNS managed to remove a greater number of lymphocytes, monocytes, eosinophils and basophiles and was able to completely remove RBC without the need for the lysis step. Therefore, IMNS-isolated neutrophils show more efficiency in GAT as a functional test for which activated or apoptotic neutrophils are not eligible cells.

Similarly, in multiple studies where neutrophil functions are investigated, IMNS-isolated neutrophils have been applied to investigate the migration through the binding of CD177 [22] and also the connection between neutrophil extracellular traps and COVID-19 severity [23]. Although more expensive it has been reported that IMNS-isolated neutrophils show high purity and less activation or apoptosis, resulting in a good alternative for neutrophil isolation applied in serological analysis.

In conclusion, anti-FcγRIIIb isoantibodies are rare in the population and may be found in asymptomatic to severe cases of NAIN. The current study presents two cases of maternal isoimmunization against FcγRIIIb expressed on the newborn neutrophils; however, the antibodies were not associated with clinical or laboratory diagnosis of NAIN. In addition, our results introduced the IMNS neutrophil isolation technique as a fast and easy method with a high cell yield, which can be useful in diagnostic laboratories involved in serological techniques for the detection of anti-HNA antibodies.

ACKNOWLEDGEMENTS

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) that provided scholarship and financial aid for the accomplishment of the study.

J.O.M. and E.M. designed the research study, collected and analysed the data and wrote the paper; S.A.A. collected samples from patients and families; B.B. contributed with additional tests, reviewed and edited the manuscript; M.M.O.B. reviewed the clinical data; R.M. and R.F.M. gave instructions on the immunomagnetic technique, acquired and analysed the viability data; J.O.B. supervised the research, reviewed and edited the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

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How to cite this article: Martins JO, Moritz E, Abbas SA, Bayat B, Barros MMO, de Marco R, et al. Analysis of maternal Fc gamma receptor IIIb isoantibodies using immunomagnetic negative selected neutrophils. Vox Sang. 2024;119:712–9.

Received: 1 November 2023 | Revised: 16 March 2024 | Accepted: 24 March 2024

DOI: 10.1111/vox.13632

ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Impact of human leucocyte antigen class II polymorphism on anti-red blood cell antibody development: Correlations and indications

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Funding information
Government of Republika Srpska

Abstract

Backround and Objectives: Blood transfusion therapy is vital for many patient groups. They can cause many complications, and the development of anti-red blood cell (RBC) antibodies is of significant importance. Molecules of class II human leucocyte antigens (HLA) are one of the several factors that influence antibody development in patients.

Materials and Methods: In this study, we investigated 108 patients who developed antibodies against different erythrocyte antigens and 115 patients on multiple transfusion therapies who did not develop anti-RBC antibodies. The HLA loci HLA-DRB1 and HLA-DQB1 were typed using commercial molecular assays routinely used in HLA laboratories.

Results: An increased frequency of the HLA-DRB1*04 allele group was observed in patients who developed antibodies. Additionally, HLA-DRB1*09 was also significant for anti-E development and in patients with multi-specific alloimmunization. It was found that the HLA-DRB1*07 allele group is associated with antibodies to antigents of the Rh and MNS systems but also lacks an association with anti-K development. The HLA-DRB1*11 and -DRB1*01 allele groups displayed a protective mechanism for anti-E development, similar to that of HLA-DQB1*02 for anti-K.

Conclusion: There is an association between various HLA class II alleles and anti-RBC development.

Keywords

antibodies, blood therapy, human leucocyte antigens, immune response, red blood cell

Highlights

- This study demonstrated the protective role of HLA-DQB1*02 in anti-K and HLA-DRB*11 and HLA-DRB*01 in anti-E development.
- The HLA-DRB1*04 allele group generally indicated a strong association with the anti-red blood cell antibody development.
- We present a novel approach for evaluating differences in HLA frequencies in transfusion therapy patients according to the number of components received.

INTRODUCTION

Therapies involving the transfusion of blood or blood products are vital for diverse patient groups. Although the most clinically important red blood cell (RBC) antigens have been identified within the ABO system, RBC transfusions can cause serious complications in patients when they are incompatible with various non-ABO antigens [1–5]. According to the International Society of Blood Transfusion (ISBT), 45 blood group systems have been recognized since the middle of 2023, which contain 360 antigens determined by 50 genes [6]. With this variability, it is hard to expect full donor–patient compatibility. Antigen matching is the basis for identifying suitable blood products. In practice, if not completely impossible to achieve, it would definitely feature complex logistic and financial challenges [7, 8]. Extended matching is performed only for patients at high risk of anti-RBC antibody (RBC Ab) development.

To develop RBC Ab, the person must be exposed to at least non-self RBC antigen(s) and have the appropriate human leucocyte antigen (HLA) groove that would present the non-self antigen-derived peptide to the immune system [9]. Even though >99% of transfused individuals are capable of producing RBC Abs with at least two different specificities, only 2%–5% of transfused individuals develop detectable RBC Abs [10]. Approximately 25% of RBC Abs vanish after a month, 50% after 6 months and 60%–70% up to 5 years after formation [11]. The clinical importance of RBC Abs is reflected in preparations for subsequent transfusion therapy, pregnancy and transplantation (especially critical for haematopoietic stem cell transplantation).

Therefore, we investigated the influence of HLA class II antigens on RBC Ab formation. We aimed to determine the HLA class II alleles responsible for these antigens from the two HLA loci (HLA-DRB1 and HLA-DQB1) as predictors for more accurate extended screening in the preparation of blood products for patients who should avoid the formation of RBC Abs with the utmost necessity.

MATERIALS AND METHODS

The study was approved by the Ethics Committee of the University Clinical Centre of Republika Srpska and the Institute for Transfusion Medicine of Republika Srpska in Banja Luka, Bosnia and Herzegovina. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Patients

The study was performed in two patient groups:

The test group consisted of patients who developed RBC Abs (responders), which were verified, and the control group comprised patients who received multiple transfusion therapies without developing RBC Abs (non-responders).

The ABO phenotypes and RhD antigens were determined for all study participants. For any RhD-negative samples, additional Rh phenotyping was performed (CcEe).

Test group

In 2019, samples were collected from 108 patients (62 women, 46 men) who developed anti-RBC antibodies. Samples were collected from gynaecological clinics (32), surgery departments (15), internal medicine departments (51) and blood donors (10).

The specificities of the RBC Abs were analysed in all patients. If the specificity of RBC Ab was not determined, they were designated as cold (the stronger positive reactions occurred at 22°C) or warm (the stronger positive reactions occurred at 37°C).

The test group was subdivided according to the number of previously received RBC components relative to the developed RBC Abs:

1. One or more RBC components received before the RBC Ab formation;
2. No received blood products or history of pregnancies before the RBC Ab formation;
3. After one or multiple pregnancies, either in the study period or earlier.

Control group

Samples of non-responder patients who received multiple transfusion therapies and were exposed to foreign RBC antigens but without RBC Ab formation were collected in 2019 and 2020. This group consisted of 115 patients who fulfilled all of the following criteria:

1. After receiving a request from the clinician for blood component preparation, it was established that the last transfusion was at least 60 days before that date by consulting our institute's information system. To obtain participants who received as many RBC products as possible during treatment, we selected patients with a minimum of two RBC products (deplasmated, resuspended, or both).
2. An indirect antiglobulin test (IAT) was also performed on the same day. Only the IAT-negative patients were included in the subsequent selection phase.
3. During the study period (until the end of 2022), the information system was consulted to check whether the patients continued with their transfusion therapy and whether any RBC Abs were formed in the meantime, which would exclude them from the control group. This was performed because IAT was obligatory before each subsequent RBC component administration (the typical interval between two consecutive IATs was 35 days). Notably, six more patients were initially included in this group but developed RBC Abs during the course of the study. Consequently, the results were linked to the test group.

The control group patients were followed up for 2 years after sampling: 24 from surgery departments and 91 from internal medicine departments (haematology, 39; oncology, 27; other, 25).

The control group was subdivided according to the number of transfusion therapies received (RBC, plasma blood components, or both): (a) 1–20, (b) 21–50, (c) 51–100 and (d) >100 blood products.

Methods

The methods used for blood collection, storage and laboratory testing at the Institute for Transfusion Medicine of Republika Srpska were in accordance with the recommendations of the Council of Europe and the EU for providing quality and safe blood [12]. IAT was performed using the gel method with appropriate microgel column cards (Biorad Laboratories, USA) on instruments IH-500 (Biorad) and Erytra (Grifols, Spain). RBC Abs were identified using commercial standard panels to detect RBC Abs (Biorad and Grifols), partially on an Erytra instrument (Grifols) or by manual pipetting on ID-card LISS-Coombs (Biorad).

Genomic DNA was extracted from 200 μL of peripheral blood samples using the Ready DNA Spin Kit (Inno-train Diagnostik, Germany).

The Inno-train's molecular FluoGene system based on PCR-sequence-specific primers (PCR-SSP) with fluorometric signal detection was used as a typing method for HLA class II alleles, specifically the HLA-FluoGene DRDQ kit (Inno-train Diagnostik). Within the allelic groups of HLA-DRB1*03 and HLA-DQB1*03, it was possible to separate alleles in two-field (high) resolution (serological equivalents to DR17, DR18, DQ7, DQ8 and DQ9).

Some samples were additionally confirmed using the PCR-sequence-specific oligonucleotides (PCR-SSO) method (Thermofisher Scientific, USA).

Data processing

Data on HLA-DRB1 and -DQB1 allele groups/alleles were collected by direct counting. HLA allele frequencies were compared between the groups. The HLA-DRB1∼DQB1 haplotypes were deduced from linkage disequilibrium data for the populations of European origin and described as presumed haplotypes, as no family HLA typing was performed [13]. The absolute frequencies were obtained by direct counting. Odds ratios (OR), standard errors and 95% confidence interval (CI) were calculated using the MedCalc software (OR calculator; https://www.medcalc.org/calc/odds_ratio.php, MedCalc Software, Ostend, Belgium, Version 22.001; accessed 24 February 2023). The chi-squared test was used to determine statistical differences in the examined alleles between groups and among subgroups. Significance was set at $p < 0.05$. Because of the small population size and correspondingly low sample size, we also paid attention to p-values that could show a tendency to be within the significant range ($0.05 \leq p < 0.15$).

HLA typing of the test-group patients with specific RBC Ab and multiresponders was compared with that of the entire control group. The HLA typing comparison for subgroups with ≥10 patients carrying a specific RBC Ab and multiresponders (two or more RBC Abs) is presented here.

RESULTS

Fifteen specific RBC Abs were detected in the test group. Two or more RBC Abs were found in 17 of 108 (15.74%) patients. Non-specific RBC Abs were described in 13 of 108 (12.04%) patients.

Molecular typing of the HLA-DRB1 locus revealed the alleles responsible for all HLA-DR serological specificities except HLA-DR18 (HLA-DRB1*03:02). The most frequent allelic group was HLA-DRB1*11 with an allelic frequency of 17.59% (38/216 alleles), followed by -DRB1*01 (32/216, 14.81%), -DRB1*04 (31/216, 14.35%), -DRB1*13 (23/216, 10.65%) and -DRB1*15 (22/216, 10.19%). All other HLA-DRB1 allelic groups/alleles were reported at frequencies <10%. For the HLA-DQB1 locus, the most common allelic group was HLA-DQB1*05 (62/216, 28.70%), followed by -DQB1*03:01 (48/216, 22.22%), -DQB1*06 (45/216, 20.83%) and -DQB1*02 (28/216, 12.96%). Other HLA-DQB1 alleles observed (HLA-DQB1*03:02, -DQB1*03:03 and -DQB1*04) had frequencies <10%.

The control group patients received 2–321 (average, 53; median, 34) different units of RBC products from different blood donors. Similar to the test group, the allele groups responsible for all serological HLA-DR specificities, except HLA-DR18, were observed. The most frequent allele was—also similar to the test group—HLA-DRB1*11, with 37/230 alleles (16.09%), followed by -DRB1*16 (30/230, 13.04%), -DRB1*01, -DRB1*13 and -DRB1*15 (all three alleles were 26/230, 11.30%). The HLA-DRB1*03:01 allele was present at a frequency of 10.00% (23/230); for the remaining HLA-DRB1 alleles, the frequency was lower. Within the HLA-DQB1 locus, the alleles that were encountered at a frequency >10% were HLA-DQB1*06 (50/230, 21.74%), -DQB1*03:01 (47/230, 20.43%) and -DQB1*02 (35/230, 15.22%).

The major observed differences in HLA allele frequencies between the test and control groups, all of which belong to the possible relevant dataset, are presented in Table 1.

There were no significant differences in the presumed haplotype frequencies between the two groups. There were two presumed haplotypes with frequency differences that fell within the possibly relevant dataset ($0.05 \leq p < 0.15$): HLA-DRB1*08∼DQB1*04 ($p = 0.097$) and HLA-DRB1*16–DQB1*05 ($p = 0.138$). Test group HLA typings in association with RBC Abs detected in the corresponding patients are shown in Figure 1.

We compared the HLA results between the test and control groups. Table 2 lists the results of HLA allele groups or alleles and HLA-DRB1∼DQB1 haplotypes in test-group patients carrying the most frequently detected specific Abs (≥10 patients per specific RBC Ab) and the multiresponders.

Data on the history of transfusion therapy (received transfusion products) and/or previous pregnancies among the test group participants are shown in Table 3.

TABLE 1 Major differences in HLA allele frequencies between the test and control groups.

	Test group		Control group			
HLA-alelle	N_1	**Allelic frequency, %**	N_2	**Allelic frequency, %**	**Odds ratio (95% confidence interval)**	***p*-Value**
HLA-DRB1*04	31	14.35	21	9.13	0.6 (0.3–1.1)	0.127
HLA-DRB1*08	4	1.85	12	5.22	0.3 (0.1–1.1)	0.065
HLA-DRB1*16	17	7.87	30	13.04	0.5 (0.3–1.1)	0.109

Note: N_1, number of alleles in the test group; N_2, number of alleles in the control group.
Abbreviation: HLA, human leucocyte antigen.

FIGURE 1 Patient human leucocyte antigen (HLA) alleles associated with the red blood cell (RBC) Abs detected in the same individual. The results of patients with multiple antibodies are shown individually in all specific RBC Ab groups that they developed. (a) Test group HLA-DRB1 results stratified per RBC Ab. (b) Test group RBC Abs stratified per HLA-DRB1 allele of the corresponding patient. (c) and (d), respectively, show the equivalent HLA-DQB1 results.

RBC Abs developed in 65 participants after transfusion therapies (subdivision 1); 8 participants were voluntary blood donors with no previous transfusion therapies or pregnancies (subdivision 2) and 35 participants were women with one or more previous pregnancies and no history of receiving transfusion therapies (subdivision 3). The most frequent RBC Abs among patients after transfusion therapy (subdivision 1) included 19 anti-E (29.23%), 18 anti-K (27.69%), 7 non-specific (10.77%), 5 anti-D (7.69%) and 11 multiresponders (16.92%). Among patients with no previous transfusion therapies, pregnancies, or both (subdivision 2), we found five anti-M (62.5%) and one each (12.5%) of anti-A1, anti-Cw and anti-Le^a, with an additional one patient (12.5%) being a multiresponder. Of the 35 women with a

TABLE 2 Comparison of the test and control group HLA results (alleles and haplotypes) in the most frequently occurring anti-RBC antibody groups and multiresponders.

RBC Ab, number of patients	HLA allelic group/allele	Test group frequency, %	Odds ratio alleles (95% confidence interval)	*p*-Value allele	HLA-DRB1–DQB1 haplotype	Odds ratio haplotypes (95% confidence interval)	*p*-Value haplotype
Anti-D, $n = 23$	DRB1*04	10.87	2.1 (0.9–5.3)	0.111	DRB1*07∼DQB1*03:03	10.4 (0.9–117.3)	0.022
	DRB1*07	6.52	1.2 (0.3–4.6)	0.113			
	DQB1*03:03	4.35	13.9 (2.6–74.1)	0.079			
Anti-E, $n = 28$	DRB1*01	1.92	0.1 (0.0–1.1)	0.052	DRB1*01∼DQB1*05	0.2 (0.0–1.2)	0.052
	DRB1*04	21.15	2.6 (1.1–5.9)	0.033			
	DRB1*07	17.31	3.8 (1.5–9.5)	0.007	DRB1*04∼DQB1*03:02	3.0 (1.2–7.2)	0.025
	DRB1*09	3.85	9.1 (0.8–103.0)	0.034			
	DRB1*11	5.77	0.3 (0.1–1.1)	0.085	DRB1*07∼DQB1*03:03	4.4 (0.3–72.9)	0.012
	DQB1*03:02	17.31	4.1 (1.7–9.1)	0.035			
	DQB1*03:03	5.77	6.9 (1.1–42.9)	0.019	DRB1*09∼DQB1*03:03	9.1 (0.8–103.0)	0.034
	DQB1*05	15.38	0.4 (0.2–0.9)	0.093	DRB1*11∼DQB1*03:01	0.3 (0.1–1.1)	0.085
Anti-K, $n = 21$	DRB1*03:01	0	NC	NA	DRB1*16∼DQB1*05	0.2 (0.0–1.3)	0.072
	DRB1*07	0	NC	NA			
	DRB1*16	2.38	0.2 (0.0–1.4)	0.065			
	DQB1*02	0	NC	NA			
Anti-M, $n = 10$	DRB1*07	15.00	3.2 (0.8–12.5)	0.109	DRB1*07∼DQB1*02	3.5 (0.9–13.8)	0.083
Multi RBC Ab, $n = 17$	DRB1*04	20.59	2.5 (1.1–6.6)	0.079	DRB1*04∼DQB1*03:02	2.5 (0.8–7.3)	0.129
	DRB1*09	2.94	6.9 (0.4–113.6)	0.122	DRB1*07∼DQB1*03:03	6.9 (0.4–113.6)	0.122
	DQB1*03:03	5.88	7.2 (0.9–52.3)	0.031	DRB1*09∼DQB1*03:03	6.9 (0.4–113.6)	0.122

Abbreviations: HLA, human leucocyte antigen; NA, not applicable; NC, not calculated; RBC, red blood cell.

TABLE 3 Most important HLA alleles and haplotypes per the test group subdivisions according to the number of previously received blood products relative to the development of RBC Abs.

Subdivision	HLA allelic group/allele	Odds ratio alleles (95% confidence interval)	*p*-Value allele	HLA-DRB1–DQB1 haplotype	Odds ratio haplotypes (95% confidence interval)	*p*-Value haplotype
One or more RBC components received before the formation of RBC Abs, $n = 65$	DRB1*04 DQB1*03:02	1.7 (0.8–3.3) 0.4 (0.1–1.5)	0.158 0.119	DRB1*04∼DQB1*03:02	2.6 (1.2–5.9)	0.087
Neither any blood products received nor history of pregnancies before the formation of RBC Abs, $n = 8$	DRB1*07	4.1 (1.05–16.7)	0.051	DRB1*07∼DQB1*02	4.3 (1.08–17.6)	0.037
After one or multiple pregnancies, $n = 35$	DRB1*09 DQB1*03:03	6.7 (0.6–75.4) 6.9 (1.2–38.5)	0.079 0.014	DRB1*07∼DQB1*03:03 DRB1*09∼DQB1*03:03	6.7 (0.6–75.4) 6.7 (0.6–75.4)	0.079 0.079

Abbreviations: HLA, human leucocyte antigen; RBC, red blood cell.

history of one or more pregnancies (subdivision 3), 18 (51.43%) developed anti-D and 17 (48.57%) had antibodies from other blood group systems. In total, five women were multiresponders (14.28%).

There were 23 patients in the test group who developed anti-D. Only five (21.74%) had previous transfusion therapy, while the majority (18; 78.26%) were women with one or more pregnancies. Of the 23 anti-D patients, four were multiresponders, with additional RBC-Ab(s), whose HLA-DRB1 typing included the following alleles among others: HLA-DRB1*04, -DRB1*07 and -DRB1*13.

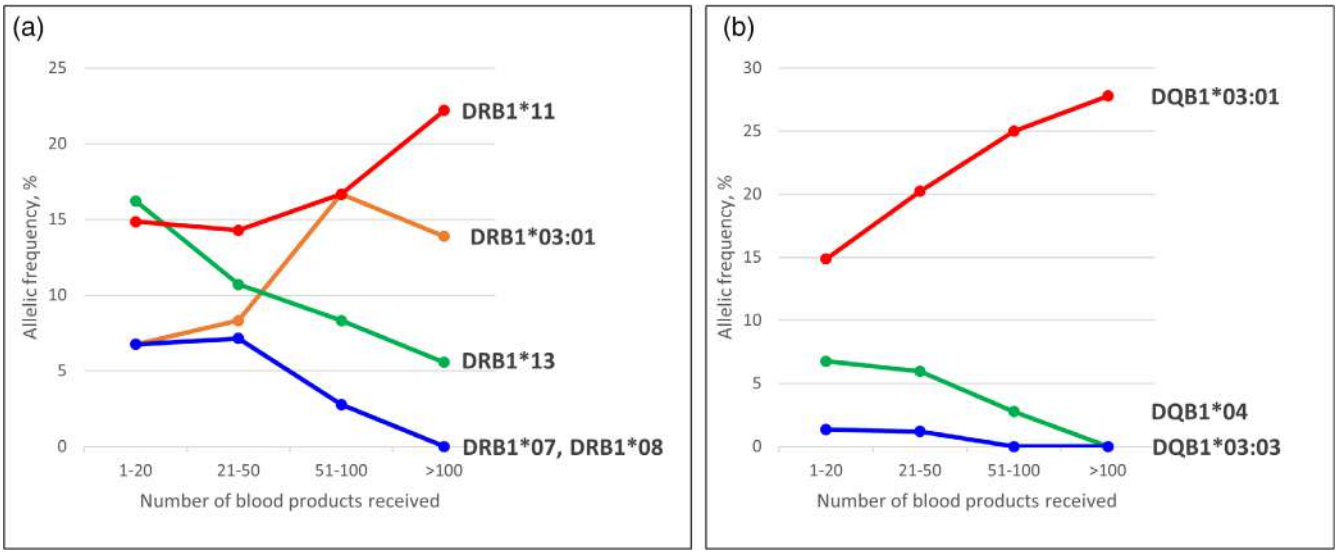

FIGURE 2 Control group human leucocyte antigen (HLA) alleles with increasing and decreasing frequencies pertaining to the increase in the number of received blood products. (a) HLA-DRB1 alleles. (b) HLA-DQB1 alleles.

Table 3 shows the HLA alleles and haplotypes from the test-group patients, stratified per subdivision, whose frequency comparison with the control group revealed *p*-values <0.15 (the odds ratio is also shown).

The frequencies of HLA alleles and HLA-DRB1∼DQB1 haplotypes in the control group stratified by the number of transfusion therapies received are presented in the Table S1. The tendency of increasing or decreasing frequencies of certain HLA allele groups/alleles, depending on the number of blood products received, is shown in Figure 2.

DISCUSSION

To the best of our knowledge, there has been no research on the general presence and frequency of HLA alleles in our population, which may have served as a control group for this study. We compared HLA frequencies in the control group with population studies in neighbouring countries to assess their significance. Our control group data on HLA class II alleles are mostly in accordance with data from Serbia [14] and Croatia [15].

Subsequent blood transfusion increases the probability of RBC Ab formation. As we chose non-responders as our control group, we were interested in examining the frequency of HLA alleles in patients who received a large number of blood products (especially 50 or more). Therefore, we divided our control group into four subgroups based on the number of blood products received by the patients. We explored the trend of each HLA allele frequency across an increasing number of blood products received. In this context, some alleles (HLA-DRB1*03:01, -DRB1*11 and -DQB1*03:01) showed an increasing trend in frequency, indicating a decreased risk of producing RBC Abs and suggesting that they might have a protective role in RBC Ab development. In contrast, some alleles (HLA-DRB1*07, -DRB1*08, -DRB1*13, -DQB1*03;03 and -DQB1*04) showed a decreasing frequency trend, indicating an increased risk of producing RBC Abs. These intriguing findings would be interesting to investigate further in a larger patient population.

One of the discrepant results in our test group is the frequency of HLA-DRB1*04 of 14.35%, which is not a relatively common allelic group in the compared neighbouring countries [14, 15]. The HLA-DRB1*04∼DQB1*03:02 haplotype was more frequent in the test group. Several studies have strongly associated the HLA-DRB1*04 allele with antibody anti-Fy^a [16, 17]. Schonewille et al. reported similar data and suggested further investigation of HLA-DRB1*04 and -DRB1*15 when anti-c+K and anti-K+Fy^a were developed [18]. Only four patients in this study developed an anti-Fy^a antibody: two had the HLA-DRB1*15 allele and one was homozygous for HLA-DRB1*04. In their review, Wong et al. labelled HLA-DRB1*04 and -DRB1*15 alleles as frequent in patients with sickle cell disease (SCD); these patients were responders in 40%–50% of SCD cases [19]. A significant difference was observed in frequencies between the test and control groups in patients with HLA-DRB1*04 who developed an anti-E antibody ($p = 0.033$). With an anti-D antibody, this difference was possibly relevant ($p = 0.111$). Among multiresponders, the most frequent alleles were HLA-DRB1*04 and -DRB1*13, each at 20.59%. On average, patients with HLA-DRB1*04 in transfusion therapy developed RBC Abs already after the third transfused component and RBC Abs against Rh antigens immediately after the first incompatible component or did not develop any RBC Abs. The immune system of these patients may not be presented with peptides derived from unknown RBC antigens because of HLA-DRB1*04 exon 2 polymorphisms.

Although the allele frequency of HLA-DRB1*07 in the test group was only 6.48% (comparable to the population of the region [13]), we derived several conclusions from the data. Most notably, HLA-DRB1*07 was detected only in patients with developed Rh and

MNS antibodies. The possibly relevant *p*-values for anti-D ($p = 0.113$), anti-M ($p = 0.109$), and multiresponders ($p = 0.169$), as well as the significant *p*-value for anti-E ($p = 0.007$, the lowest in the entire study), testify to its importance. HLA-DRB1*07 was undetected in patients with anti-K antibody, which was the third most common antibody in the study. This is comparable to the results of De Souza et al., who demonstrated a very low frequency of HLA-DRB1*07 in individuals with anti-K [20]. The association of this HLA allele with RBC Ab development has been demonstrated in patients who develop RBC Abs with neither a history of receiving blood components nor previous pregnancies. In addition, all patients had chronic diseases. In the control group, the HLA-DRB1*07 allele showed the highest frequency among patients who received the least number of components. Its frequency gradually decreased within the subgroups that received more components, and the allele was completely absent in patients who received >100 components. In contrast to the HLA-DRB1*04 allele, HLA-DRB1*07 allele carriers may require extended exposure to foreign RBC antigens or inflammation.

As more blood donations were received, the frequency of HLA-DRB1*13 decreased. It is known to be associated with anti-D, anti-C, anti-c, anti-K [18] and anti-E+c antibodies in the Czech population [21]. HLA-DRB1*13 and HLA-DRB1*07 behaved similarly, as shown by their frequencies in multiresponders in this study. Our study results disclose the strong association between HLA-DRB1*09 and anti-E ($p = 0.034$). The same allele was possibly relevant in previously pregnant patients without a history of receiving blood components ($p = 0.079$). This was predominantly seen in RhD-negative patients in previous studies, which was also the case in our study [22, 23]. Maluskova et al. suggested the association of HLA-DRB1*09 with anti-E because the trend was clearly demonstrated, although the sample size was too small to show any significance [21]. The common haplotype association of HLA-DRB1*09 with HLA-DQB1*03:03 suggests that the latter allele may be associated with anti-E. Our data corroborated this claim (Table 2), as the comparison of the test and control groups of HLA-DQB1*03:03 patients was significant for anti-E ($p = 0.019$) and multiresponders ($p = 0.031$), as well as possibly relevant for anti-D ($p = 0.079$). Tatari-Calderone et al. did not investigate HLA-DQB1*03:03 because of its low frequency; hence, insufficient data are available for comparison with European populations [24]. The decrease in HLA-DQB1*03:03 frequencies observed within the control group in this study as the number of blood products received increased suggests its importance in transfusion therapy patients (Figure 2). Consequently, we assigned the allele as seemingly efficient for RBC Ab development in our study population.

The most frequent allele in this study was HLA-DRB1*11. It is seldom associated with the development of anti-E antibody. Although previous studies have shown a strong association with anti-K antibody development [21], our study could not replicate these results (Table 2) precisely because of the high frequency of this allele in both groups. However, it was most frequent in anti-K patients (23.81%); therefore, we cannot rule out its importance in our population. Our results for the HLA-DRB1*11 allele demonstrate its protective role in anti-E antibody development. This explains the increase in its frequency with an increase in the number of blood products received. The HLA-DQB1*03:01 allele behaved similarly owing to its haplotype connection with HLA-DRB1*11.

The third most frequent HLA-DRB1 allele, HLA-DRB1*01, showed a possibly relevant association with anti-E ($p = 0.052$). Comparable behaviour was shown by the HLA-DQB1*05 allele ($p = 0.093$), resulting in the haplotype HLA-DRB1*01∼DQB1*05 act similarly ($p = 0.052$). Schonewille et al. found a greater influence of HLA-DRB1*01 on anti-D development [18]. Our results do not mirror this finding, as a high frequency was reported exclusively in patients with a history of previous pregnancies.

Finally, HLA-DQB1*02, the fourth most frequent in both study groups, was associated with anti-D formation. Unusually, it was not encountered among anti-K patients, which was the third most frequent RBC Ab (Table 2). We can attribute that to the protective function of HLA-DQB1*02 for anti-K development in our population. This was confirmed indirectly by the absence of HLA-DRB1*07 and HLA-DRB1*03:01 due to their common haplotype association with HLA-DQB1*02. A similar function of this allele has been shown in a SCD patient alloimunization study [22].

Although the precise mechanism of the association between HLA antigens and RBC Ab development is still unresolved, there is certainly one, which is also found in this study. We showed that the HLA-DRB1*04 allele should be considered when preparing blood components for patients in our population, as should HLA-DRB1*07 for the Rh and MNS blood group systems. The protective function of HLA-DRB1*11 in the development of anti-E should also be considered when selecting blood components. We have definitely confirmed the protective effect of HLA-DQB1*02 in anti-K development.

ACKNOWLEDGEMENTS

This study was supported (in part) by research funding from the Government of Republika Srpska.

M.M. contributed substantially to the conception and design; acquisition, analysis and interpretation of data; and drafting and revising the manuscript critically for important intellectual content; M.L. contributed substantially to the conception and design; acquisition, analysis, evaluation and interpretation of data; and drafting and revising the manuscript critically for important intellectual content; Z.A. contributed to the acquisition, analysis and interpretation of data and critically revising the manuscript for important intellectual content.

CONFLICT OF INTEREST STATEMENT

M.L. is employed as a scientific advisor by a local distributor of Inno-Train Diagnostik, which was not involved scientifically, financially or in the writing of this paper. All other authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The anonymized data that support the findings of this study are available on request. For original data, please contact milomilanka@gmail.com. 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.

How to cite this article: Milosavić M, Lilić M, Andrić Z. Impact of human leucocyte antigen class II polymorphism on anti-red blood cell antibody development: Correlations and indications. Vox Sang. 2024;119:720–7.

Received: 6 December 2023 | Revised: 19 March 2024 | Accepted: 24 March 2024

DOI: 10.1111/vox.13633

ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Laboratory approach for vaccine-induced thrombotic thrombocytopenia diagnosis in the Netherlands

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Funding information
Sanquin PPOC22-14/L2714 (to Rick Kapur)

Abstract

Background and Objectives: Vaccine-induced thrombotic thrombocytopenia (VITT) is a rare adverse effect characterized by thrombocytopenia and thrombosis occurring after COVID-19 vaccination. VITT pathophysiology is not fully unravelled but shows similarities to heparin-induced thrombocytopenia (HIT). HIT is characterized by the presence of antibodies against platelet factor 4 (PF4)/heparin complex, which can activate platelets in an FcγRIIa-dependent manner, whereas IgG-antibodies directed against PF4 play an important role in VITT.

Materials and Methods: We characterized all clinically suspected VITT cases in the Netherlands from a diagnostic perspective and hypothesized that patients who developed both thrombocytopenia and thrombosis display underlying mechanisms similar to those in HIT. We conducted an anti-PF4 ELISA and a functional PF4-induced platelet activation assay (PIPAA) with and without blocking the platelet-FcγRIIa and found positivity in both tests, suggesting VITT with mechanisms similar to those in VITT.

Results: We identified 65 patients with both thrombocytopenia and thrombosis among 275 clinically suspected VITT cases. Of these 65 patients, 14 (22%) tested positive for anti-PF4 and PF4-dependent platelet activation. The essential role of platelet-FcγRIIa in VITT with mechanisms similar to those in HIT was evident, as platelet activation was inhibited by an FcγRIIa-blocking antibody in all 14 patients.

Conclusion: Our study shows that only a small proportion of clinically suspected VITT patients with thrombocytopenia and thrombosis have anti-PF4-inducing,

FcγRIIa-dependent platelet activation, suggesting an HIT-like pathophysiology. This leaves the possibility for the presence of another type of pathophysiology ('non-HIT like') leading to VITT. More research on pathophysiology is warranted to improve the diagnostic algorithm and to identify novel therapeutic and preventive strategies.

Keywords

COVID-19, platelet factor 4, thrombocytopenia, thrombosis, vaccination

Highlights

- Twenty-two percent of patients with thrombocytopenia and thrombosis had platelet factor 4 (PF4) antibodies with PF4-induced platelet activation, indicative of vaccine-induced thrombotic thrombocytopenia (VITT) with mechanisms similar to those in heparin-induced thrombocytopenia (HIT).
- The majority of patients with thrombocytopenia and thrombosis may have other VITT mechanisms, as 78% did not display VITT with mechanisms similar to those in HIT.
- More research on non-HIT-like and HIT-like pathophysiology is warranted to improve the diagnostic algorithm and to identify novel therapeutic and preventive strategies.

INTRODUCTION

Shortly after the start of the national vaccination strategy to control SARS-CoV-2, the first reports of patients with vaccine-induced thrombotic thrombocytopenia (VITT) after vaccination with adenovirus vector-based vaccines ChAdOx1 nCoV-19 and Ad26.COV2 emerged [1–5]. VITT is clinically characterized by the occurrence of thrombocytopenia and thrombosis, sometimes at unusual sites such as in cerebral venous sinus thrombosis (CVST), occurring 5–30 days post vaccination.

According to the Dutch National Institute for Public Health and the Environment (RIVM), 12,860,272 individuals were vaccinated between the start of the Dutch vaccination campaign (6 January 2021) and 26 November 2021 [6]. During this time period, a minority of people received adenovirus-based vaccines as a first dose (1,307,732 individuals received ChAdOx1 nCoV-19 and 779,212 received Ad26.COV2.S), whereas the majority of individuals were vaccinated with mRNA-based vaccines BTN162b2 ($N = 9{,}756{,}681$) or mRNA-1273 ($N = 1{,}016{,}647$).

Platelet factor 4 (PF4; CXCL4) is released from the α-granules of platelets upon platelet activation and appears to play an important role in VITT. In VITT, immunoglobulin G (IgG) antibodies directed against PF4 are produced and mediate platelet activation [1–4, 7, 8]. Interestingly, anti-PF4-associated VITT shows similarities to heparin-induced thrombocytopenia (HIT) [9–11]. HIT is also characterized by thrombocytopenia and/or thrombosis, which develops upon treatment with the negatively charged anticoagulant heparin binding to the positively charged PF4. This results in a PF4 conformational change, apparently leading to recognition by antibodies formed against the PF4–heparin complexes. If these antibodies bind to platelets, they subsequently activate platelets via the IgG-Fc receptor expressed by platelets, namely FcγRIIa, leading to HIT [10, 12].

Between 22 March and 26 November (2021), 275 samples of patients who were diagnosed with thrombocytopenia and/or thrombosis, and were recently vaccinated with COVID-19 vaccines and therefore suspected of VITT, were sent to the Platelet/Leukocyte Serology Laboratory of Sanquin Diagnostic Services in the Netherlands for VITT diagnostic testing. At that time, there was very limited knowledge regarding the pathophysiology of VITT, and the methods for diagnosing VITT were not well established, but diagnostic analyses were deemed urgent for clinical decision making regarding the initiation of treatments such as with heparin.

Initially, due to the clinical resemblance with HIT, established HIT diagnostic tests, namely the anti-PF4/heparin ELISA and the heparin-induced platelet activation assay (HIPAA) [13], were used to support the clinical diagnosis of VITT [14]. However, it soon became clear that, in contrast to HIT, the presence of heparin was not necessary for the binding of antibodies [2]. Therefore, HIT tests were modified by testing for anti-PF4 IgG in an ELISA and testing for functional platelet activation in the presence of PF4 [14, 15].

Although, over time, guidelines for the diagnosis of VITT have been established, there is no gold standard diagnostic algorithm for VITT diagnostics [14, 16, 17]. In addition, VITT pathophysiology has not been investigated extensively. Here we describe a unique nationwide cohort with clinically suspected VITT patients, where we utilize clinical characteristics and diagnostic tests in order to improve the diagnostic algorithm for VITT, and further investigate the pathophysiology.

We stratified our cohort consisting of 275 clinically suspected VITT patients based on whether they experienced thrombocytopenia and/or thrombosis, as we hypothesized that patients who experienced both thrombocytopenia and thrombosis upon vaccination might predominantly have FcγRIIa-dependent platelet activating antibodies, like in HIT [1, 2]. By testing for anti-PF4 IgG antibodies by ELISA and the PF4-induced platelet activation assay (PIPAA) with and without

blockade of platelet-FcγRIIa, we aimed to determine whether these patients experienced VITT with mechanisms similar to those in HIT.

MATERIALS AND METHODS

Sample and data collection

After the first potential VITT reports in the Netherlands in the beginning of 2021, it was agreed upon and included in national guidelines that blood samples of patients who were suspected of having VITT be sent to the Platelet/Leukocyte Serology Laboratory of Sanquin Diagnostic Services, which is the national reference laboratory responsible for HIT and VITT diagnostics [18]. Blood samples and clinical data were obtained from 69 hospitals in the Netherlands, between 22 March 2021 and 26 November 2021. All patients received at least one dose of the mRNA vaccines BTN162b2 (Pfizer-BioNTech) or mRNA-1273 (Moderna), or adenovirus vector vaccines ChAdOx1 nCoV-19 (Oxford-AstraZeneca) or Ad26.COV2.S (Janssen/Johnson & Johnson), with the exception of 22 patients of whom the received vaccine was unknown. Control plasma samples and donor platelets were obtained from healthy volunteers who had given informed consent through Sanquin Blood Supply. Data were collected for the purpose of improving VITT diagnostics, and were provided by physicians and clinical chemists and manually reviewed by physicians from the Platelet/Leucocyte Serology Laboratory. We included only patients who were 18 years of age or older; patients treated with heparin for VITT-related symptoms prior to sampling ($N = 13$) were excluded because we could not rule out HIT. Data on vaccination status of the healthy blood donors were not available to us. Data from patients who were sent in for follow-up were not included in our cohort. This research was performed in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Anti-PF4 IgG ELISA

Similar to previously described methods, an in-house-developed anti-PF4 IgG ELISA, which does not involve the complexation of PF4 with heparin or any other negatively charged molecules, was used to determine the presence of anti-PF4 [19, 20]. A 96-well plate (Thermo Fisher Scientific, Waltham, MA) was coated with PF4 (3 μg/mL in phosphate buffered saline [PBS], ChromaTec, Greifswald, Germany) for 90 min at 37°C and washed with 0.05% Tween in PBS. After washing, diluted patient serum was added and incubated for 1 h at room temperature. After incubation, plates were washed, and GaH-HRP IgG (IgG-HRP + TRIS washing buffer/BSA 0.2%; Jackson ImmunoResearch, Westgrove, PA) was added and incubated for 60 min. The plate was washed, and 100 μL OPD solution (2.5 mL phosphate–citrate buffer +1 tablet of OPD [2 mg, O-phenylenediamine; Sigma-Aldrich, Zwijndrecht, The Netherlands] and 0.001% H_2O_2) was added to stain HRP. The plate was incubated for 5 min at room temperature. After staining, the reaction was stopped with H_2SO_4. Extinction was measured at 492 nm using the Epoch 2 microplate reader (BioTek). Results were expressed in optical density (OD) units. We defined an OD < 1.0 as negative, indicating the absence or low levels of anti-PF4 IgG; 1.0 ≤ OD < 2.0 as weakly positive, indicating intermediate levels of IgG; and OD ≥ 2.0 as positive, indicating high levels of anti-PF4 IgG [2].

Functional FcγRIIa-mediated PF4-dependent platelet activation assay

The PIPAA was performed as previously described by Greinacher et al. [13], with slight modifications. Whole blood was obtained from four healthy donors. Platelets were purified in the presence of adenine citrate dextrose (ACD) solution A. Platelet-rich plasma was resuspended in 11.1% ACD and 5% Apyrase (Sigma-Aldrich, Zwijndrecht, The Netherlands) after centrifugation for 7 min at 650*g* and washing with washing buffer (2.5 IU/mL Apyrase and 1 U/mL Hirudin in Tyrode's buffer containing 0.4% bovine serum albumin [Bayer Pentex], 0.1% glucose and 5% bicarbonate buffer). Platelets (75 μL) with and without FcγRIIa (CD32)-blocking monoclonal antibody clone IV.3 (final concentration 1.9 μg/mL; Sanquin Research, Amsterdam, The Netherlands) were incubated in a microtitre plate for at least 45 min at 37°C, and were subsequently incubated with heparin 2 U/mL (Leo Pharma, Amsterdam, The Netherlands), heparin 1000 U/mL (excess heparin), hirudin 500 U/mL (control; Sigma-Aldrich, Zwijndrecht, The Netherlands) or PF4 10 μg/mL (final concentration; Chromatec, Germany) in a microtitre plate. The microtitre plate was placed on a magnetic stirrer (50 min, 500–600 rpm) with two steel spheres in each well, and heat-inactivated patient serum was added. Every 5 min, transparency of the suspension was assessed using an indirect light source. Platelet activation was established if aggregates formed within 45 min or less. As a positive control, we included pooled human sera from patients diagnosed with HIT, with strong reactive heparin/PF4-antibodies and platelet activation occurring within 10 min in the PIPAA. As a negative control, we used inert sera from healthy donors with blood group AB. FcγRIIa-mediated inhibition was concluded if PF4-dependent platelet activation was delayed by at least 15 min. Activation was assessed up to 45 min, and 50 min in case of inconclusive results (when inactivated patient serum causes agglutination with platelets from two out of four different donors). Results are reported as negative, positive or inconclusive. The PIPAA is considered positive if platelet activation occurs in the presence of hirudin (buffer) and PF4 with at least three donors showing activation, and when there is no platelet activation in the presence of FcγRIIa (CD32)-blocking antibody clone IV.3 and excess heparin. Activation was measured by time the elapsed until platelet agglutination. We report time until platelet activation as the mean of time elapsed until agglutination in isolated platelets from four different donors. Platelet donors were selected randomly, and we did not control for FcγRIIa polymorphisms.

Although platelet activation in the presence of 2 U/mL heparin was tested in PIPAA, these results were not used in our diagnostic algorithm [21].

Data analysis

Data analysis was performed with Stata version 16.1 (StataCorp, College Station, TX) and R (R version 4.1.2). Analyses were considered significant if the *p*-value was <0.05. In order to analyse the difference in occurrence of VITT with mechanisms similar to those in HIT between males and females with thrombocytopenia and thrombosis, we used a chi-squared test of independence.

RESULTS

Baseline and clinical characteristics of 275 clinically suspected VITT patients

The baseline clinical characteristics of 275 patients with clinical suspicion of VITT are presented in Table 1, and grouped according to the occurrence of thrombocytopenia and/or thrombosis. In our cohort, 221 of 275 (80%) clinically suspected VITT patients presented with thrombocytopenia (platelet counts $<150 \times 10^9$/L) and 90 of 275 (33%) with thrombosis.

A total of 65 out of 275 (24%) clinically suspected VITT patients had both thrombocytopenia and thrombosis. These patients had a median age of 63 years, and 49% of them were female. Of these 65 patients, 43 (66%) were vaccinated with adenovirus vector vaccines (ChAdOx1 nCoV-19 or Ad26.COV2.S).

A total of 156 out of 275 (57%) patients presented with isolated thrombocytopenia, and 25 out of 275 (9%) with thrombosis without thrombocytopenia.

For 26 out of 275 (10%) patients, no clinical characteristics were reported. Three out of the 275 patients did not experience thrombocytopenia or thrombosis and were considered not likely to have VITT.

Anti-PF4 IgG ELISA and FcγRIIa-dependent PIPAA

In our diagnostic algorithm, we first performed the anti-PF4 IgG ELISA (Figure S1), followed by the FcγRIIa-dependent PIPAA (Figure S2, panel A). This ELISA was validated by testing it with samples from 49 healthy blood donors, in whom it did not produce a positive result (Figure S1). Out of the 65 patients with thrombocytopenia and thrombosis, 47 tested negative in the anti-PF4 IgG ELISA, 1 tested weakly positive and 17 tested positive (Table 1). In 14 out of the 65 (22%) patients with thrombocytopenia and thrombosis testing positive in the anti-PF4 ELISA, FcγRIIa-dependent platelet activation was observed (Figure S2, panels A and B, respectively). Since FcγRIIa-dependent platelet activation is also observed in HIT, we classified this group of patients as 'VITT with mechanisms similar to those in HIT'. In this group, 6 out of 14 patients (43%) presented with intracranial thrombosis, of whom 2 had CVST (Table S1).

One patient who tested positive in the anti-PF4 IgG ELISA and FcγRIIa-dependent PIPAA had been tested for VITT earlier. At the time of the initial test, this patient presented with pulmonary embolism, mesenterial thrombosis and CVST, and tested positive in the anti-PF4 ELISA. However, this patient did not test positive in the initial PIPAA, likely due to the sample not being diluted before testing because initial testing was done at an early stage of implementing the PIPAA [22]. After 22 days, the anti-PF4 IgG ELISA and FcγRIIa-dependent PIPAA were performed with new samples, and both tests were found to be positive. This data was included in this report.

In the Netherlands, ChAdOx1 nCoV-19 was the first vaccine to be used during the Dutch vaccination campaign. The first groups vaccinated with adenovirus vector vaccines primarily consisted of healthcare professionals, the majority of whom were likely female, followed by individuals over the age of 60. Therefore, we tested whether there was any association between sex and VITT with mechanisms similar to those in HIT in patients with thrombocytopenia and thrombosis; however, we did not observe a statistically significant association (χ^2 [1, $N = 65$] $= 0.447$, $p = 0.504$). We also found no significant association between the age and VITT with mechanisms similar to those in HIT ($W = 2092$, $p = 0.3612$, 95% CI [−2.999937 to 10.000036]).

Type of vaccines in clinically suspected VITT patients

All 19 patients with both a positive anti-PF4 IgG ELISA and positive FcγRIIa-dependent PIPAA received adenoviral vector vaccines (Table S2). Of the 14 VITT patients in our cohort, 11 patients received ChAdOx1 nCoV-19 and 3 received Ad26.COV2.S.

Overall, we observed PF4 antibodies in a larger proportion of clinically VITT suspected patients who received adenovirus-based vaccines: in 23 out of 127 (18%) of those who received ChAdOx nCoV-19 and 4 out of 8 (50%) who received Ad26.COV2.S; and in 1 out of 36 (3%) who had received mRNA-1273 and 1 out of 81 (1%) of BTN162b2 recipients. We observed FcγRIIa-dependent PF4-mediated platelet activation in 19 out of 127 (15%) of ChAdOx nCoV-19 vaccinees and 4 out of 8 (50%) of Ad26.COV2.S vaccinees (Table S2). Although less frequent, FcγRIIa-dependent PF4-mediated platelet activation in the absence of anti-PF4 was observed in mRNA-based vaccinees: in 1 out of 36 (3%) mRNA-1273 vaccinees and 2 out of 82 (2%) BTN162b2 vaccinees (Table S2, panel C). Notably, two mRNA-based vaccine recipients tested weakly positive in the anti-PF4 ELISA but negative in the PIPAA.

Platelet activation in suspected VITT patients treated with heparin

Although patients treated with heparin prior to sampling were excluded, we analysed whether serum of these patients induced platelet activation. We performed an FcγRIIa-dependent PIPAA with the serum of these patients, and found that in three patients there was platelet activation in the presence of PF4 and heparin (Figure S2, panel D). Only two heparin-treated patients tested positive in both the FcγRIIa-dependent PIPAA and anti-PF4 ELISA; one of these patients had both thrombocytopenia and thrombosis (and could be

TABLE 1 Baseline and clinical characteristics of 275 VITT-suspected patients.

	Clinically VITT-suspected patients ($N = 275$)	Subgroups, based on clinical characteristics				
		Thrombocytopenia and thrombosis ($N = 65$)	Thrombocytopenia ($N = 156$)	Thrombosis ($N = 25$)	No clinical characteristics known ($N = 26$)	No thrombocytopenia, no thrombosis ($N = 3$)
Demographics						
Median age (IQR)	63 (54–69)	63 (59–72)	63 (52–72)	62 (47–64)	64 (59–70)	64 (64–65)
Female sex, no. (%)	132 (48)	32 (49)	71 (45)	12 (48)	14 (54)	3 (100)
Male sex, no. (%)	143 (52)	33 (51)	85 (55)	12 (52)	12 (46)	–
Vaccination						
Vaccine type, no. (%)						
ChAdOx1 nCoV-19	127 (46)	40 (62)	57 (37)	14 (56)	13 (50)	3 (100)
Ad26.COV2.S	8 (3)	3 (5)	4 (2.6)	1 (4)	–	–
mRNA-1273	36 (13)	5 (8)	24 (15)	3 (12)	4 (15)	–
BTN162b2	82 (30)	14 (22)	60 (39)	4 (16)	4 (15)	–
No data available	22 (8)	3 (5)	11 (7)	2 (12)	5 (19)	–
Days between admission and vaccination						
Mean (standard deviation)	22 (18)	17 (11)	24 (20)	29 (20)	30 (43)	–
Interquartile range	10–28	11–22	9–32	18–37	10–29	–
Number of vaccination, no. (%)						
First dose	45 (16)	12 (19)	25 (16)	4 (16)	4 (15)	–
Second dose	74 (27)	14 (22)	52 (33)	3 (12)	5 (19)	–
Third dose	3 (1)	1 (2)	1 (1)	1 (4)	–	–
No clinical data available	153 (56)	38 (59)	78 (50)	16 (68)	17 (65)	3 (100)
Clinical presentation, no. (%)						
Thrombocytopenia (<150 $\times 10^9$/L)	221 (80)	65 (100)	156 (100)	–	–	–
No thrombocytopenia	18 (7)	–	–	15 (60)	–	3 (100)
No clinical data available	36 (36)	–	–	10 (40)	26 (100)	–
Thrombosis	90 (33)	65 (100)	–	25 (100)	–	–
No thrombosis	140 (51)	–	130 (83)		7 (27)	3 (100)
No clinical data available	45 (16)	–	26 (17)		19 (73)	–

TABLE 1 (Continued)

	Clinically VITT-suspected patients ($N = 275$)	Subgroups, based on clinical characteristics				
		Thrombocytopenia and thrombosis ($N = 65$)	Thrombocytopenia ($N = 156$)	Thrombosis ($N = 25$)	No clinical characteristics known ($N = 26$)	No thrombocytopenia, no thrombosis ($N = 3$)
Site of thrombosis, no. (%)						
Deep vein thrombosis (DVT)	8 (3)	7 (11)	—	1 (4)	—	—
Mesenteric thrombosis	4 (1)	2 (3)	—	2 (8)	—	—
Intercranial	12 (4)	6 (9)	—	6 (24)		
Cerebral venous sinus thrombosis	7 (3)	2 (3)	—	5 (20)	—	—
Vena porta thrombosis	6 (2)	6 (9)	—	—	—	—
Pulmonary embolism[a]	23 (8)	15 (23)	—	8 (32)	—	—
Multiple sites	6 (2)	4 (6)	—	2 (8)	—	—
Other	16 (6)	14 (22)	—	2 (8)	—	—
No clinical data available	15 (5)	11 (17)	26 (17)	4 (16)	26 (100)	—
Laboratory tests						
Anti-PF4 ELISA negative	243 (88)	47 (72)	145 (93)	24 (96)	24 (92)	3 (100)
PIPAA negative	236 (85)	45 (69)	141 (90)	23 (90)	24 (92)	3 (100)
PIPAA positive	7 (3)	2 (3)	4 (3)	1 (4)	—	—
Anti-PF4 ELISA weakly positive	11 (4)	1 (2)	9 (6)	—	1 (4)	—
PIPAA negative	10 (4)	1 (2)	8 (5)	—	1 (4)	—
PIPAA positive	1 (0.4)	—	1 (1)	—	—	—
Anti-PF4 ELISA positive	21 (8)	17 (26)	2 (1)	1 (4)	1 (4)	—
PIPAA negative	3 (1)	3 (5)	—		—	—
PIPAA positive	18 (7)	14 (22)[b]	2 (1)	1 (4)	1 (4)	—

Note: Patients were stratified based on whether patients experienced thrombocytopenia and/or thrombosis. Other thrombosis sites in VITT patients include aorta, arm, vein, leg and neck.
Abbreviations: PIPAA, PF4-induced platelet activation assay; VITT, vaccine-induced thrombotic thrombocytopenia.
[a]Thrombosis marked by physician as pulmonary embolism, but specific primary site of thrombosis is not known.
[b]Considered VITT patients with HIT characteristics.

considered as VITT with mechanisms similar to those in HIT), while the other patient had isolated thrombocytopenia.

DISCUSSION

In the context of the national Dutch VITT policy program, we analysed clinical and laboratory data of clinically suspected VITT patients in the Netherlands between 22 March (time of the first report of a suspected VITT patients in the Netherlands) and 26 November 2021 [18]. By categorizing patients based on post-vaccination thrombocytopenia and/or thrombosis and conducting the recommended set of laboratory tests based on regular testing for HIT but adjusted for VITT, an anti-PF4 IgG ELISA and FcγRIIa-dependent PIPAA, we evaluated the presence of VITT with an underlying HIT mechanism. Among 275 patients, 24% had both thrombocytopenia and thrombosis. Of these, 22% ($N = 14$) tested positive in both tests, indicating an HIT-like pathophysiology. We observed an incidence of 1 case per 118.884 after a first dose of ChAdOx1 nCoV-19 and 1 case per 259.737 after a first dose of Ad26.COV2.S. These findings are in line with reported incidences ranging from 1 case per 26,500 to 127,300 after a first dose of ChAdOx1 nCoV-19 and 1 case per 26,300 after a dose of Ad26.COV2.S [23].

We found two patients with thrombocytopenia and thrombosis, four patients with isolated thrombocytopenia and one patient with thrombosis tested positive for FcγRIIa-dependent PF4-dependent platelet activation without detectable levels of anti-PF4 antibodies (Table 1). Interestingly, we observed three thrombocytopenic patients vaccinated with mRNA vaccines, which are highly unlikely to cause VITT (weak), positive in the FcγRIIa-dependent assay while lacking anti-PF4 (Table S2). The fact that patients tested positive for anti-PF4 but negative in the FcγRIIa-dependent platelet activation assay could be explained by the presence of non-pathogenic anti-PF4 described in 5%–8% of healthy blood donors and other patients groups [17]. Considering that 51 out of 65 (78%) patients with thrombocytopenia and thrombosis did not show PF4 antibodies or FcγRIIa-dependent platelet activation, the presence of alternative mechanisms beyond the anti-PF4-mediated HIT-like pathway that can affect platelet activation in clinically suspected VITT are plausible. These results could be explained by other underlying conditions such as immune thrombocytopenia (ITP) or COVID-19, both hallmarked by thrombocytopenia and/or thrombosis [24–26]. However, vaccine constituents [27–29] or neutrophil–platelet interaction [30], along with the presence of other antibodies such as those against platelet-specific chemokine neutrophil-activating peptide 2 (NAP2; CXCL7) [31], could explain anti-PF4-independent platelet activation observed in some patients. We believe that PF4 itself also contributes to FcγRIIa-dependent PF4-mediated platelet activation. This is supported by a recent study, which showed that PF4 activates platelets via the cellular myeloproliferative leukaemia protein (c-Mpl)-Janus kinase 2 (Jak2) pathway, supporting a model of PF4-based immune complexes that activate platelets through binding of the Fc domain to FcγRIIA and PF4 to c-Mpl [32].

Nine patients exhibited intermediate anti-PF4 levels with no FcγRIIa and PF4-dependent platelet activation. This might stem from non-vaccination events like surgery, infections (COVID-19 or non-COVID-19) [33–36] or treatments such as IVIg which is known to diminish antibody-induced platelet activation, potentially affecting PIPAA outcomes [15].

We performed both the anti-PF4 ELISA and PIPAA in accordance with early VITT guidelines [14, 37]. In the meantime, more detailed algorithms have been developed in which either a positive anti-PF4 or a positive functional assay is considered sufficient to support a VITT diagnosis [17, 38]. Given the discrepancies in our diagnostics test, combining an ELISA with a functional assay—although not widely available—could better distinguish genuine antibody-mediated VITT cases from others testing positive for anti-PF4/polyanion, thus ensuring appropriate treatment [16, 39–41]. The large proportion of patients with thrombocytopenia and/or thrombosis in individuals who did not test positive for anti-PF4 may be partially, but not entirely, explained by other underlying conditions such as cancer [17]. Given the apparent and significant relevance of non-HIT-like mechanisms in clinically suspected VITT patients as revealed in our study, the diagnostic algorithm of VITT could potentially be strengthened in this respect in follow-up studies.

Our nationwide cohort provides substantial amounts of data but lacks information on D-dimer levels, underlying conditions and treatment. Consequently, patients with clinical features unrelated to vaccination may have inadvertently been included and could have hindered VITT patient identification, and treatments such as IVIg can modulate platelet function, further hindering identification of VITT patients [15]. D-dimer levels, which could aid in the early detection of VITT, were not collected, which prevented us from adhering to previously established VITT cases [7]. Additionally, we analysed samples taken only at the time of VITT suspicion and not in a longitudinal manner. It may be that the assays did not detect anti-PF4 IgG or platelet activation due to low levels of antibodies or consumption of antibodies.

In summary, we studied 275 clinically suspected VITT patients, identifying at least 14 cases with VITT with mechanisms similar to those in HIT, based on the occurrence of both thrombocytopenia and thrombosis and positive results in both the anti-PF4 IgG ELISA and FcγRIIa-dependent PIPAA. All patients were vaccinated with adenovirus vector vaccines, and 2 of the 14 VITT patients presented with CVST. However, 51 out of 65 (78%) patients with thrombocytopenia and thrombosis did not display a mechanism similar to that in HIT, suggesting possible alternative mechanisms involved in VITT pathophysiology, or perhaps a different diagnosis than VITT. Therefore, more research on the non-HIT-like and HIT-like pathophysiology is warranted to not only increase the accuracy of the diagnostic algorithm but also to identify novel therapeutic targets and preventive strategies.

ACKNOWLEDGEMENTS

We thank Sanquin Diagnostic Services (Platelet-leukocyte laboratory) for processing the samples and C. Caram-Deelder for her advice and

help in processing the data. This work was supported by Grant Sanquin PPOC22-14/L2714 (to Rick Kapur).

R.M. and L.P. interpreted and analysed the data, wrote the manuscript and developed the VITT database; S.H.-v.E. designed the VITT database and performed diagnostic tests; Y.H., J.C., M.K., A.S., J.Z., J.v.d.B., E.v.d.S. and M.d.H. contributed to the interpretation of the results; R.K. obtained research funding, interpreted the data and wrote the manuscript.

CONFLICT OF INTEREST STATEMENT

M.K. has an unrestricted research grant from Sobi, and receives speaker fees from Roche, Sobi and BMS. All other authors have no relevant 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.

How to cite this article: Meier RT, Porcelijn L, Hofstede-van Egmond S, Henskens YMC, Coutinho JM, Kruip MJHA, et al. Laboratory approach for vaccine-induced thrombotic thrombocytopenia diagnosis in the Netherlands. Vox Sang. 2024;119:728–36.

Received: 24 December 2023 | Revised: 26 March 2024 | Accepted: 5 April 2024

DOI: 10.1111/vox.13641

ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

The influence of HLA allele and haplotype on RhE alloimmunization among pregnant females in the Chinese Han population

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Funding information
CAMS Innovation Fund for Medical Sciences, Grant/Award Number: 2021-1-I2M-060; Foundation of the First Affiliated Hospital of Chengdu Medical College, Grant/Award Number: CYFY-GQ52; School fund of Chengdu Medical College, Grant/Award Number: CYZYB22-05; 2020 Natural Science Key Project of Chengdu Medical College Foundation, Grant/Award Number: CYZZD20-05

Abstract

Background and Objectives: Anti-E alloantibody is the most common and important red blood cell (RBC) alloantibody during pregnancy. The study aimed to determine the correlation between RhE alloimmunization and human leukocyte antigen (HLA) allele polymorphism, as well as haplotype diversity, among pregnant individuals in the Chinese Han population.

Study Design and Methods: All individuals included in our study were RhE-negative pregnant women of Chinese Han ethnicity, confirmed through serological testing. Pregnancy could be the only potential stimulating factor in RBC alloimmunization. Given the serological testing, the participants were divided into anti-E (responders) and non-anti-E-producing group (non-responders). The class I and II classical HLA genotyping were determined using next-generation sequencing, and the HLA genotype and haplotype frequencies were compared between the responders and non-responders.

Results: In total, 76 responders and 94 non-responders were enrolled in this study. Comparison results showed that all HLA class I alleles had no difference between the two groups. For HLA class II phenotypes, responders had higher frequencies of HLA-DRB1*09:01, HLA-DQA1*03:02 and HLA-DQB1*03:03 phenotypes than non-responders, and the differences were statistically significant ($p_c < 0.05$). In addition, the haplotype frequency of *HLA-DRB1*09:01-DQA1*03:02-DQB1*03:03* in the RhE responders was significantly higher than in the non-responders (31.58% vs. 12.77%; odds ratio, 3.154; 95% confidence interval, 1.823–5.456; p_c value, 1.25×10^{-3}).

Conclusion: Our findings indicated that *HLA-DRB1*09:01*, *HLA-DQA1*03:02* and *HLA-DQB1*03:03* might be susceptible alleles for RhE alloimmunization among

Yujie Kong and Jie Xiao contributed equally to this study.

Chinese Han pregnant females. These three susceptible alleles constituted the unique three-locus haplotype in the RhE responders and collaborated to RhE alloimmunization.

Keywords
haplotype, HLA allele, pregnancy, RBC alloimmunization, RhE

Highlights

- Anti-E is the most common alloantibody in the Chinese population, with reported frequencies ranging from 26.4% to 41.0%.
- Our findings suggest that HLA-DRB1*09:01, HLA-DQA1*03:02 and HLA-DQB1*03:03 might be susceptible alleles for RhE alloimmunization among Chinese Han pregnant females.
- We showed that the three susceptible alleles constitute the unique three-locus haplotype in the RhE responders and together contribute to RhE alloimmunization.

INTRODUCTION

Red blood cell (RBC) alloimmunization refers to an immune response against any non-autologous RBC antigens, resulting in the generation of RBC alloantibodies. Due to the complexity of blood group systems, blood transfusion, foetal–maternal haemorrhage during pregnancy or parturition, stem cell or organ transplantation or other allogeneic blood exposure are all potential factors affecting RBC alloantibody production [1, 2]. Most RBC alloantibodies may lead to serious or fatal clinical consequences, including acute or delayed haemolytic reactions, anaemia and haemolytic disease of the newborn [1, 3]. The incidence of RBC alloimmunization after blood transfusion is the most reported at present and different studies with specific populations have come to different conclusions, with data between 1% and 10% [4–6].

From the reported incidence of RBC alloimmunization, it can be seen that not all individuals have strong immunologic responses to foreign RBCs, and the factors influencing RBC alloimmunization are constantly investigated. The established influencing factors include specific features of donors and recipients, genetic factors, immune system status and immune cell characteristics of recipients [3, 7]. The induction of alloimmunization depends on alloantigenic peptide presentation at the cell surface to T cells by human leukocyte antigen (HLA) transmembrane molecules, subsequently triggering an immune response [8]. *HLA* genes are subdivided into *HLA* class I and class II genes. The classical *HLA* class I and class II genes (*HLA-A*, *HLA-B*, *HLA-C*, *HLA-DR*, *HLA-DQ* and *HLA-DP*) are highly polymorphic [9], and different *HLA* alleles present different alloantigenic peptides, potentially influencing the T-cell immune response [10]. Therefore, *HLA* gene polymorphism may influence the recipients' immune susceptibility to RBC alloantigens [11–13].

Anti-E is the most common alloantibody in Chinese population, with reported frequencies ranging from 26.4% to 41.0% [14, 15]. Anti-E antibodies can cause severe haemolytic transfusion reactions and haemolytic disease of the foetus and newborn (HDFN) [16–19]. Recently, it was confirmed that the HLA molecule is a genetic susceptibility factor for RhE alloimmunization. *HLA-DRB1*11*, *HLA-DRB1*12*, *HLA-DRB1*15* and *HLA-DRB1*09:01* alleles were all reported to be related to RhE alloimmunization [13, 20–22]. *HLA-DQB1*06* and *HLA-DQB1*02* might also be potential risk alleles for anti-E production [23].

Currently, most of the studies focused on RhE alloimmunization caused by blood transfusion exposure, but pregnancy was another important contributor to RhE alloimmunization in Chinese women. Given that the production of anti-E antibodies is one of the main factors associated with HDFN, studies on the susceptibility of RhE alloimmunization in pregnant females seem necessary, and the corresponding literature is limited at present. Thus, our study aims to explore the relationship between *HLA* allele and haplotype diversity and RhE alloimmunization among Chinese Han pregnant females in Sichuan province, and the results can be valuable in the management of HDFN.

STUDY DESIGN AND METHODS

Study population

All pregnant females enrolled in this study were of Han nationality and come from Sichuan Provincial Maternity and Child Health Care Hospital. Written informed consent was obtained from all study participants before entering this study. The ethic was approved by the Institute of Blood Transfusion of the Chinese Academy of Medical Sciences and Sichuan Provincial Maternity and Child Health Care Hospital.

Criteria for selecting the individuals were as follows: pregnant females who had two or more pregnancies, maternal RhE negative with foetal RhE positive, without blood transfusion history, without transplantation history, without blood system diseases, without autoimmune diseases that may affect the production of RBC alloantibodies. Pregnancy could be the only potential stimulating factor in RBC alloimmunization. Eligible pregnant females who matched the inclusion criteria were divided into two groups based on the results of anti-E antibodies detection: one group with anti-E production (responders) and the other group without anti-E production

(non-responders). The responders had only anti-E alloantibody without any other alloantibodies or autoantibodies.

RBC antigen and antibody testing techniques

Screening of RBC antigens in pregnant females and their umbilical cord blood by saline agglutination test tube method. RBC alloantibodies were detected and identified by routine indirect antiglobulin testing in a low ionic strength saline with column agglutination technique (Micro Typing System, DiaMed-ID).

DNA extraction and HLA typing methods

After obtaining the informed consent form of the individual, genomic DNA was isolated from blood samples using TIANamp Blood DNA KIT (Tiangen, China). *HLA* genotyping was performed using AllType™ NGS Reagents (One Lambda, USA), with the Ion Chef™ and Ion S5™ system (Roche, Switzerland). Full gene (*HLA-A*, *HLA-B*, *HLA-C*, *HLA-DPA1* and *HLA-DQA1*) and partial gene (*HLA-DPB1*, *HLA-DQB1* and *HLA-DRB1*) were sequenced. HLA genotypes were analysed using TypeStream Visual 3.0 software (One Lambda, USA).

Haplotype estimation

The estimation of HLA haplotypes among alleles from *HLA-DRB1*, *HLA-DQA1* and *HLA-DQB1* was carried out by the Arlequin program version 3.1. For *HLA-DRB1-DQA1-DQB1* haplotypes, the haplotype frequencies were compared between RhE responders and non-responders.

Statistical analysis

Frequencies of *HLA* alleles, phenotypes and haplotypes were compared between the responders and non-responders using Chi-square test or Fisher's exact test. Comparison results were described as odds ratio (OR), 95% confidence intervals (CIs) and p value. Bonferroni inequality method was used to correct p values, to avoid a type I error. Between-group differences with results of corrected p values (p_c) less than 0.05 were considered statistically significant.

RESULTS

Study population

From 1 January 2020 to 31 December 2022, a total of 170 Chinese Han pregnant females were included in this study, and they all signed informed consent forms for this study. Among these pregnant females, 76 showed responses to RhE antigen and the others showed no response. In RhE responders and non-responders, the *HLA* class I (*HLA-A*, *HLA-B* and *HLA-C*) and class II (*HLA-DRB1*, *HLA-DQA1*, *HLA-DQB1*, *HLA-DPA1* and *HLA-DPB1*) allele distributions of the polymorphisms studied were confirmed with Hardy–Weinberg equilibrium ($p > 0.05$). In total, 172 alleles were identified among study subjects, which comprised 19 *HLA-A*, 44 *HLA-B*, 24 *HLA-C*, 31 *HLA-DRB1*, 15 *HLA-DQA1*, 15 *HLA-DQB1*, 6 *HLA-DPA1* and 18 *HLA-DPB1*.

Comparing the HLA-A, HLA-B and HLA-C phenotype frequencies between RhE responders and non-responders in Chinese pregnant females

The allele and phenotype frequencies of HLA-A, HLA-B and HLA-C between RhE responders and non-responders were calculated, and the top 10 highest differences in phenotypes are shown in Table 1. The phenotype frequency of HLA-C*14:02 was higher, and HLA-C*03:04 was lower in the RhE responders, when compared with the RhE non-responders, but it did not reach statistical significance upon correction by the number of alleles (p_c value). There was no significant difference in phenotype frequencies of the HLA-A and HLA-B among the two groups. The most prominent phenotypes in RhE responders and non-responders were HLA-A*11:01 (40.79% and 53.19%), HLA-B*46:01 (38.16% and 36.17%) and HLA-C*01:02 (51.32% and 42.55%), and their prevalence did not show any statistically significant differences between the two groups. No contribution from *HLA* class I alleles was observed for RhE alloimmunization.

Comparing the HLA-DRB1, HLA-DP and HLA-DQ phenotype frequencies between RhE responders and non-responders

Distribution in the phenotype frequencies of HLA-DRB1, HLA-DPA1, HLA-DPB1, HLA-DQA1 and HLA-DQB1 among RhE responders and non-responders are shown in Table 2. Comparing HLA phenotypes, frequencies of HLA-DRB1*09:01, HLA-DRB1*07:01, HLA-DQA1*03:02, HLA-DQA1*02:01, HLA-DQB1*03:03 and HLA-DQB1*02:02 were higher, and frequencies of HLA-DRB1*15:01, HLA-DRB1*11:01, HLA-DQA1*01:01, HLA-DQB1*06:01 and HLA-DPB1*14:01 were lower in the responders than non-responders, and the differences were statistically significant ($p < 0.05$). However, after p value correction by the number of alleles, the frequencies of HLA-DRB1*09:01 ($p_c = 1.18 \times 10^{-3}$), HLA-DQA1*03:02 ($p_c = 2.85 \times 10^{-4}$) and HLA-DQB1*03:03 ($p_c = 5.70 \times 10^{-4}$) were the significantly different phenotypes. From the particular HLA class II phenotype, the HLA-DRB1*09:01, HLA-DQA1*03:02 and HLA-DQB1*03:03 contributed the most to RhE alloimmunization.

Haplotype frequencies among *HLA-DRB1*, *HLA-DQA1* and *HLA-DQB1*

The haplotype frequencies of *HLA-DRB1-DQA1-DQB1* among RhE responders and non-responders were shown in Table 3. The

TABLE 1 The top 10 highest differences in *HLA* class I phenotypes between RhE responders and non-responders in Chinese Han pregnant females.

	RhE responders ($n = 76$[a])		RhE non-responders ($n = 94$[a])				
HLA class I allele	No. of alleles (%)	No. of HLA phenotypes (%)	No. of alleles (%)	No. of HLA phenotypes (%)	Odds ratio (95% CI)	*p* value	p_c value[b]
HLA-A							
A*11:01	36 (23.7)	31 (40.8)	56 (29.8)	50 (53.2)	0.61 (0.33–1.12)	0.107	-
A*30:01	8 (5.3)	8 (10.5)	5 (2.7)	4 (4.3)	2.65 (0.77–9.16)	0.112	-
A*11:02	2 (1.3)	2 (2.6)	10 (5.3)	9 (9.6)	0.26 (0.05–1.22)	0.130	-
A*24:02	34 (22.4)	30 (39.5)	28 (14.9)	27 (28.7)	1.62 (0.85–3.07)	0.140	-
A*33:03	6 (4.0)	6 (7.9)	13 (6.9)	13 (13.8)	0.53 (0.19–1.48)	0.222	-
A*26:01	4 (2.6)	4 (5.3)	9 (4.8)	9 (9.6)	0.53 (0.16–1.78)	0.293	-
A*02:05	3 (2.0)	3 (4.0)	1 (0.5)	1 (1.1)	3.82 (0.39–37.51)	0.469	-
A*02:07	28 (18.4)	27 (35.5)	31 (16.5)	29 (30.9)	1.24 (0.65–2.35)	0.519	-
A*02:01	10 (6.6)	9 (11.8)	9 (4.8)	9 (9.6)	1.27 (0.48–3.37)	0.633	-
A*31:01	6 (4.0)	6 (7.9)	5 (2.7)	5 (5.3)	1.53 (0.45–5.21)	0.715	-
HLA-B							
B*13:01	7 (4.6)	6 (7.9)	16 (8.5)	16 (17.0)	0.42 (0.16–1.13)	0.078	-
B*15:01	12 (7.9)	12 (15.8)	7 (3.7)	7 (7.5)	2.33 (0.87–6.25)	0.086	-
B*51:02	5 (3.3)	5 (6.6)	1 (0.5)	1 (1.1)	6.55 (0.75–57.31)	0.090	-
B*51:01	11 (7.2)	10 (13.2)	6 (3.2)	6 (6.4)	2.22 (0.77–6.42)	0.133	-
B*40:06	5 (3.3)	5 (6.6)	2 (1.1)	2 (2.1)	3.24 (0.61–17.19)	0.244	-
B*55:02	8 (5.3)	8 (10.5)	6 (3.2)	6 (6.4)	1.73 (0.57–5.21)	0.329	-
B*13:02	6 (4.0)	6 (7.9)	5 (2.7)	4 (4.3)	1.93 (0.52–7.10)	0.345	-
B*15:02	5 (3.3)	5 (6.6)	9 (4.8)	9 (9.6)	0.67 (0.21–2.08)	0.480	-
B*35:01	3 (2.0)	3 (4.0)	7 (3.7)	7 (7.5)	0.51 (0.13–2.05)	0.515	-
B*58:01	7 (4.6)	7 (9.2)	11 (5.9)	11 (11.7)	0.77 (0.28–2.08)	0.600	-
HLA-C							
C*03:04	10 (6.6)	9 (11.8)	25 (13.3)	24 (25.5)	0.39 (0.17–0.90)	0.025	0.600
C*14:02	10 (6.6)	10 (13.2)	4 (2.1)	4 (4.3)	3.41 (1.02–11.34)	0.036	0.846
C*06:02	10 (6.6)	10 (13.2)	6 (3.2)	5 (5.3)	2.70 (0.88–8.26)	0.073	-
C*15:02	2 (1.3)	2 (2.6)	9 (4.8)	9 (9.6)	0.26 (0.05–1.22)	0.114	-
C*01:02	42 (27.6)	39 (51.3)	45 (23.9)	40 (42.6)	1.42 (0.78–2.61)	0.255	-
C*03:03	13 (8.6)	13 (17.1)	13 (6.9)	13 (13.8)	1.29 (0.56–2.97)	0.555	-
C*02:02	2 (1.3)	2 (2.6)	1 (0.5)	1 (1.1)	2.51 (0.22–28.26)	0.587	-
C*03:02	7 (4.6)	7 (9.2)	11 (5.9)	11 (11.7)	0.77 (0.28–2.08)	0.600	-
C*12:03	5 (3.3)	4 (5.3)	3 (1.6)	3 (3.2)	1.69 (0.37–7.77)	0.702	-
C*07:02	21 (13.8)	20 (26.3)	29 (15.4)	27 (28.7)	0.89 (0.45–1.75)	0.727	-

Abbreviation: CI, confidence interval.

[a]*n* denotes number of pregnant females enrolled in the group.

[b]*p* value corrected by the Bonferroni inequality method. $p_c = p$ value corrected by 19, 44 and 24 for HLA-A, HLA-B and HLA-C, respectively. p_c value was calculated only for *p* value <0.05.

haplotype of *HLA-DRB1*09:01-DQA1*03:02-DQB1*03:03*, which consisted of all three susceptible alleles, was the most frequent haplotype in both groups, and the frequencies of other haplotypes were less than 10%. The haplotype frequency of *HLA-DRB1*09:01-DQA1*03:02-DQB1*03:03* in the RhE responders was significantly higher than in the non-responders (31.58% vs. 12.77%; odds ratio, 3.154; 95% confidence interval, 1.823–5.456; p_c value, 1.25×10^{-3}). The frequencies of *HLA-DRB1*15:01-DQA1*01:02-DQB1*06:01* and *HLA-DRB1*11:01-DQA1*05:05-DQB1*03:01* were lower, and the frequency of *HLA-DRB1*07:01:01-DQA1*02:01-DQB1*02:02* was higher in the responders than non-responders and the differences were statistically significant ($p < 0.05$). After *p* value correction by the number of haplotypes, they did not reach statistical significance.

TABLE 2 The top 10 highest differences in *HLA* class II phenotypes between RhE responders and non-responders in Chinese Han pregnant females.

	RhE responders ($n = 76$[a])		RhE non-responders ($n = 94$[a])				
HLA class II allele	No. of alleles (%)	No. of HLA phenotypes (%)	No. of alleles (%)	No. of HLA phenotypes (%)	Odds ratio (95% CI)	*p* value	p_c value[b]
HLA-DRB1							
DRB1*09:01	49 (32.2)	43 (56.6)	25 (13.3)	24 (25.5)	3.80 (1.99–7.27)	0.000038	0.00118
DRB1*15:01	9 (5.9)	7 (9.2)	25 (13.3)	24 (25.5)	0.30 (0.12–0.73)	0.006	0.186
DRB1*07:01	12 (7.9)	12 (15.8)	5 (2.7)	5 (5.3)	3.34 (1.12–9.94)	0.024	0.744
DRB1*11:01	4 (2.6)	4 (5.3)	15 (8.0)	14 (14.9)	0.32 (0.10–1.01)	0.042	1.302
DRB1*08:02	4 (2.6)	4 (5.3)	1 (0.5)	1 (1.1)	5.17 (0.57–47.23)	0.174	-
DRB1*12:02	8 (5.3)	8 (10.5)	17 (9.0)	16 (17.0)	0.57 (0.23–1.42)	0.227	-
DRB1*04:06	4 (2.6)	4 (5.3)	9 (4.8)	9 (9.6)	0.53 (0.16–1.78)	0.293	-
DRB1*13:01	3 (2.0)	3 (4.0)	1 (0.5)	1 (1.1)	3.82 (0.39–37.51)	0.326	-
DRB1*04:05	10 (6.6)	10 (13.2)	8 (4.3)	8 (8.5)	1.63 (0.61–4.36)	0.328	-
DRB1*16:02	8 (5.3)	8 (10.5)	6 (3.2)	6 (6.4)	1.73 (0.57–5.21)	0.329	-
HLA-DQA1							
DQA1*03:02	49 (32.2)	43 (56.6)	24 (12.8)	23 (24.5)	4.02 (2.09–7.71)	0.000019	0.000285
DQA1*02:01	12 (7.9)	12 (15.8)	5 (2.7)	5 (5.3)	3.34 (1.12–9.94)	0.024	0.360
DQA1*01:01	1 (0.7)	1 (1.3)	8 (4.3)	8 (8.5)	0.14 (0.02–1.17)	0.043	0.645
DQA1*05:05	7 (4.6)	7 (9.2)	16 (8.5)	15 (16.0)	0.53 (0.21–1.39)	0.193	-
DQA1*01:02	21 (13.8)	20 (26.3)	38 (20.2)	33 (35.1)	0.66 (0.34–1.28)	0.219	-
DQA1*01:04	11 (7.2)	10 (13.2)	17 (9.0)	17 (18.1)	0.69 (0.29–1.60)	0.382	-
DQA1*06:01	8 (5.3)	8 (10.5)	15 (8.0)	14 (14.9)	0.67 (0.27–1.70)	0.399	-
DQA1*01:03	12 (7.9)	12 (15.8)	21 (11.2)	19 (20.2)	0.74 (0.33–1.64)	0.458	-
DQA1*03:03	10 (6.6)	10 (13.2)	9 (4.8)	9 (9.6)	1.43 (0.55–3.72)	0.461	-
DQA1*05:01	5 (3.3)	5 (6.6)	9 (4.8)	9 (9.6)	0.67 (0.21–2.08)	0.480	-
HLA-DQB1							
DQB1*03:03	50 (32.9)	43 (56.6)	25 (13.3)	24 (25.5)	3.80 (1.99–7.27)	0.000038	0.00057
DQB1*06:01	10 (6.6)	10 (13.2)	30 (16.0)	28 (29.8)	0.36 (0.16–0.79)	0.010	0.150
DQB1*02:02	10 (6.6)	10 (13.2)	4 (2.1)	4 (4.3)	3.41 (1.02–11.34)	0.036	0.540
DQB1*03:01	20 (13.2)	19 (25.0)	40 (21.3)	34 (36.2)	0.59 (0.30–1.15)	0.118	-
DQB1*06:02	4 (2.6)	4 (5.3)	11 (5.9)	11 (11.7)	0.42 (0.13–1.37)	0.141	-
DQB1*05:01	2 (1.3)	2 (2.6)	8 (4.3)	8 (8.5)	0.29 (0.06–1.41)	0.188	-
DQB1*06:03	3 (2.0)	3 (4.0)	1 (0.5)	1 (1.1)	3.82 (0.39–37.51)	0.326	-
DQB1*04:01	9 (5.9)	9 (11.8)	8 (4.3)	8 (8.5)	1.44 (0.53–3.94)	0.472	-
DQB1*02:01	5 (3.3)	5 (6.6)	9 (4.8)	9 (9.6)	0.67 (0.21–2.08)	0.480	-
DQB1*05:02	18 (11.8)	18 (23.7)	22 (11.7)	19 (20.2)	1.23 (0.59–2.54)	0.586	-
HLA-DPA1							
DPA1*02:02	91 (59.9)	66 (86.8)	98 (52.1)	73 (77.7)	1.90 (0.83–4.33)	0.123	-
DPA1*02:01	12 (7.9)	12 (15.8)	19 (10.1)	19 (20.2)	0.74 (0.33–1.64)	0.458	-
DPA1*02:07	1 (0.7)	1 (1.3)	3 (1.6)	3 (3.2)	0.40 (0.04–3.97)	0.629	-
DPA1*04:01	5 (3.3)	5 (6.6)	7 (3.7)	7 (7.5)	0.88 (0.27–2.88)	0.826	-
DPA1*01:03	43 (28.3)	40 (52.6)	60 (31.9)	50 (53.2)	0.98 (0.53–1.79)	0.942	-
DPA1*01:11	0 (0.0)	0 (0.0)	1 (0.5)	1 (1.1)	-	1.000	-
HLA-DPB1							
DPB1*14:01	2 (1.3)	2 (2.6)	10 (5.3)	10 (10.6)	0.23 (0.05–1.07)	0.043	-
DPB1*13:01	15 (9.9)	14 (18.4)	10 (5.3)	10 (10.6)	1.90 (0.79–4.55)	0.147	-

(Continues)

TABLE 2 (Continued)

HLA class II allele	RhE responders ($n = 76$[a]) No. of alleles (%)	RhE responders ($n = 76$[a]) No. of HLA phenotypes (%)	RhE non-responders ($n = 94$[a]) No. of alleles (%)	RhE non-responders ($n = 94$[a]) No. of HLA phenotypes (%)	Odds ratio (95% CI)	*p* value	p_c value[b]
DPB1*04:02	4 (2.6)	4 (5.3)	10 (5.3)	10 (10.6)	0.47 (0.14–1.55)	0.205	-
DPB1*04:01	7 (4.6)	7 (9.2)	12 (6.4)	12 (12.8)	0.69 (0.26–1.86)	0.464	-
DPB1*03:01	5 (3.3)	5 (6.6)	9 (4.8)	9 (9.6)	0.67 (0.21–2.08)	0.480	-
DPB1*05:01	62 (40.8)	52 (68.4)	77 (41.0)	60 (63.8)	1.23 (0.65–2.33)	0.530	-
DPB1*135:01	2 (1.3)	2 (2.6)	1 (0.5)	1 (1.1)	2.51 (0.22–28.26)	0.587	-
DPB1*02:02	14 (9.2)	14 (18.4)	15 (8.0)	15 (16.0)	1.19 (0.53–2.65)	0.671	-
DPB1*02:01	31 (20.4)	28 (36.8)	34 (18.1)	32 (34.0)	1.13 (0.60–2.13)	0.704	-
DPB1*19:01	1 (0.7)	1 (1.3)	2 (1.1)	2 (2.1)	0.61 (0.06–6.90)	1.000	-

Abbreviation: CI, confidence interval.
[a]*n* denotes number of pregnant females enrolled in the group.
[b]*p* value corrected by the Bonferroni inequality method. $p_c = p$ value corrected by 31, 15, 15, 6 and 18 for HLA-DRB1, HLA-DQA1, HLA-DQB1, DPA1 and DPB1, respectively. p_c value was calculated only for *p* value <0.05.

TABLE 3 The top 10 highest differences in haplotype frequencies of *HLA-DRB1-DQA1-DQB1* between RhE responders and non-responders in Chinese Han pregnant females.

Haplotype	RhE responders ($n = 152$[a]) No. of haplotypes (%)	RhE non-responders ($n = 188$[a]) No. of haplotypes (%)	Odds ratio (95% CI)	*p* value	p_c value[b]
DRB1*09:01-DQA1*03:02-DQB1*03:03	48 (31.6)	24 (12.8)	3.15 (1.82–5.46)	0.000024	0.00125
DRB1*15:01-DQA1*01:02-DQB1*06:01	1 (0.7)	10 (5.3)	0.12 (0.02–0.93)	0.016	0.832
DRB1*07:01-DQA1*02:01-DQB1*02:02	10 (6.6)	4 (2.1)	3.24 (1.00–10.54)	0.040	2.08
DRB1*11:01-DQA1*05:05-DQB1*03:01	4 (2.6)	14 (7.5)	0.34 (0.11–1.04)	0.049	2.548
DRB1*15:01-DQA1*01:02-DQB1*06:02	4 (2.6)	11 (5.9)	0.44 (0.14–1.39)	0.151	-
DRB1*12:02-DQA1*06:01-DQB1*03:01	7 (4.6)	15 (8.0)	0.56 (0.22–1.40)	0.209	-
DRB1*08:03-DQA1*01:03-DQB1*06:01	9 (5.9)	18 (9.6)	0.59 (0.26–1.36)	0.215	-
DRB1*04:06-DQA1*03:01-DQB1*03:02	4 (2.6)	9 (4.8)	0.54 (0.16–1.78)	0.303	-
DRB1*01:01-DQA1*01:01-DQB1*05:01	1 (0.7)	4 (2.1)	0.31 (0.03–2.75)	0.385	-
DRB1*14:54-DQA1*01:04-DQB1*05:03	1 (0.7)	4 (2.1)	0.31 (0.03–2.75)	0.385	-

Abbreviation: CI, confidence interval.
[a]*n* denotes number of haplotypes.
[b]*p* value corrected by the Bonferroni inequality method. $p_c = p$ value corrected by 52. p_c value was calculated only for *p* value <0.05.

DISCUSSION

RBC alloimmunization to RhD antigens used to be the main cause of HDFN. However, the administration of Rh immunoglobulin to pregnant RhD-negative females can effectively reduce RBC sensitization rates and prevent HDFN [24]. Therefore, except for RhD, other Rh antigens like C, c, E, e antigens have emerged as an important cause of HDFN. High-frequency screening rate of anti-E antibodies in pregnant females makes anti-E one of the main influencing factors of HDFN [25, 26]. Diversity of HLA class molecules in individuals have been reported to be important factors influencing RBC alloimmunization [12]. Therefore, *HLA* genotypes were investigated between RhE responders and non-responders among Chinese Han pregnant females in this study, with the aim of elucidating relationships between HLA diversity and RhE alloimmunization during pregnancy. The results showed that phenotype frequencies of HLA-DRB1*09:01, HLA-DQA1*03:02 and HLA-DQB1*03:03 were significantly higher among the pregnant females with anti-E antibodies production, suggesting that *HLA-DRB1*09:01*, *HLA-DQA1*03:02* and *HLA-DQB1*03:03* alleles were associated significantly with RhE alloimmunization among Chinese pregnant females in Sichuan province.

The association between *HLA-DRB1*09:01* and RhE alloimmunization susceptibility has been reported in our previous study [13]. In Chinese individuals with transfusion history, we identified that the frequency of HLA-DRB1*09:01 phenotype in the anti-E group (76.9% vs. 27.6%) was significantly higher than in the control group (OR, 8.7; 95% CI, 4.7–16.2; $p_c < 0.0034$). In addition, HLA-DRB1*09 phenotype was also confirmed to show a strong association with RhE alloimmunization in the European population [23, 27]. In our current study, although the frequency of HLA-DRB1*09:01 phenotype in pregnant

women is a little lower than that in the individuals with transfusion history in our previous study, the result supported that HLA-DRB1*09:01 phenotype was a RhE alloimmunization-susceptible phenotype in Chinese pregnant females.

The association between the HLA-DQ and RhE alloimmunization susceptibility has been determined in various studies and showed varying results. In multiple-transfused patients with sickle cell anaemia, Rodrigues discovered that *HLA-DQB1*03* allele was significantly presented in RBC alloimmunized patients, compared with non-alloimmunized patients [28]. In another study, Maluskova et al. focused on all RBC responders in the Czech population including alloimmunization caused by transfusion, pregnancy, transplantation and others, with results showing that *HLA-DQB1*06* was associated with the production of anti-E plus anti-c, and *HLA-DQB1*02* was related to the production of anti-E plus anti-Cw. However, the association between the production of single anti-E antibodies and *HLA-DQB1* alleles was not significantly observed [23]. In contrast, Tatari-Calderone et al. found that *HLA-DQB1*03* allele may have a protective effect against RBC alloimmunization among the African-American population [29]. In our study, the significant increase of *HLA-DQA1*03:02* and *HLA-DQB1*03:03* in the RhE responders was demonstrated. The haplotype estimation suggested that the *HLA-DQA1*03:02* was increased partially because of the association with *HLA-DQB1*03:03*. It suggested that HLA-DQA1*03:02 may contribute to RhE alloimmunization, by forming an antigen-binding groove of the HLA-DQ molecule together with HLA-DQB1*03:03 [30].

The haplotype *HLA-DRB1*09:01-DQA1*03:02-DQB1*03:03*, which was the most common haplotype in the RhE responders, was found at a significantly higher frequency when compared with the non-responders. It was interesting that the most common haplotype in the RhE responders consisted of all three susceptible alleles. Furthermore, several non-frequent haplotypes containing *HLA-DRB1*09:01*, *HLA-DQA1*03:02* or *HLA-DQB1*03:03* showed no increasing trend in the RhE responders. Recently, Shang reported that the *HLA-DRB1*09:01-DQA1*03:02-DQB1*03:03* haplotype showed a significant positive association with anti-E in the pregnant women [31]. These observations indicated that the contribution of *HLA-DRB1*09:01*, *HLA-DQA1*03:02* or *HLA-DQB1*03:03* could not be as an independent risk allele and must be together with the other two alleles.

In conclusion, one of the more significant findings to emerge from this study is that *HLA-DRB1*09:01*, *HLA-DQA1*03:02* and *HLA-DQB1*03:03* may be susceptible alleles for RhE alloimmunization in pregnant females of Chinese Han population. The three susceptible alleles constituted the unique 3-locus haplotype in the RhE responders and co-worked to contribute to RhE alloimmunization. However, additional research involving diverse populations is needed to discover more factors impacting RBC alloimmunization in pregnant females. Nevertheless, the findings of our study can be valuable in identifying important alloimmunization risk factors for the better management of complications caused by alloantibodies in pregnant females.

ACKNOWLEDGEMENTS

This work was supported by the 2020 Natural Science Key Project of Chengdu Medical College Foundation (Grant No. CYZZD20-05), School Fund of Chengdu Medical College (Grant No. CYZYB22-05), Foundation of the First Affiliated Hospital of Chengdu Medical College (Grant No. CYFY-GQ52) and the CAMS Innovation Fund for Medical Sciences (Grant No. 2021-1-I2M-060).

Y.K. and J.X. were responsible for performing the experiments, data collection and analysis and writing the original manuscript; L.T. and Y.X. contributed to the study design and manuscript revision; all authors read and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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How to cite this article: Kong Y, Xiao J, Tian L, Xu Y. The influence of HLA allele and haplotype on RhE alloimmunization among pregnant females in the Chinese Han population. Vox Sang. 2024;119:737–44.

Received: 10 January 2024 | Revised: 19 February 2024 | Accepted: 3 March 2024

DOI: 10.1111/vox.13617

SHORT REPORT

Vox Sanguinis ISBT International Society of Blood Transfusion

An international review of the characteristics of viral nucleic acid-amplification testing (NAT) reveals a trend towards the use of smaller pool sizes and individual donation NAT

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Funding information
The authors received no specific funding for this work.

Abstract

Background and Objectives: Nucleic acid-amplification testing (NAT) is used for screening blood donations/donors for blood-borne viruses. We reviewed global viral NAT characteristics and NAT-yield confirmatory testing used by blood operators.

Materials and Methods: NAT characteristics and NAT-yield confirmatory testing used during 2019 was surveyed internationally by the International Society of Blood

For affiliations refer to page 750

Transfusion Working Party Transfusion-Transmitted Infectious Diseases. Reported characteristics are presented herein.

Results: NAT was mainly performed under government mandate. Human immunodeficiency virus (HIV), hepatitis C virus (HCV) and hepatitis B virus (HBV) NAT was performed on all donors and donation types, while selective testing was reported for West Nile virus, hepatitis E virus (HEV), and Zika virus. Individual donation NAT was used for HIV, HCV and HBV by ~50% of responders, while HEV was screened in mini-pools by 83% of responders performing HEV NAT. Confirmatory testing for NAT-yield samples was generally performed by NAT on a sample from the same donation or by NAT and serology on samples from the same donation and a follow-up sample.

Conclusion: In the last decade, there has been a trend towards use of smaller pool sizes or individual donation NAT. We captured characteristics of NAT internationally in 2019 and provide insights into confirmatory testing approaches used for NAT-yields, potentially benefitting blood operators seeking to implement NAT.

Keywords
NAT, transfusion safety, virus

Highlights

- Human immunodeficiency virus, hepatitis C virus and hepatitis B virus nucleic acid-amplification testing (NAT) was performed on all donors and donation types, whereas selective testing was reported for West Nile virus, hepatitis E virus and Zika virus.
- In the last decade, there has been a trend towards the use of smaller pool sizes or individual donation NAT.
- Confirmatory testing for NAT-yield samples was generally performed by NAT on a sample from the same donation or by NAT and serology on samples from the same donation and a follow-up sample.

INTRODUCTION

Blood-borne viruses can be transmitted through blood transfusion. Blood operators employ a myriad of tools to reduce this risk including nucleic acid-amplification testing (NAT). Since the introduction of hepatitis C virus (HCV) and human immunodeficiency virus (HIV) NAT in the 1990s, NAT has been implemented for other viruses, including hepatitis B virus (HBV), hepatitis E virus (HEV), West Nile virus (WNV) and Zika virus (ZIKV) [1, 2]. NAT was initially performed mainly on mini-pools (MPs), but technological advances and reduced costs of NAT have led to the introduction of individual donation (ID)-NAT [1]. Procedures for confirming NAT-yield samples (donations testing reactive by NAT, but negative by serology) can be complex. Variability remains in the different approaches to NAT and indeed in the ways that NAT-yield samples are confirmed, amongst blood operators internationally.

A survey of NAT usage and yield amongst blood operators internationally was recently undertaken by the International Society of Blood Transfusion Working Party Transfusion-Transmitted Infectious Diseases (ISBT WP-TTID) [2]. The characteristics of blood donation viral NAT and NAT-yield confirmatory testing was surveyed amongst blood operators in 2019, the analysis of which is presented herein.

MATERIALS AND METHODS

This is a sub-analysis of data collected for an international review of NAT performed by ISBT WP-TTID [2]. The present study focused on reviewing international practices for viral blood donation NAT and NAT-yield confirmatory testing in 2019.

Given some regions within a country (representing different blood operators within a country) reported different responses to some survey questions, percentages were based on the proportion of survey responses, not the proportion of countries involved. For analyses of MP size, where a range of MPs were provided for a virus by the same responder or same country (for comparison with 2008 data [1]), the largest number was used. The 2008 data were not available for all countries that responded to the 2019 survey.

Descriptive analyses were performed, with reported variables expressed as frequencies and percentages. Comparisons of MP size per country in 2019 with that from 2008 [1] were performed with a Mann–Whitney test, using GraphPad Prism. Ordinal logistic regression analysis was performed to determine whether possible predictors including world bank income category (data from [3]), region, HIV incidence (data from [4]), HCV viraemic prevalence (data from [5]), HBV incidence (data from [4]) or number of donations tested for HIV were associated with the use of ID- or MP-NAT or MP size (where a MP of 1 was used for ID-NAT). For quantitative predictors, correlations were first performed to determine multi-collinearity. These analyses were performed using R Statistical Software version 2023.06.0 [6].

Confirmatory testing procedures for NAT yields were initially examined via frequency analysis; however, given the large number of separate responses, data were re-categorized by sample and assay type. Categorization by sample type was separated into three groups: same donation (re-testing the same or alternate sample only, where an alternate sample was defined as another tube from the same donation or sample from the retrieved plasma unit), donor follow-up (testing a donor follow-up sample only), donation and follow-up (any combination of testing the same/alternate sample and a donor follow-up sample). Categorization by assay type was separated into three groups: NAT (NAT assay only, where the NAT assay was defined as the same assay or an alternate NAT assay with comparable or increased sensitivity), serology (serology only) and NAT and serology (combination of NAT assays and serology testing). Responses that could not be grouped by confirmatory assay type were omitted.

RESULTS

In 2019, 38 survey participants, representing 27 countries, performed blood donation NAT for at least one virus (a total of 43 survey responses were received from 32 countries; 5 countries indicated they were not performing NAT [2]). Of these survey responders, 25/38 were from high-income countries, 10/38 were from upper-middle-income countries and 3/38 were from lower-middle-income countries. Most responders performing NAT did so under government mandate (Figure 1a).

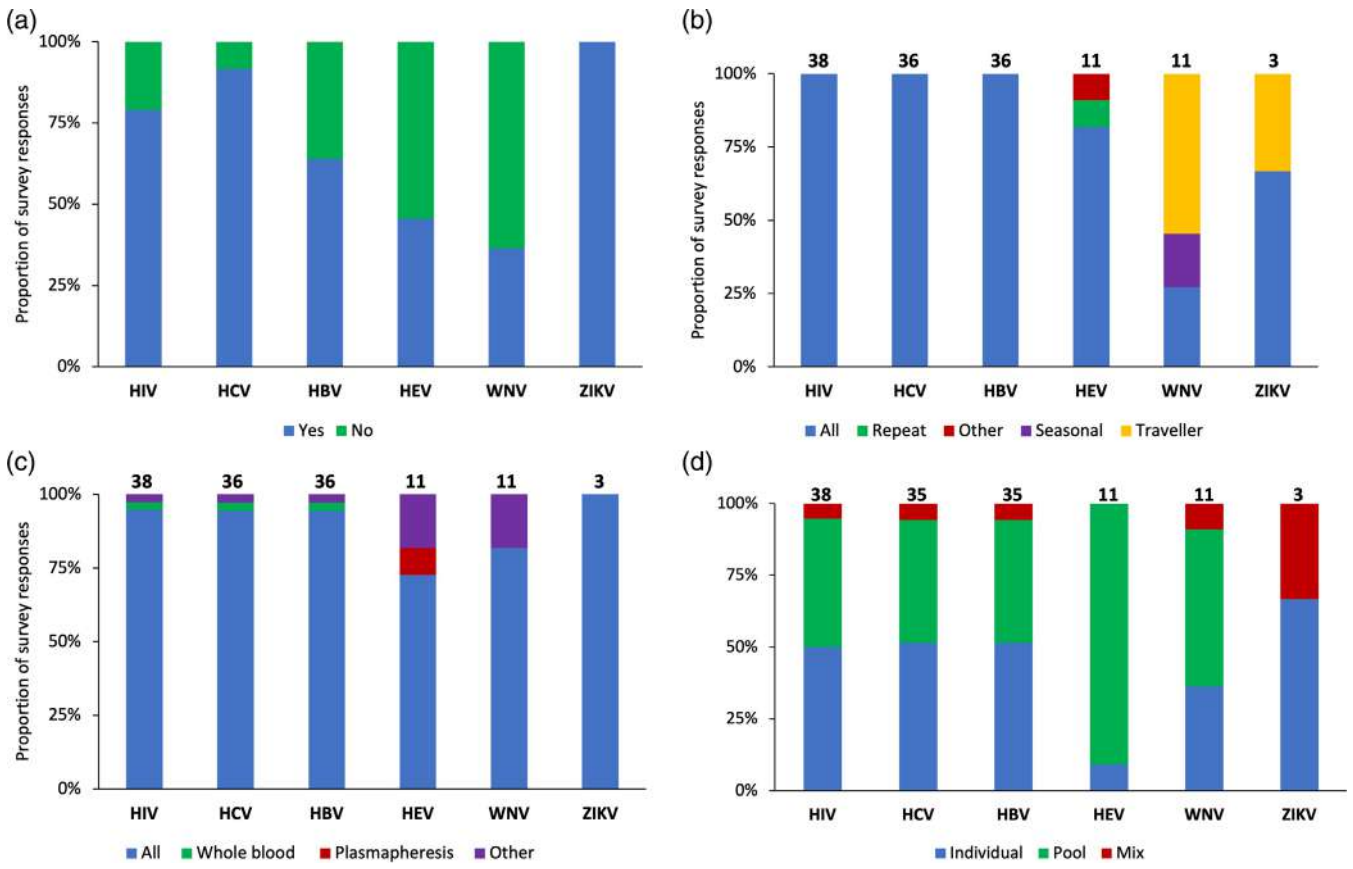

FIGURE 1 Characteristics of blood screening nucleic acid-amplification testing (NAT), 2019, amongst survey responders, organized by (a) whether NAT is government mandated, (b) by donor type, (c) by donation type and (d) by sample type (individual, pooled or a mix of individual and pooled). The number of survey responders performing NAT for each virus with suitable data available for analysis is shown above each bar. Complete data were not available from all respondents. HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus; HIV, human immunodeficiency virus; WNV, West Nile virus; ZIKV, Zika virus.

All survey participants performed HIV, HBV and HCV on all donor types (Figure 1b), whereas 82% ($n = 9$) of responders tested all donor types for HEV (in one country where HEV RNA screening is undertaken, it was only performed on donors of plasma intended for fractionation). WNV and ZIKV testing was performed on donors who travelled to endemic regions (55% ($n = 6$) and 33% ($n = 1$), respectively), whereas 18% ($n = 2$) of responders performed WNV NAT only on a seasonal basis.

For all viruses, most responders indicated that all donation types were tested (Figure 1c). One responder indicated that HIV, HCV and HBV NAT were not performed on plateletpheresis or granulocyte donations, which are short shelf-life products. HEV NAT was performed only on whole blood and plasma donations by one responder. For WNV, one responder indicated that all donation types except plasmapheresis donations for fractionation were tested.

For HIV, HCV, HBV and WNV, approximately 50% of responders performed ID-NAT (Figure 1d). For responders in which donations were pooled prior to testing, MP sizes ranged from 4 to 96, with 6 being the most frequently reported pool size for HIV, HBV, HCV and WNV (Table S1). For WNV NAT, responders from one country reported a combination of ID and MP testing, switching between the two testing approaches depending on circumstances (e.g., triggering conversion to individual donation NAT based on detection of WNV RNA by MP-NAT in defined geographical regions in the United States). For HEV NAT, most responders performed MP-NAT with a similar range in pool sizes, but with MPs of 16 samples most frequently reported (Table S1). ZIKV was primarily tested by ID-NAT by responders.

Comparing ID- and MP-NAT usage for HIV within countries between 2008 and 2019, approaches were either maintained (ID-NAT [$n = 5$]; same MP-NAT size [$n = 2$]), changed from MP- to ID-NAT ($n = 7$) or reduced MP size ($n = 7$) (Figure 2). No country moved from ID- to MP-NAT or increased MP size. These observations were consistent for all regions. The median MP size for HIV NAT in 2019 was smaller than in 2008 (6; range: 6–96 versus 16; range: 6–96, respectively; $p = 0.0331$).

We wanted to explore whether using ID- or MP-NAT, as well as MP size, was associated with income category, region, HIV incidence in the general population, HCV viraemic prevalence in the general population, HBV incidence in the general population or the number of donations tested for HIV. No significant correlations were observed between the quantitative variables, hence no multi-collinearity

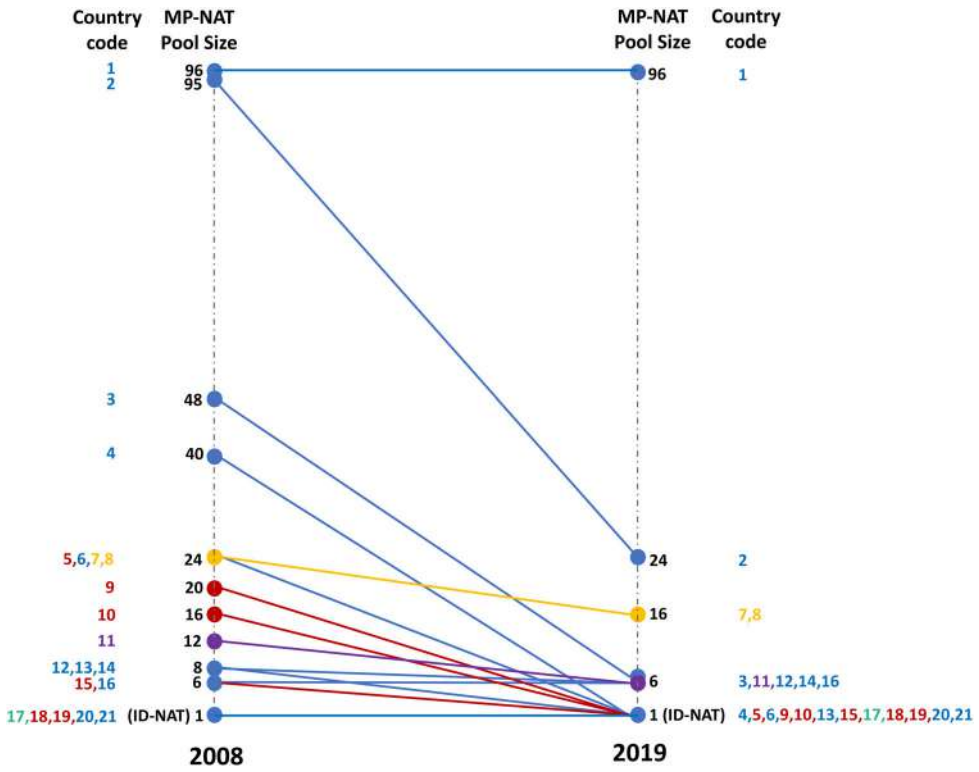

FIGURE 2 Schematic showing changes in the use of individual donation (ID) and mini-pool (MP) blood donation human immunodeficiency virus (HIV) nucleic acid-amplification testing (NAT) in 2008 and 2019. Country data from 2008 [1] were compared with country data from 2019 survey responses ($n = 21$). Where a combination of ID- and MP-NAT or a range of MPs were provided by the same responder, or by the same country, the highest number was used. Data from 2008 were not available for all countries that responded to the 2019 survey. Country codes: 1, Germany; 2, United Kingdom; 3, Netherlands; 4, Switzerland; 5, Republic of Korea; 6, France; 7, Canada; 8, United States; 9, Japan; 10, Australia; 11, Brazil; 12, Belgium; 13, Ireland; 14, Spain; 15, Thailand; 16, Poland; 17, South Africa; 18, New Zealand; 19, Singapore; 20, Denmark; 21, Greece. Colour-coding of countries by region: Africa, green; Asia/Pacific, red; Europe, blue; North America, yellow; South America, purple. Schematic adapted from one made with Flourish (available from https://flourish.studio).

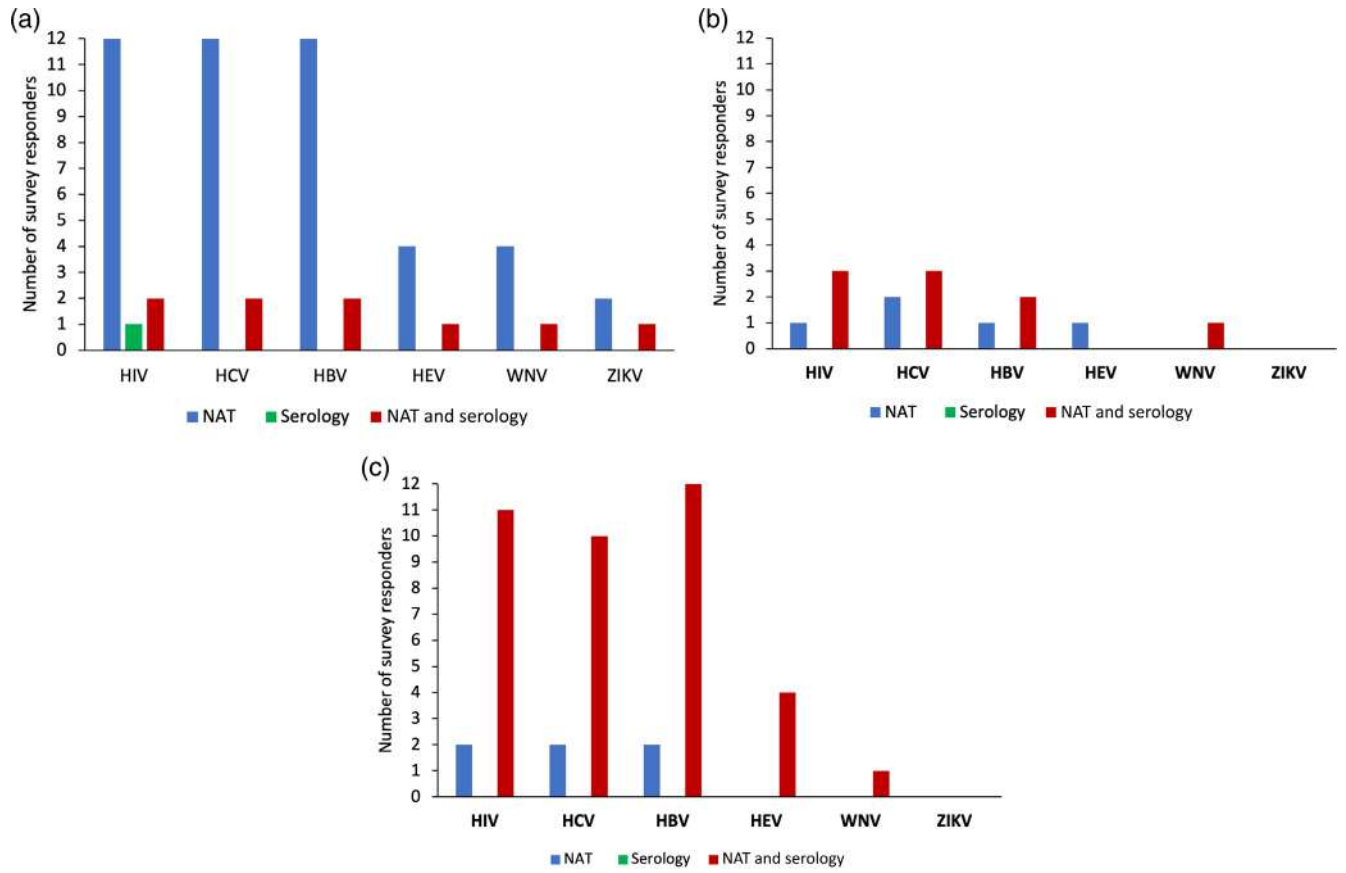

FIGURE 3 Confirmatory testing approaches for nucleic acid-amplification testing (NAT) yield samples, 2019, amongst survey responders, organized by whether testing is performed on a sample from (a) the same donation ($n = 15$), (b) a follow-up donation ($n = 4$) or (c) both the same donation and a follow-up donation ($n = 13$). Complete data were not available from all respondents. HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus; HIV, human immunodeficiency virus; WNV, West Nile virus; ZIKV, Zika virus.

observed, and no association was observed between each outcome and the predictors ($p > 0.05$ for all; data not shown).

Confirmatory testing for NAT-yield samples were classified based on the sample type tested and types of assays used as described above. Confirmatory testing was generally performed on a sample from the same donation using NAT (Figure 3a) or on both a sample from the same donation and a follow-up sample using NAT and serology-based assays (Figure 3c). Using NAT and serology-based assays on the same donation or only on a follow-up sample with any assay type were less frequent (Figure 3a,b). Timing of follow-up samples ranged from 72 h following a reactive result up to 6 months (data not shown).

DISCUSSION

We outlined characteristics of blood donation viral NAT and NAT-yield confirmatory testing used by 38 blood operators from 27 countries. HIV, HCV and HBV NAT were primarily undertaken under government mandate and were performed on all donor and donation types. More selective testing was performed for other viruses, such as HEV, in the absence of a government mandate, or WNV and ZIKV, for donors who travelled to countries not endemic for these viruses. The tailored approaches used for HEV, WNV and ZIKV reflect localized factors such as regional epidemiology, viral incidence, lack of screening mandates, resource availability and use of other risk management strategies.

There appears to be a move towards use of smaller MP sizes or ID-NAT since 2008. This may be driven by the introduction of individual triplex HIV/HCV/HBV NAT assays on higher throughput instruments and/or the higher sensitivity of ID-NAT assays compared to their MP counterparts. Our observation is consistent with reports of blood operators transitioning from MP- to ID-NAT, whereby such a transition can result reduction in reported transfusion-transmitted infections as recently reported by Japan for HBV [7]. Moreover, there is interest in moving to ID-NAT for HEV in a combined HIV-1/HIV-2/HCV/HBV/HEV assay that has comparable sensitivities to existing assays [8]. Future studies could investigate the impact of this transition to ID-NAT on the occurrence of transfusion-transmitted infections internationally. Income, region, HIV incidence in the general population, HCV viraemic prevalence in the general population and HBV incidence in the general population or the number of donations tested were not associated with whether a blood operator performs ID- or MP-NAT or with MP size; however, only a small number of

lower-middle-income countries ($n = 3$) reported performing NAT in this survey. Other factors, such as individualized level of risk acceptance, history or additional cost may be responsible for the decision to perform ID- or MP-NAT, and if the latter, pool size.

Confirmatory testing, when done, for NAT-yield samples is complex, with a large amount of highly diverse testing algorithms. Our study suggests that such testing was generally performed by NAT on a sample from the same donation or by NAT and serology on samples from the same donation and also a follow-up sample. When follow-up samples were used for confirmatory testing, the timing of sampling was also varied. Although we have attempted to summarize current confirmatory testing approaches for NAT-yield samples, given the high degree of variability used amongst blood operators, additional studies focusing on the specifics of confirmatory testing algorithms, and how this relates to donor management, are needed.

Our study is not without limitations, including those outlined previously [2]. In addition, where different ID- and MP-NAT sizes were reported by a survey responder or within a country, the larger MP size was used, which may have led to estimates of larger MP sizes thus underestimating the reduction in MP size. Comparisons of ID- and MP-NAT between 2008 and 2019 were made for a whole country rather than for a specific blood operator; in instances where a different blood operator responded to the 2019 survey compared with the 2008 survey, changes may have been incorrectly assigned; however, this may have impacted only a small number of responders. The operating characteristics of the assays used may have differed between survey responders as well as over time; however, investigation of this was not a focus of the present study.

To our knowledge, this is the only comprehensive assessment of NAT characteristics and confirmatory testing approaches used by blood operators in the past 10 years. It is anticipated that the data presented herein will assist blood operators planning to implement viral NAT to augment blood transfusion safety in their local setting.

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ACKNOWLEDGEMENTS

We acknowledge the contribution of Clive Seed to the initial stages of this project and also Thi Thanh Dung, Konstantinos Stamoulis, Roberta Fachini and Ratti Ram Sharma, for supplying data. We thank ISBT WP-TTID members not listed as authors and also Susan Galel, Jean Stanley and Laura Fryza who facilitated distribution of the survey.

H.M.F., C.O., B.C., M.B. and S.L.S. conceived the study and prepared the survey; E.V. performed statistical analyses; H.M.F. prepared the first draft of the manuscript; all authors contributed to study

design, data analysis, data interpretation, manuscript editing and approval of the final manuscript. Open access publishing facilitated by University of the Sunshine Coast, as part of the Wiley - University of the Sunshine Coast agreement via the Council of Australian University Librarians.

CONFLICT OF INTEREST STATEMENT

Helen M. Faddy has received research funding and/or honoraria from Grifols Diagnostic Solutions Inc. and Roche Diagnostic Solutions in the past. Brian Custer and/or the organization he is employed by have received research funding from Grifols Diagnostic Solutions Inc. and Roche Diagnostic Solutions. Susan L. Stramer has received research funding and/or honoraria from Grifols Diagnostic Solutions Inc. and Roche Diagnostic Solutions in the past. Christian Erikstrup has received unrestricted research grants from Abbott Diagnostics and Novo Nordisk, which are both administered by Aarhus University Hospital and Aarhus University, respectively. Christian Erikstrup has not received any personal fees from these or other entities. Silvia Sauleda has received research funding from Grifols Diagnostic Solutions in the past. Pierre Gallian has received honoraria for lectures from Grifols Diagnostic Solutions Inc. and Roche Diagnostic Solutions in the past. Aneta Kopacz has received honoraria for lectures from Grifols Diagnostic Solutions Inc. and Roche Diagnostic Solutions in the past. Magdalena Łętowska has received honoraria for lecture from Grifols Diagnostic Solutions Inc. in the past. The remaining authors have no relevant conflict of interest 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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SUPPORTING INFORMATION

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

How to cite this article: Faddy HM, Osiowy C, Custer B, Busch M, Stramer SL, Dean MM, et al. An international review of the characteristics of viral nucleic acid-amplification testing (NAT) reveals a trend towards the use of smaller pool sizes and individual donation NAT. Vox Sang. 2024;119:745–51.

Received: 11 January 2024 | Revised: 13 March 2024 | Accepted: 24 March 2024

DOI: 10.1111/vox.13630

SHORT REPORT

Vox Sanguinis ISBT International Society of Blood Transfusion

The use of intracellular dyes to create a multiplexed flow cytometry-based red blood cell phenotyping assay

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Funding information
The authors received no specific funding for this work.

Abstract

Background and Objectives: Flow cytometry can be used to phenotype red blood cell antigens, allowing for high-throughput testing while using low reagent volumes. This article utilizes intracellular dyes to pre-label red blood cells to further multiplex flow cytometry-based red blood cell antigen phenotyping.

Materials and Methods: Red blood cells were pre-labelled using the intracellular dyes V450 and Oregon Green. These dyes are detected fluorescently via flow cytometry. Four combinations of intracellular staining were used to allow four patient or donor red blood cells to be analysed in a single test well. Antigen phenotyping was then performed via flow cytometry using a previously described method.

Results: The intracellular dyes showed uniform staining when measured in mean fluorescence intensity and allowed the red blood cells to be clearly distinguished from one another. The presence or absence of red blood cell antigens was determined with 100% accuracy.

Conclusion: The use of intracellular dyes allowed a fourfold increase in the throughput of our previously described flow cytometry-based red blood cell antigen phenotyping method. The described method allows up to 48 patients to be simultaneously phenotyped using a single 96-well microplate. Furthermore, additional fluorescent dyes could potentially increase the throughput exponentially.

Keywords

antigen testing, flow cytometry, RBC antigen, RBC phenotyping, red blood cell phenotype, transfusion

Highlights

- Intracellular dyes were used to allow increased multiplexing of our existing flow cytometry-based red blood cell (RBC) phenotyping assay.
- Intracellular staining was uniform, reproducible and had no impact on the ability to perform phenotyping for RBC antigens.
- The use of intracellular dyes is a simple way to greatly increase the throughput of flow cytometry-based RBC phenotyping methods.

INTRODUCTION

Presently, routine testing in transfusion medicine is predominantly performed via agglutination-based test platforms [1, 2]. These test platforms rely on visual detection of red blood cell (RBC) agglutination, which is a surrogate for the detection of antigen–antibody interactions. The downside of this testing methodology is that it requires very large volumes of both RBCs and serum to create visually detectable reactions. In addition, RBCs are not individually interrogated in agglutination-based assays and therefore cannot be multiplexed, as only a single antigen–antibody interaction can be assessed per test well.

To overcome these limitations, we developed a series of blood transfusion tests using flow cytometry, which allows RBCs to be individually analysed via a laser-based detection system. This allows for extremely low volumes of reagents to be used to perform highly sensitive and specific testing with the possibility of highly multiplexed assays.

Previously, we published a phenotyping method that used 1%–2% of the antisera compared with our traditional agglutination-based phenotyping method and allowed up to 12 samples to be tested on a single 96-well microplate [3]. This assay employed some degree of multiplexing by utilizing cocktails of immunoglobulin G (IgG)- and immunoglobulin M (IgM)-based antisera in some wells with reaction detection using anti-human IgM and IgG secondary antibodies conjugated to distinct fluorochromes.

These flow cytometric principles of detecting antibody binding and multiplexing were leveraged to develop flow cytometry-based RBC antibody screen and identification assays by our group. This was successfully done utilizing the intracellular dyes, Oregon Green and Violet Proliferation Dye (V450), to label test RBCs to allow up to four antibody screen or identification panel RBCs to be evaluated in a single microtitre plate well. This resulted in highly multiplexed RBC antibody screens and panels via flow cytometry, which has been reported separately [4].

Oregon Green and V450 have been used as cell tracers and in proliferation studies [5]. These dyes passively enter viable cells where they are cleaved by intracellular esterases to yield fluorescent products that stably bind intracellular proteins, which can be detected via flow cytometry making them ideal candidates for labelling RBCs for use in a variety of contexts in flow cytometry in blood transfusion.

Importantly, as these dyes are intracellular stains, they do not interfere with surface antibody binding. This was found to be the case in our RBC antibody screen and identification assays, and therefore, we sought to adapt this technique to greatly increase the throughput of our previously described phenotyping assay. Using a combination of dyes (unstained, V450+, Oregon Green+ and dual stained for V450 and Oregon Green), this approach creates the potential to increase throughput of our existing RBC antigen phenotyping assay by fourfold. In this article, we describe the successful utilization of these intracellular dyes to allow 48 samples to be phenotyped simultaneously by flow cytometry using minimal volumes of antisera and patient RBCs.

STUDY DESIGN AND METHODS

Reagents

RBC phenotyping by flow cytometry was performed using commercially available direct-typing antisera (Table 1). Cocktails of antiserum were prepared by mixing combinations of antisera with Dulbecco's phosphate buffered saline (DPBS; Life Technologies Inc., Carlsbad, CA) as shown in Table 1. Similarly, a cocktail of secondary detection antibodies was produced using allophycocyanin (APC)-conjugated polyclonal goat anti-human IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted to 1:200 with DPBS and phycoerythrin (PE)-conjugated polyclonal donkey anti-human IgM (One Lambda, Canoga Park, CA) diluted to 1:100 in DPBS. Flow wash buffer (FWB) composed of DPBS and 2% (v/v) fetal calf serum (Life Technologies Inc., Carlsbad, CA) was used for all washes during the phenotyping procedure. 0.8% RBC suspensions and all antisera and secondary antibody cocktails were prepared in DPBS (Life Technologies Inc., Carlsbad CA). Screen panel RBCs (0.8% suspension) were purchased from Ortho Clinical Diagnostics (Markham, ON), and four antibody identification panel RBCs (0.8% suspension) were purchased from Ortho Clinical Diagnostics (Markham, ON) and Bio-Rad (Mississauga, ON).

Intracellular staining of RBCs was performed using cell permanent dyes CellTrace Oregon Green 488 carboxylic acid diacetate succinimidyl ester (ThermoFisher Scientific, Waltham, MA) at a final concentration of 1.0 μM and Violet Proliferation Dye 450 (V450; BD Biosciences, Franklin Lakes, NJ) at a final concentration of 0.2 μM. Quenching buffer (QB) composed of DPBS and 10% (v/v) fetal calf serum was used for all washes in the intracellular staining procedure.

RBC intracellular staining

RBCs were stained using the intracellular dye staining protocol utilized and described in our RBC antibody screen and panel assay [4]. A total of 48 reagent RBCs obtained from 4 separate 11-cell antibody identification panels plus 3 different screen panel cells with 1 being run in duplicate were added to individual wells of a 96-well U-bottom Falcon microplate as shown in Figure 1a. The number of cells positive for each antigen is as follows: C (16), c (36), E (9), e (43), K (10), Jk^a (32), Jk^b (29), S (26), s (36), k (48), Fy^a (22) and Fy^b (29). For RBC staining, 75 μL of 0.8% screening or identification panel RBC suspension was added to each well and washed twice in 200 μL of DPBS at 1800×*g* for 30 s. Red cells were resuspended in 200 μL of DPBS, 200 μL of V450 intracellular staining solution (final concentration 0.2 μM), 200 μL of Oregon Green intracellular staining solution (final concentration of 1.0 μM) or 200 μL of V450 and Oregon Green intracellular staining solution (final concentration of 0.2 μM for V450 and 1.0 μM for Oregon Green) and incubated for 10 min at room temperature (RT). Red cells were washed twice in 200 μL of QB at 1800×*g* for 30 s and resuspended in 75 μL of DPBS. Finally, 70 μL of each red cell suspension was combined such that four separate samples were included per test well as shown in Figure 1a. For example, cells 1, 13,

TABLE 1 Antisera cocktail suspensions.

	IgM antisera			IgG antisera			
Cocktail	Specificity	Catalogue number	Volume (µL)	Specificity	Catalogue number	Volume (µL)	DPBS (µL)
A	Anti-C	Ortho. CAT: 713080	30	Anti-S	Ortho. CAT: 723030	60	210
B	Anti-c	Ortho. CAT: 714080	30	Anti-s	Ortho. CAT: 721830	30	240
C	Anti-E	Ortho. CAT: 713180	30	—		—	270
D	Anti-e	Bio-Rad. CAT: BT802370	30	—		—	270
E	Anti-K1	Ortho. CAT: 713129	30	Anti-K2	Ortho. CAT: 721030	60	210
F	Anti-Jk^a	Ortho. CAT: 721105	30	Anti-Fy^a	Immucor. CAT: 4816	60	210
G	Anti-Jk^b	Ortho. CAT: 721480	30	Anti-Fy^b	Ortho. CAT: 725230	30	240

Note: Each test required 20 µL of diluted antisera. This recipe will allot enough for approximately 60 tests.
Abbreviations: DPBS, Dulbecco's phosphate buffered saline; IgG, immunoglobulin G; IgM, immunoglobulin M.

25 and 37 were combined to create the M1 cell suspension. This process was repeated to create suspensions M1 through M12 as shown.

Red cell phenotyping by flow cytometry

RBC phenotyping was performed using our previously described method [3]. Briefly, 20 µL of the appropriate antisera cocktail labelled A–G (see Table 1) or DPBS (background control) was added to the bottom of each well of a 96-well U-bottom Falcon microplate in same manner as is shown in Figure 1b. Next 10 µL of each RBC cell suspension (M1 through M12) was added to the 8 wells containing cocktail or DPBS according to the schema in Figure 1b. Trays were then centrifuged at 1800×*g* for 10 s and agitated by vortexing to ensure appropriate mixing of the cells and antisera. Trays were incubated at RT for 5 min and washed three times in 200 µL of FWB at 1800×*g* for 1 min. Then, 80 µL of the secondary antibody cocktail (anti-IgM PE and anti-IgG APC in DPBS) was added to each well (as shown in Figure 1c), mixed by vortexing and incubated at RT for 5 min. Cells were washed once in 200 µL FWB at 1800×*g* for 1 min and resuspended in 150 µL of FWB before acquisition and analysis by flow cytometry.

Flow cytometry acquisition and analysis

Red cell events were acquired on FACSLyric flow cytometers (BD Biosciences, Mississauga, ON) and analysed with BD FACSDIVA™ software (BD Biosciences, Mississauga, ON) using the 5-log median fluorescence intensity (MFI) scale. For anti-IgG–APC and anti-IgM–PE fluorescence data, MFI values were normalized to the relative background fluorescence obtained with the DPBS control. Positive cut-offs were defined as MFI >100 above background fluorescence based on the DPBS-negative control staining.

RESULTS

A total of 48 reagent antibody screen or identification panel RBCs were tested, and we obtained 100% concordance with expected phenotype results (Figure S1). Although, for a given antigen, homozygous cells did show slightly higher MFI on average, there was considerable overlap in MFI between homozygous and heterozygous antigen expression which precludes assessment of zygosity based on MFI. A representative example of the gating strategy and histograms of MFI intensity for detection of the S and C antigens are shown in Figure 2 for four RBC antibody identification panel cells.

Intracellular staining was uniform across cells and allowed clear distinction of the cellular populations within each cell mixture (Figure S2). MFI for double-negative RBCs was 2133.7±969.2 for Oregon Green and 124.7±8.6 for V450. Results for Oregon Green-positive and V450-negative RBCs were 34583.7±7715.3 and 116.2±11.0, respectively. Results for Oregon green-negative and V450-positive RBCs were 1542.2±656.1 and 11676.9±3830.8, respectively. Finally results for Oregon Green-positive and V450-positive dual stained RBCs were 24194.8±5415.0 and 11545.33±4277.1, respectively.

DISCUSSION

In this article, we demonstrate the successful use of intracellular dyes to increase throughput of our existing flow cytometry-based RBC phenotyping method fourfold from 12 to 48 patients per test run using a 96-well microtitre plate. Additionally, throughput could be further increased in an exponential fashion via the use of additional intracellular dyes. We achieved 100% concordance with expected phenotyping results based on comparison with known antigen expression of commercially available donor RBCs.

The intracellular dyes demonstrated uniform staining characteristics that provided clear separation of the RBC populations allowing accurate gating and identification of these populations. The absence of uniform staining in this context would be a potential source of error as gating on erroneous cellular populations could result in falsely increased or decreased MFI. Similarly, staining did not transfer between cells once the cell mixtures were combined, and the double-negative cellular population acted as a control in this regard.

This study demonstrates intracellular staining had no impact on the accurate antibody-mediated detection of RBC surface antigens via the use of commercially available antisera and is corroborated by our

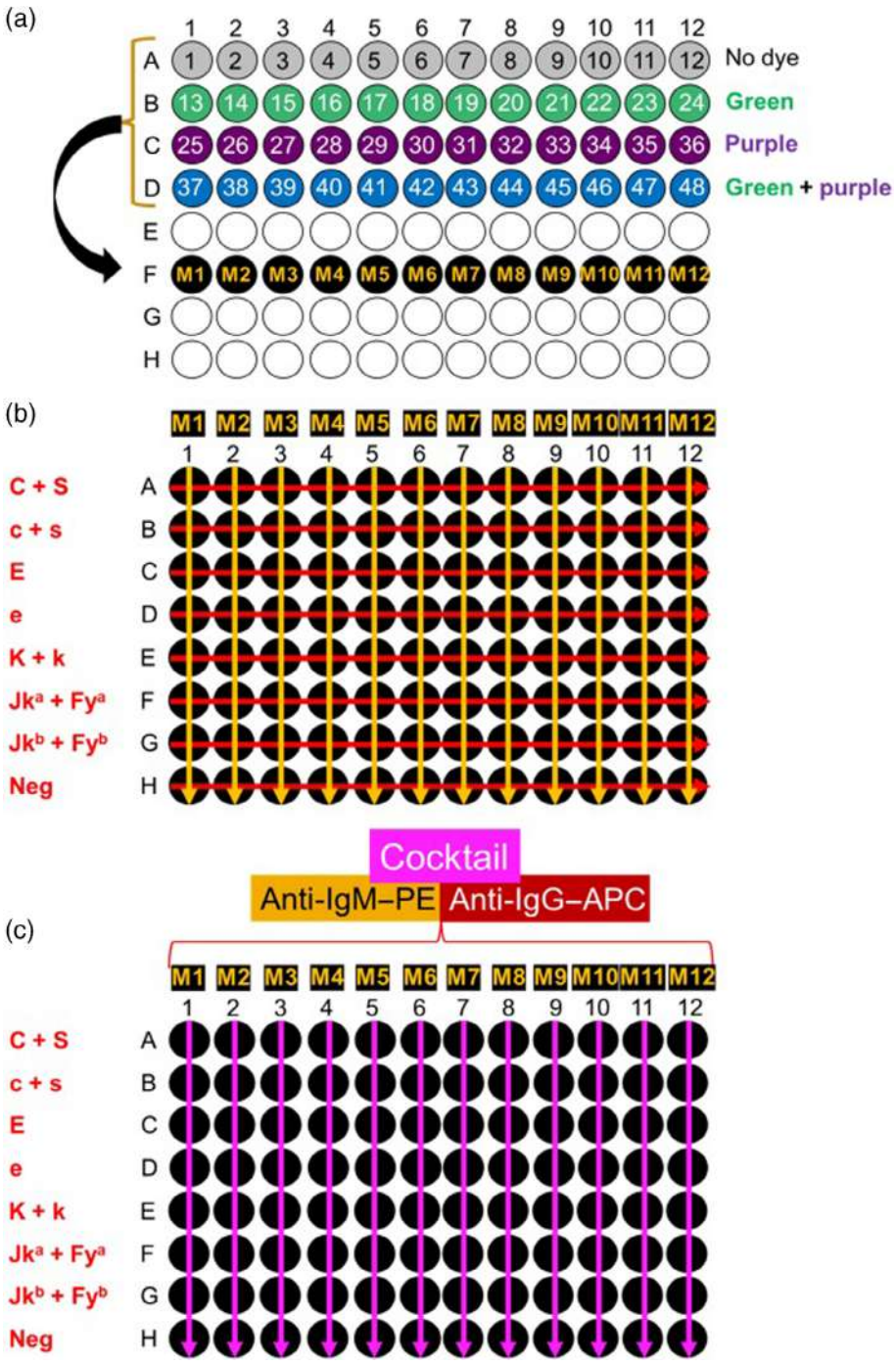

FIGURE 1 Panel (a) illustrates the approach to staining 48 separate red blood cell (RBC) samples simultaneously. Cells 1–12 do not contain any intracellular dyes. Cells 13–24 were stained with Oregon Green (labelled as green in the figure), cells 25 and 26 were stained with V450 (labelled purple in the figure) and cells 37–48 were stained with both V450 and Oregon Green (labelled purple + green). Once stained, the four cells in each column were then combined into a single well labelled M1–M12 in (a). Panel (b) shows that RBC mixtures (M1–M12) were added to every well in their corresponding column of a new microplate (wells 1–12). Antisera was then added across rows as shown by the red line and arrow in (b). Panel (c) shows the final step of the process in which a cocktail of anti-IgM–phycoerythrin (PE) and anti-IgG–allophycocyanin (APC) was added to every well in the plate as illustrated by the purple lines and arrows in (c). IgG, immunoglobulin G; IgM, immunoglobulin M; Neg, negative control.

previous report, which showed the accurate detection of alloantibodies on stained RBC in our antibody screen and panel assay [4].

We have previously posited that the ability to provide high-throughput, low reagent volume RBC phenotyping could make RBC antigen phenotyping more broadly utilized and could facilitate a variety of paradigm-changing innovations, such as routine phenotype matching as standard of care in transfusion medicine [3]. This article strengthens that case given we have further increased

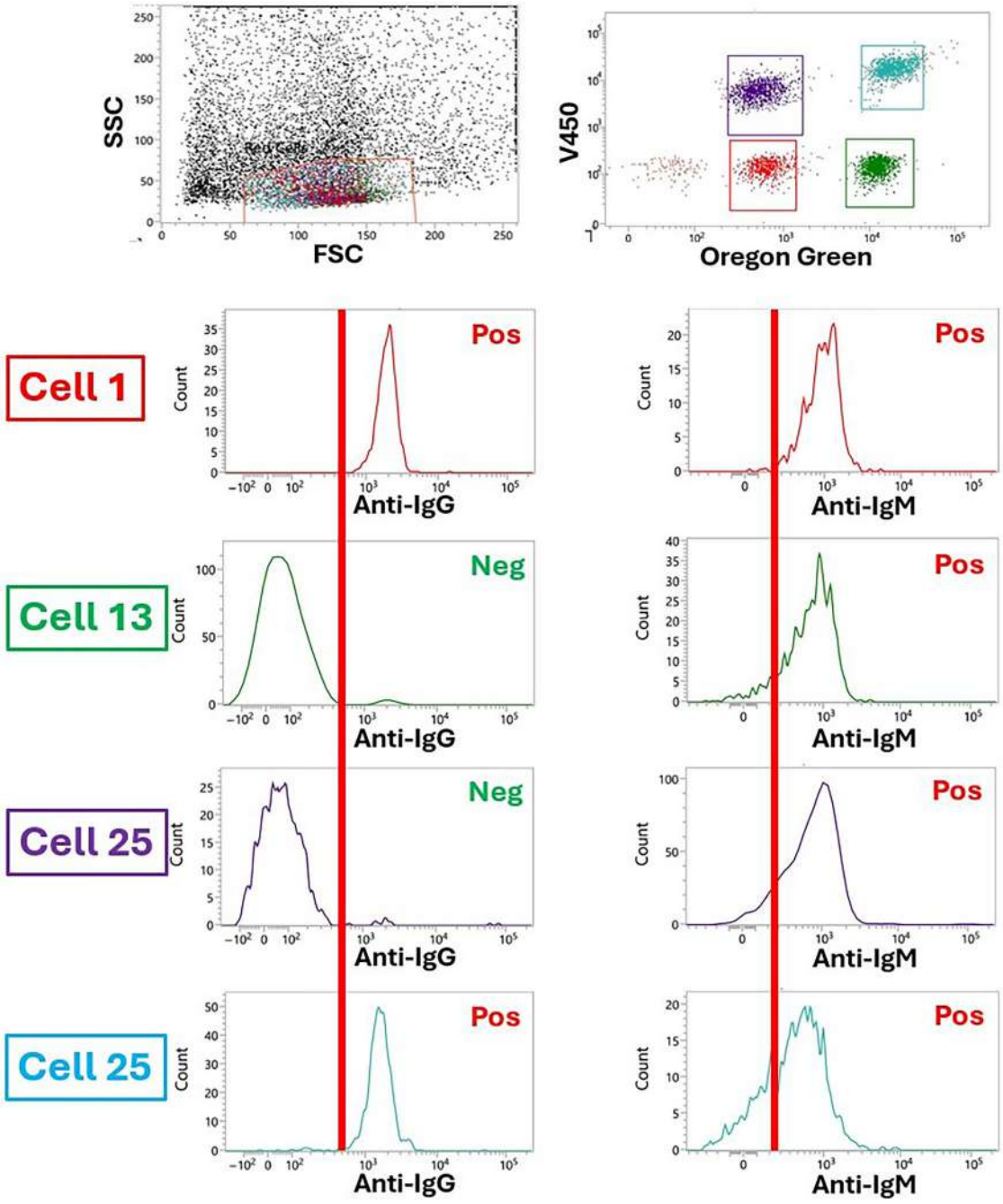

FIGURE 2 The top left panel shows the gating strategy based on side scatter (SSC) and forward scatter (FSC) followed by gating on red blood cells based on their staining of V450 and Oregon Green intracellular dyes (top right panel). The gating boxes are as follows: V450 positive (purple box), Oregon Green (green box), double positive (blue box) and double negative (red box). The panels below show median fluorescence intensity on the *x*-axis for anti-IgG–allophycocyanin (labelled Anti-IgG) labelling the S antigen and anti-IgM–phycoerythrin (labelled Anti-IgM) labelling the C antigen. The red line illustrates the positive cut-off. IgG, immunoglobulin G; IgM, immunoglobulin M; Neg, reaction graded negative; Pos, reaction graded positive.

throughput. Future directions for this work include the utilization of additional intracellular dyes to further increase throughput, optimizing the assay for the given numbers of dyes used with respect to number of RBCs stained, volume of stain and volume of antisera used, potentially reducing reagent volumes and associated costs.

ACKNOWLEDGEMENTS

J.Q. was involved in design, experimental work, data acquisition, analysis and writing of manuscript; R.L. was involved in design, experimental work, data acquisition, analysis and manuscript writing; I.G. was involved in data acquisition, data analysis and manuscript preparation; A.G. was involved in some aspects of design, experimental work and

manuscript review; C.R. was involved in data acquisition and manuscript review; C.C. was involved in interpretation of results and manuscript review.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

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

How to cite this article: Liwski R, Greenshields A, Grace I, Rourke C, Cheng C, Quinn JG. The use of intracellular dyes to create a multiplexed flow cytometry-based red blood cell phenotyping assay. Vox Sang. 2024;119:752–7.

Received: 15 December 2023 | Revised: 10 April 2024 | Accepted: 11 April 2024

DOI: 10.1111/vox.13643

SHORT REPORT

Vox Sanguinis ISBT International Society of Blood Transfusion

Predicting haemoglobin deferral using machine learning models: Can we use the same prediction model across countries?

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Funding information
Stichting Sanquin Bloedvoorziening, Grant/Award Number: 21-10/L2590; Punainen Risti Veripalvelu (FRCBS); Australian Government

Abstract

Background and Objectives: Personalized donation strategies based on haemoglobin (Hb) prediction models may reduce Hb deferrals and hence costs of donation, meanwhile improving commitment of donors. We previously found that prediction models perform better in validation data with a high Hb deferral rate. We therefore investigate how Hb deferral prediction models perform when exchanged with other blood establishments.

Materials and Methods: Donation data from the past 5 years from random samples of 10,000 donors from Australia, Belgium, Finland, the Netherlands and South Africa were used to fit random forest models for Hb deferral prediction. Trained models were exchanged between blood establishments. Model performance was evaluated

Amber Meulenbeld and Jarkko Toivonen shared first authorship.

Mart Janssen and Mikko Arvas shared last authorship.

using the area under the precision–recall curve (AUPR). Variable importance was assessed using SHapley Additive exPlanations (SHAP) values.

Results: Across the validation datasets and exchanged models, the AUPR ranged from 0.05 to 0.43. Exchanged models performed similarly within validation datasets, irrespective of the origin of the training data. Apart from subtle differences, the importance of most predictor variables was similar in all trained models.

Conclusion: Our results suggest that Hb deferral prediction models trained in different blood establishments perform similarly within different validation datasets, regardless of the deferral rate of their training data. Models learn similar associations in different blood establishments.

Keywords

donor health, haemoglobin deferral, haemoglobin measurement, prediction

Highlights

- Within one validation dataset, haemoglobin deferral prediction models from different blood establishments show similar predictive performance.
- Performance of haemoglobin deferral prediction models is more influenced by the characteristics of the validation dataset than by the origin and characteristics of the training data.
- Despite differences in the origin of training data, the importance of predictor variables remains largely consistent across all trained models and validation datasets.

INTRODUCTION

To mitigate the risk of anaemia in whole blood donors, blood establishments are by law required to conduct haemoglobin (Hb) tests. Deferral from donation based on these tests protects donor health but may be demoralizing for the donors and costly for the blood establishments [1]. Predicting deferral or iron status can help guide donation strategies. For example, by postponing donor invitations for which a model would predict deferral. However, donor data that are collected varies across blood establishments, in terms of consistency, number and type of variables. This complicates the development of universal prediction models. Here, we assess the potential of exchanging prediction models using a common set of predictors of Hb deferral between blood establishments.

The SanguinStats group has previously presented a comparison of various Hb prediction model architectures in Australia, Belgium, Finland, the Netherlands and South Africa. We found that the performance of different types of models applied within a blood establishment was very similar, but models overall perform better in countries with a high deferral rate [2].

In the past, various model structures of Hb prediction models were constructed and validated within each blood establishment and external validation of regression models was performed, but transferring of machine learning models between multiple blood establishments remains unexplored [2–4]. Our set up, in which similar datasets and model architectures were used across blood establishments, provides an opportunity to assess how well-performing models (trained on data from blood banks with high deferral rates) perform on datasets that yielded poor performing models, and vice versa. The motivation for studying the transferability of Hb prediction models between blood establishments is twofold: first, we investigate whether the training or validation data is the most important determinant of the performance of our prediction models. Second, it holds the potential to investigate whether or not blood establishments that lack resources to independently develop and train such models can adopt existing prediction models.

METHODS

Data sources

We used previously described data collected over a period of 5 years among whole blood donors from Australian Red Cross Lifeblood, the Finnish Red Cross Blood Service, Dutch national blood bank Sanquin, the Red Cross Flanders in Belgium and the South African National Blood Service [2].

Prediction models

In each setting, we trained a random forest (RF) model to predict Hb deferral in males and females separately. Because Australian Red Cross Lifeblood and the South African National Blood Service apply a lower Hb deferral cut-off (women 120 and men 125 g/L) than the EU countries (women 125 and men 135 g/L), we compiled two validation datasets for these blood establishments: using the national and EU cut-off.

Models are trained using the same variables in each setting, namely donor age; days since donation (for each of the 5 previous donations); Hb (for each of the 5 previous donations); Hb at first ever donation; season (warm vs. cold); number of consecutive deferrals since the last successful donation; number of successful donations in the last 5 years and the number of low Hb measurements in the last 2 years ('Recent low Hb'). The outcome is a dichotomous variable: deferral or non-deferral based on Hb level. To account for the difference in deferral cut-offs, we trained two models in Australia and South Africa: using the national and European threshold.

The training, validation and test sets consisted of 6400, 1600 and 2000 donors, respectively, based on a common but arbitrary way of partitioning data in proportions 0.64, 0.16 and 0.20. The RF hyperparameters were tuned on Finnish training data using grid search with receiver operating characteristic (ROC) as the objective function. The trained RF models were distributed among all blood establishments and applied to their validation datasets.

To mimic our analysis in a controlled setting, we additionally conducted a simulation in 10,000 fictional donations. We trained and tested classifier models for predicting Hb deferral in datasets with different deferral rates (1%, 2%, 4%, 8%, 16%) and levels of measurement error (0.25 and 0.5 mmol/L). The simulation is further described in Data S1.

Performance metrics

A widely adopted metric for assessing prediction model performance is the area under the ROC (AUROC) curve. However, given the class imbalance in our datasets, we opted to evaluate model performance using the area under the precision–recall curve (AUPR), a metric better suited to such imbalanced data [5]. This curve shows the precision versus recall of the models as a function of the classification threshold, and the area under this curve represents the average precision across all thresholds. A higher AUPR value indicates a better overall model performance. We subtracted the deferral rate from the AUPR, to adjust for the difference in deferral rates between validation datasets which would systematically bias this estimate [2]. We generated confidence intervals of the AUPR using boot.ci (boot R package, 1.3.28). To obtain stable interval estimates, we used a normal approximation with 2000 bootstrap samples. As the AUROC is a commonly used metric, we have included those results in Data S2. To compare variable importance across models and settings, we calculated the mean absolute attribution derived from absolute SHapley Additive exPlanations (SHAP) values [6].

Software and code

To ensure that the same RF model architecture is trained in every blood establishment, we used a Docker container (version 0.34). A Docker container is a self-contained package including all necessary components to run an application, facilitating consistent deployment across environments. For this study, we adjusted a previously developed container to export trained RF models without the training data [2, 7]. The Docker container image and source code are available through Github (https://github.com/FRCBS/Hb_predictor_container). Analyses of the results were performed using R (4.1.1) with packages dplyr (1.1.2), tidyr (1.3.0), ROCR (1.0–11), ggplot2 (3.4.2) and cowplot (1.1.1).

RESULTS

Characteristics of the donors included for the analysis are displayed in Data S3.

Comparison of model performance

In Figure 1 per validation dataset, the AUPR values are shown for each of the seven RF prediction models. In Data S2, the model performance in each validation dataset is expressed as AUROC. For women, in most cases, the highest performance in each validation dataset was obtained by the model that was trained at that blood establishment. Exceptions are Australian women (EU Hb deferral cut-off), where the model trained in South Africa (EU Hb deferral cut-off) performed similarly; Finnish women, where the model trained in the Netherlands performed better and South African women (national Hb deferral cut-off), where the model trained on the Australian data (national Hb deferral cut-off) performed better. For men, we observe a similar pattern, with some exceptions as well: for Australian men (national Hb deferral cut-off) the Belgian model performed best and in both the Netherlands and South Africa (national Hb deferral cut-off), the models trained in the Netherlands and South Africa (national Hb deferral cut-off) performed equally well. When grouping the prediction models per validation dataset, the confidence intervals of the AUPR in both men and women largely overlap, indicating that the performance of all seven prediction models does not significantly differ when applied to the same validation dataset.

The Australian and South African setting provide the opportunity to compare performance on two validation datasets using two Hb deferral cut-off levels. In both the blood establishments, the prediction models for both sexes perform better on the validation dataset compiled with the EU Hb cut-off level than their own, lower, Hb cut-off level. Moreover, in our simulation, we found that classifier prediction models generally perform better on datasets with higher deferral rates. Performance is further improved when more accurate measurements are used for prediction (Data S1).

Comparison of variable importance

In Figure 2, per validation dataset, the top six SHAP values per model and the average of all models are shown to indicate variable importance in the prediction of Hb deferral. Importance of all variables can

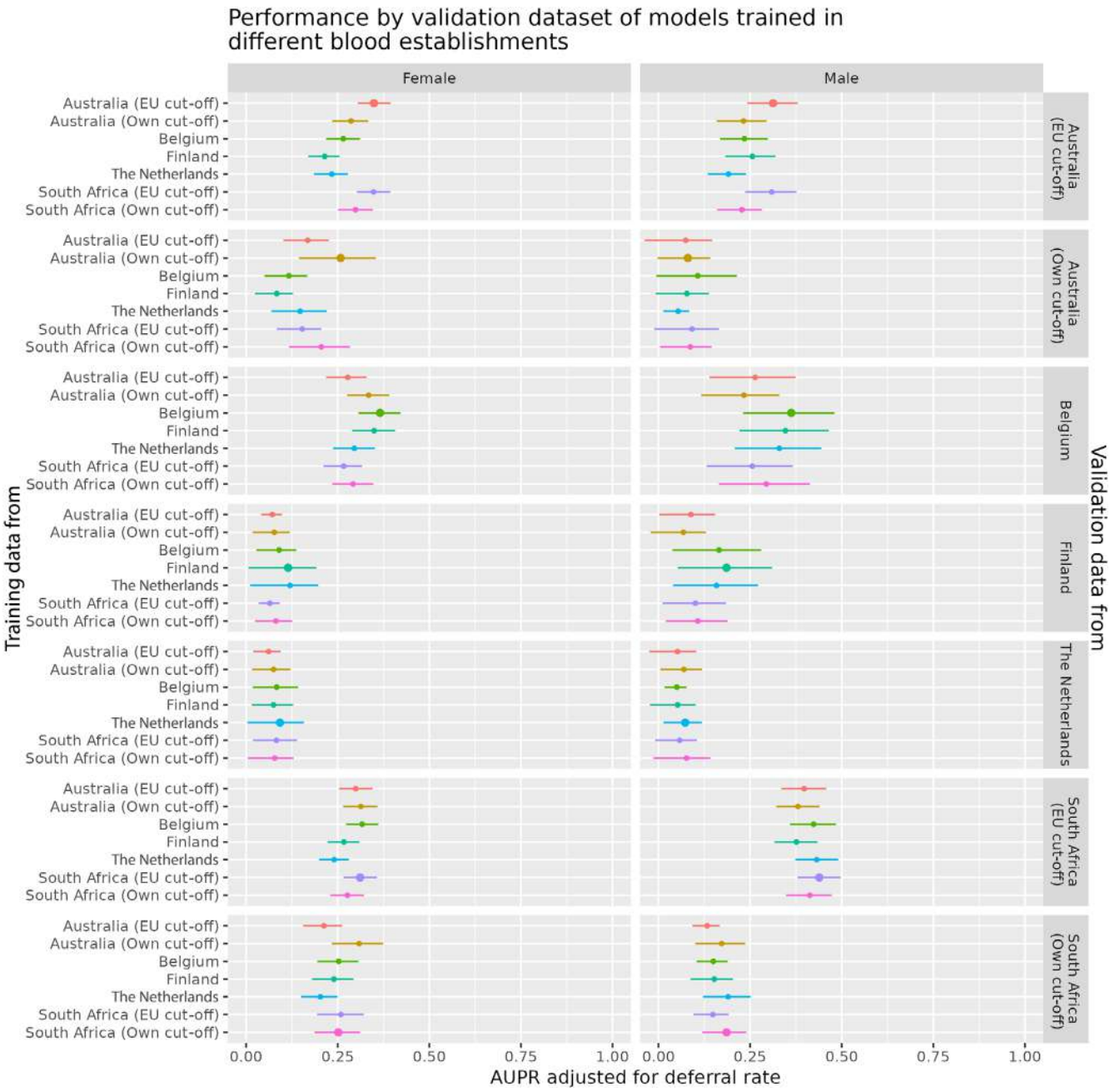

FIGURE 1 Area under the precision–recall curve (AUPR) and confidence intervals of prediction models trained in different settings by validation dataset. Larger points indicate that the origin of the training and validation data matches.

be found in Data S4. Overall, within and between validation datasets, variable importance was very similar. Although Hb measurements at previous visits were considered more important in the prediction of deferral, the timing of these measurements was less important. On average, in all validation datasets, previous Hb was the most important predictor of Hb deferral. However, across validation datasets, the importance of this variable was especially high for predictions by the model trained on the Belgian data. Across validation datasets, recent donations contributed to the prediction more when the model trained on Dutch data was used than when other models were used for both men and women.

DISCUSSION

In this article, seven prediction models trained on datasets from five blood establishments were applied to validation datasets from all blood establishments to assess the performance of exchanged models. In most cases, a match between the origin of the validation and training dataset yielded the highest performance. The seven models performed similarly and yielded modest results, although new variables representing four earlier Hb measurements and their timing were added to the RF model [2]. Across all models and datasets, previous Hb is the most important predictor for donor deferral,

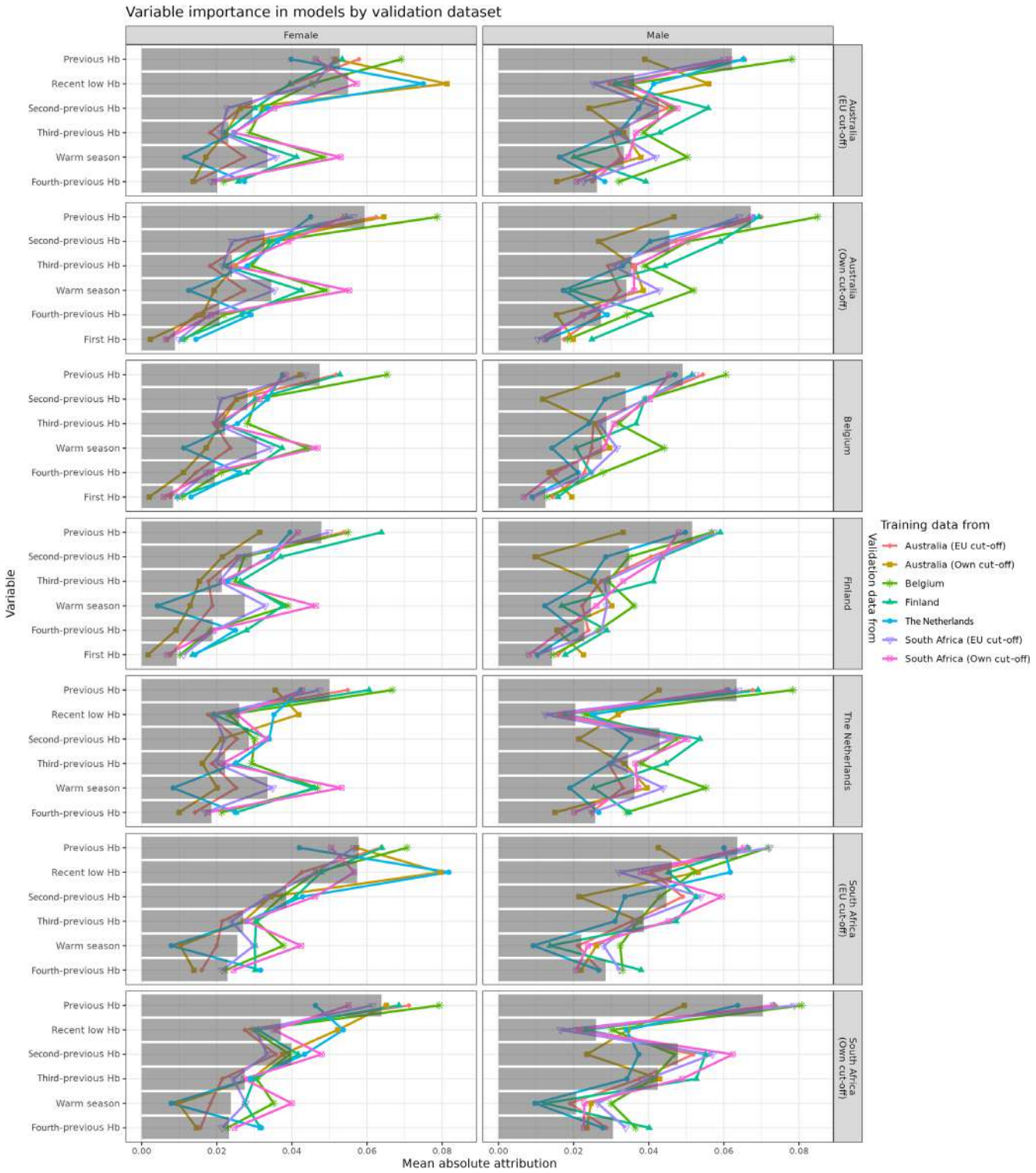

FIGURE 2 Six most important variables of prediction models from different blood establishments by validation dataset. The grey bars represent the mean importance of the variable across models for the validation dataset. Hb: haemoglobin.

and variable importance is similar both within and between validation datasets.

Generally, performance measures indicate that models perform better on validation datasets with higher deferral rates (Australia, Belgium and South Africa), and this performance is evident even when using models that are trained on datasets with low deferral rates (the Netherlands and Finland) [2]. Model performance and variable importance are similar within the validation datasets, independent of the training dataset. These results suggest that donor deferral is easier to predict in some blood establishments because of differences in

pre-analytical procedures and measurement accuracy, reflected in the validation dataset, and not because prediction models learn more about the associations when trained on datasets with high deferral rates.

The finding that performance depends on the validation data rather than the training data is supported by the simulation conducted in Data S1: the adjusted AUPR increased with higher donor deferral rates, with the adjusted AUPR being more sensitive to changes in positive predictive value. In addition, these findings emphasize the significance of the accuracy of Hb measurements and its impact on the predictive capabilities of the models, as illustrated by improved performance when measurement error was reduced.

To our knowledge, this is the first paper to exchange trained machine learning models between blood banks, whereas previous research has focussed mainly on external validation of regression models [2–4]. Using a prediction model trained elsewhere could be beneficial for blood banks without the data volume or technical expertise required for training such models, as long as the required variables are available. There are, however, limitations to these results. The predictive performance of the specific RF models presented here and thus their use in practice is limited. Moreover, possibilities of exchanging machine learning models without sharing sensitive data are limited. In our previous project, we also trained support vector machines, but we were unable to exchange these models as they required partially sharing the training data. The RF models do not store any training data whilst performing equally well as the support vector machine models and are therefore ideal for model exchange [2]. Ensemble modelling, a machine learning technique that combines individual prediction models, was explored to further improve predictive performance. However, probably as a result of the similarity in main predictor variables, the benefits of this approach were found to be limited in this case.

This article has shown that it is possible to exchange trained RF prediction models for Hb deferral between blood establishments and that performance is determined mainly by the deferral rate in the validation dataset. The performance differences between prediction models was negligible within validation datasets. For blood establishments without resources to train prediction models that want to apply them in practice, it can be beneficial to use a model that was trained elsewhere. In particular for a population with a Hb deferral rate of for example over 10%, implementation of donation intervals based on a prediction model could be a useful way to reduce Hb deferrals.

ACKNOWLEDGEMENTS

Supported by Sanquin Blood Supply Foundation, PPOC grant 21-10/L2590, VTR (Valtion tutkimusrahoitus) funding from the Australian Governments fund Australian Red Cross Lifeblood to provide blood, blood products and services to the Australian community.

M.J., M.A., A.M., J.T. and J.v.R. designed the study; J.T. contributed to software development; A.M., J.T., D.d.C., T.B., M.W. obtained results from local data; M.J. and A.M. contributed to simulations and code for Data S1; A.M. aggregated the results and drafted the paper; M.J., M.A., A.M., J.T., M.V., J.v.R., R.S., E.L., V.C., S.K. and K.v.d.H. reviewed and edited of the final paper.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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SUPPORTING INFORMATION

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

How to cite this article: Meulenbeld A, Toivonen J, Vinkenoog M, Brits T, Swanevelder R, D de Clippel, et al. Predicting haemoglobin deferral using machine learning models: Can we use the same prediction model across countries? Vox Sang. 2024;119:758–63.

Received: 7 December 2023 | Revised: 15 March 2024 | Accepted: 16 April 2024

DOI: 10.1111/vox.13646

SHORT REPORT

Vox Sanguinis ISBT International Society of Blood Transfusion

Evaluation of a method to fluorescently label platelets for in-human recovery and survival studies

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Abstract

Background and Objectives: Platelets for transfusion are evaluated for in vivo quality using recovery and survival measurements in healthy human subjects. Radiolabelling is the standard for tracing platelets post-transfusion but imposes logistical and technical limitations. This study investigates the in vitro feasibility of labelling platelets with the calcein family of fluorescent dyes as an alternative to radioisotopes or biotin.

Materials and Methods: Protocols for radiolabelling were adapted for use with calcein acetoxymethyl ester (CAM) and biotin. Labelled platelets were analysed by flow cytometry and evaluated for activation and function. We tested feasibility for labelling without manipulation of platelets and for multiplexing of samples.

Results: Labelling at 2 μg CAM/10^{10} platelets resulted in >99% of CAM^+ platelets. There was no significant difference in activation or aggregation between CAM-labelled or biotinylated platelets and vehicle controls although $\%CD62P^+$ was significantly lower in platelets that were not processed for labelling. Addition of CAM to the platelet storage bag labelled >95% of platelets. Platelet populations labelled with different dyes could be distinguished by flow cytometry.

Conclusion: These data provide a rationale for further development of CAM and other fluorescent dyes as tools for measuring post-transfusion kinetics of platelets.

Keywords

in vivo recovery and survival, platelet labelling, platelet tracking

Highlights

- Current labelling methods to prepare platelets for radiolabelling increase surface expression of P-selectin.
- Biochemical labels, such as calcein acetoxymethyl ester and biotin, can be used to rapidly and efficiently label platelets for human studies, regardless of the plasma content.
- Novel biochemical labels improve the preparation method and may eliminate the need for radioisotopes in platelet kinetic studies.

INTRODUCTION

In vivo kinetic studies are frequently required by regulators for licensure of novel or modified platelet products [1]. The gold standard method to evaluate platelet kinetics uses radioisotopes (^{51}Cr and ^{111}In) to label platelets for infusion in healthy volunteers according to standardized and validated procedures published by the Biomedical Excellence for Safer Transfusion (BEST) Collaborative [1]. Although

radiolabelling enables evaluation of recovery and survival (R&S) of platelets in circulation, the use of radioisotopes imposes significant expense and regulatory burden on clinical sites that can impede development and licensure of new products [2]. Radiolabelling further confines evaluation of therapeutic potential to healthy volunteer studies, requires extensive manipulation of platelets during labelling, results in low labelling efficiency for platelets (5%–15% with ^{51}Cr) and limits assessment of a complex cell population to a single parameter.

An alternative to radiolabelling is to tag platelets with a small molecule for single-cell enumeration and characterization by flow cytometry. This approach is in advanced development for kinetic studies using biotinylated red cells [3]. N-Hydroxysulfosuccinimide (NHS) esters of biotin label cell surface proteins by covalent binding to primary amines [4]. Biotinylated platelets are in early development, in part due to concerns about non-specific binding to plasma proteins requiring additional washing [5–7].

Advances in dye chemistry have produced diverse fluorescent reagents with potential to overcome limitations of radioisotopes and biotin [4]. Fluorescent dyes are used to trace platelets in animal models [8, 9] but have received limited attention as an alternative to radiolabelling studies [10]. Calcein acetoxymethyl ester (CAM), a membrane-permeable dye that becomes cell impermeant and brightly fluorescent upon cleavage of the AM moiety by esterase enzymes [4], has been used to trace murine platelets in vivo [8]. In this study, we present in vitro feasibility data on the calcein dyes as an alternative to radiolabelling human platelets and compare with biotinylation, another promising option.

MATERIALS AND METHODS

Apheresis platelets in 100% plasma were collected with Trima Accel (Terumo BCT, Lakewood, CO) under American Red Cross Biomedical Services (ARCBS) Institutional Review Board (IRB) approval. After a 2-hour resting period, platelets were stored with agitation (Helmer Scientific, Noblesville, IN) at room temperature for 5–7 days prior to use. Complete blood counts were performed on a Sysmex XE-2100D (Sysmex Corp., Kobe, Japan).

Platelets were labelled using a variant of the BEST method (Figure 1a) [1]. Briefly, 10 mL was removed from the platelet unit and mixed with Anticoagulant Citrate Dextrose, Solution A (ACD-A; Fenwal) prior to centrifugation. The supernatant was removed, and the pellet resuspended in 2 mL of an ACD-A/saline solution. Platelets were labelled with Sulfo-NHS-Biotin (Fisher Scientific, PIA39256), Calcein AM (CAM; Fisher Scientific, C3099), or Calcein Violet 450 AM (CVAM; Fisher Scientific 50-169-54). The equivalent volume of dimethyl sulfoxide (DMSO) or water was added to vehicle controls. Platelets were labelled at room temperature for 30 min with biotin or

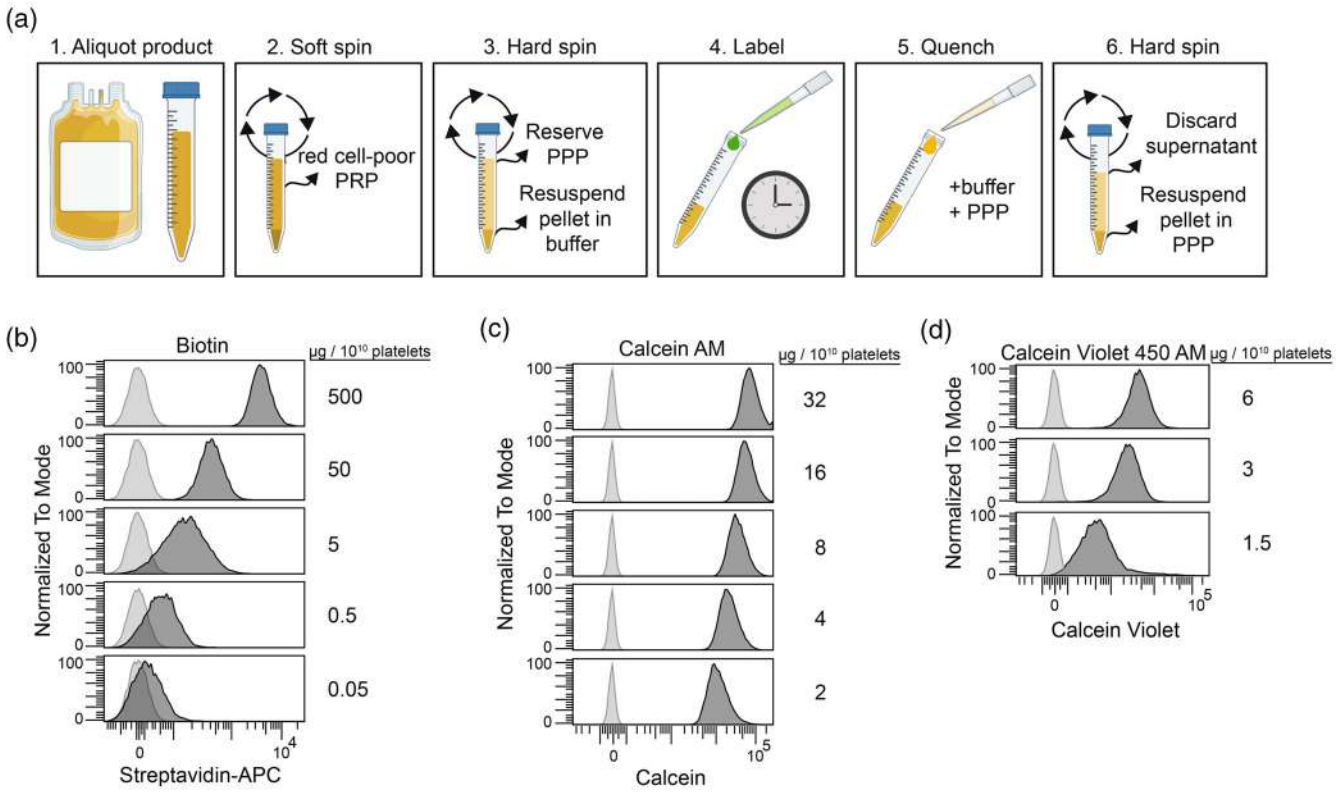

FIGURE 1 Efficient platelet labelling with biotin or calcein acetoxymethyl ester (CAM) does not affect platelet activation or aggregation. (a) Workflow used to prepare labelled platelets. (b) Histograms of fluorescence intensity for biotinylated platelets stained with APC-conjugated streptavidin (black) overlaid with an unstained control (grey). (c) Flow cytometry histograms of fluorescence intensity for CAM-labelled platelets (black) overlaid with an unstained control (grey).

for 15 min with CAM or CVAM. An additional 3.5 mL of ACD-A/saline and 0.5 mL of plasma were added prior to centrifugation. The staining solution was removed, and platelets were resuspended in plasma.

Platelets were diluted to 10^7/mL in 100 uL of 1X phosphate buffered saline (PBS) supplemented with 0.1% human serum albumin (Gemini Bio-Products, 800-120). Staining occurred for 20 min at room temperature. Single and multicolor panels used the following: mouse anti-human CD61 PerCP-Cy5.5 (BD Biosciences, 564173), mouse anti-human CD62P PE (BD Biosciences, 555524), mouse anti-human CD42b PE (BD Biosciences, 555473) or APC (BD Biosciences, 551061). A total of 20 µL of antibody was used per 100 µL test. Biotinylated platelets were detected by APC-conjugated streptavidin (BD Biosciences, 554067). Each experiment included unstained and single-stained platelets for compensation and fluorescence-minus-one (FMO) controls. Samples were analysed on a FACS Lyric cytometer (BD Biosciences).

Aggregometry was performed on an Aggregometer (Chronolog, Havertown, PA). Autologous plasma was used as a blank. Aggregation was measured in response to collagen (10 µg/mL) and adenosine diphosphate (ADP) (10 µM) dual agonists.

Stimulation of 60 x 10^6 platelets was conducted in 1 mL of PBS with 150 pg of PMA. Platelets were incubated with PMA for 10 min at room temperature prior to staining for CD62P expression.

Analysis of flow cytometry data was performed using FlowJo (version 10.9.0). GraphPad Prism (version 10.0.3) was used for statistical testing and data visualization. Repeated measures one-way analysis of variance ($n = 3$ platelet donors, two repeats per donor) was performed. Significance was defined as $p < 0.05$. Significant results were analysed by subgroups using Tukey's multiple comparison (significance of $p_{adj} < 0.05$). Descriptive statistics are reported as mean ± standard deviation unless otherwise stated.

RESULTS

Platelet labelling by the variant BEST method is described in Figure 1a [1]. Titrations of biotin, CAM and CVAM showed that 50 or 500 µg of biotin, 2 µg of CAM or 3 ug CVAM per 10^{10} platelets was sufficient to achieve bright, uniform labelling of >99% of platelets (Figure 1b–d). Subsequent experiments on platelet function used

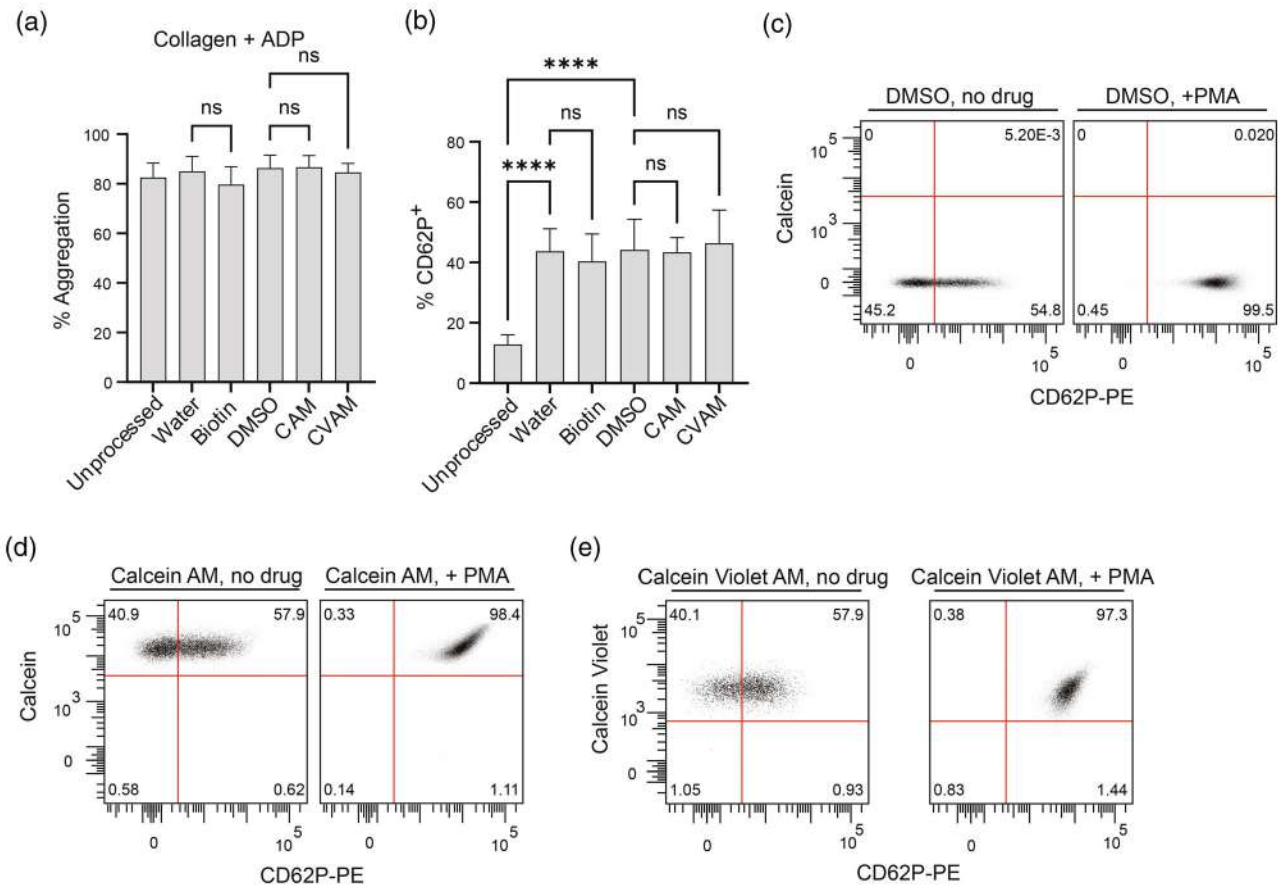

FIGURE 2 Labelling with biotin or calcein acetoxymethyl ester (CAM) does not affect platelet activation or aggregation. (a) Maximum aggregation in response to a dual agonist of 10 µg/mL collagen and 10 µM adenosine diphosphate (ADP). (b) Percentage of $CD62P^+$ platelets measured by flow cytometry. (c) Biaxial plots showing expression of $CD62P^+$ and CAM staining with and without treatment with a platelet activating drug. Data are shown for a vehicle control, (d) CAM, and (e) Calcein Violet 450 AM (CVAM)-labelled platelets. Statistical testing was performed on $n = 3$ replicates. Bars represent mean ± SD; $^{****}p_{adj} < 0.05$; ns, not significant.

75 μg of biotin per 10^{10} platelets and 2 μg of CAM or 3 μg CVAM per 10^{10} platelets, using water or DMSO as the respective vehicle control. Platelets directly from the storage bag were used to account for the effect of manipulating platelets during the labelling workflow.

There was no significant difference in platelet aggregation in response to a dual agonist of 10 μg/mL collagen and 10 μM ADP between any of the treatments (Figure 2a). The percentage of platelets expressing CD62P on the cell surface, a marker of platelet activation, was not significantly different between labelled platelets (biotin, 40.3% ± 9.1%; CAM 43.3 ± 4.9%; CVAM, 46.3 ± 11.0%) and vehicle controls (water, 43.7% ± 7.5%; DMSO, 44.1% ± 10.1%) processed by the BEST method (Figure 2b). In contrast, we found a significantly lower percentage of $CD62P^+$ platelets in the unprocessed condition (12.8% ± 3.2%). CAM- and CVAM-labelled platelets treated with phorbal-myristate-acetate (PMA), a drug known to induce CD62P expression, retained fluorescence and the percentage of $CD62P^+$ platelets increased to >98% (Figure 2c–e).

One of the limitations of NHS-biotin is non-specific binding to plasma proteins which reduces labelling efficiency and necessitates multiple wash steps, increasing activation [2]. We found that inhibition of CAM-labelling by plasma was negligible (Figure 3a,b). We hypothesized that platelets could be labelled in situ by adding CAM directly to the platelet bag. Dye was added to a 5-day stored unit in 100% plasma at a ratio of 10 μg of CAM per 10^{10} platelets and agitated for 30 min. After a single wash and resuspension in plasma, we measured CAM staining by flow cytometry and found bright, uniform staining of >99% of platelets (Figure 3c).

The current in vivo R&S method simultaneously compares novel products with fresh, autologous platelets as a control [1, 11]. Therefore, a replacement method should permit multiplexing labelled platelets. To determine whether CAM and CVAM could distinguish two distinct platelet populations in a single sample, platelets were labelled with either CAM and anti-human CD42b APC or CVAM and anti-human CD42b PE and then mixed. Of the total platelets in the sample, 53.7% were PE^+APC^- and 44.5% were PE^-APC^+ (Figure 3d). Comparably, 52.4% of platelets were CAM^+CVAM^- and 44.4% were CAM^-CVAM^+ (Figure 3e).

DISCUSSION

Despite its utility for estimating platelet kinetics, radiolabelling comes at a high cost and regulatory burden for a limited view of platelet biology post-transfusion. An ideal label and protocol would result in high labelling efficiency, minimal impact on platelet function, minimal manipulation of platelets during labelling and an option for multiplexing. We evaluated two calcein dyes, CAM and CVAM, alongside biotin for feasibility as a replacement for radiolabelling.

Adapting the BEST radiolabelling method to alternative labels produced bright, uniform labelling of platelets labelled after 5 days of storage. We observed labelling of >99% of the $CD61^+$ population,

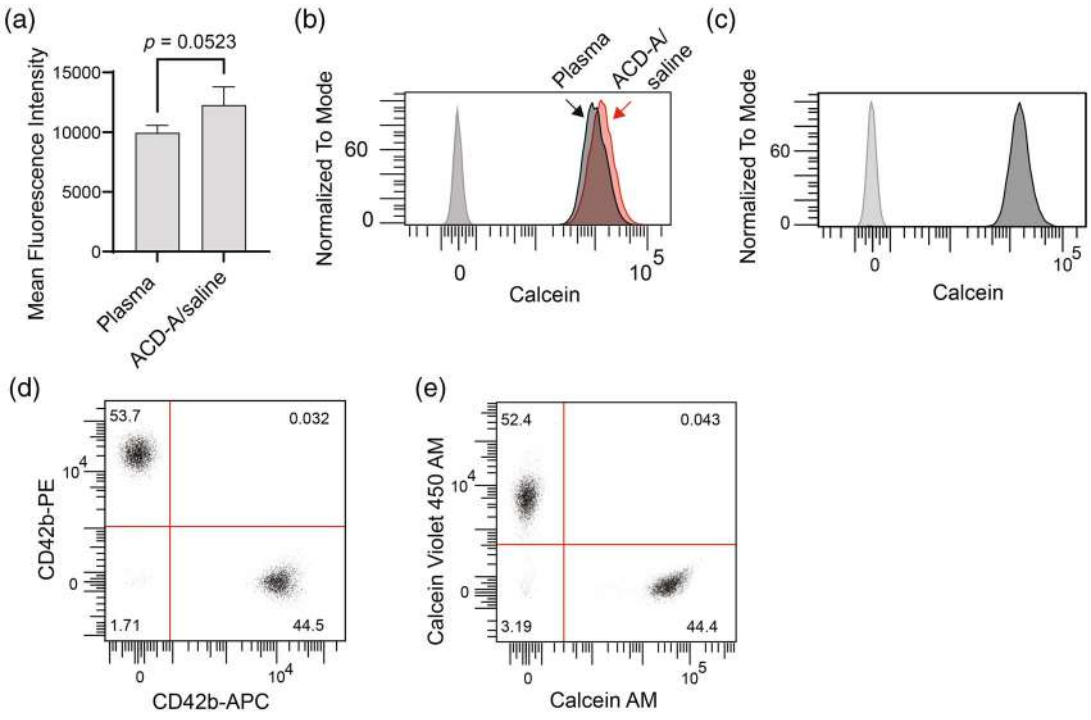

FIGURE 3 Fluorescent labelling enables multiplexing of platelet samples and labelling in the platelet bag. (a) Mean fluorescence intensity of calcein acetoxymethyl ester (CAM) platelets labelled in 100% plasma or ACD-A/saline. (b) Representative histogram showing fluorescence intensity of platelets labelled in 100% plasma or ACD-A saline. (c) Histogram showing fluorescence intensity of platelets labelled with CAM in the apheresis platelet bag ($n = 1$). (d) Platelets were stained with CD42b-APC and CAM or CD42b-PE and calcein Violet 450 AM (CVAM) prior to mixing 1:1 ($n = 1$). Two distinct populations are visible on biaxial flow cytometry plots of PE versus APC or (e) CVAM versus CAM.

suggesting that the storage lesion did not preclude intracellular retention or activation of the calcein dyes. Whether cold storage or freeze-dried preparations would affect calcein labelling is unknown. Other groups have shown that fresh and stored platelets are efficiently labelled by biotin [7]. While we found no direct effect of either label on platelet function, processing by the BEST protocol increased platelet activation as expected.

Development of alternative labels provides an opportunity to further optimize the BEST protocol. Although advances in closed system labelling have been made for biotin and indocyanine green, a fluorescent dye approved for clinical use, non-specific binding to plasma proteins necessitates washing steps and protein-free buffer to achieve efficient labelling [2, 10]. Our results suggest the effect of plasma on calcein labelling is minimal enough for labelling without prior manipulation of the platelet unit.

In vivo kinetic studies use a fresh platelet control for comparison with stored platelets and to control for donor variability [11], requiring multiplexing of at least two samples for simultaneous testing. Our results using CAM and CVAM demonstrate that related fluorescent dyes with distinct excitation and emission spectra can be used to distinguish multiple platelet populations in the same sample. Others have shown that the same can be achieved by labelling with biotin at different densities [6]. Either labelling strategy would enable characterization of labelled platelets by flow cytometry, increasing the value of in vivo studies.

Our in vitro feasibility study demonstrates that the calcein dyes and biotin merit further investigation as replacements for radiolabelling. Future studies on calcein labelling must address functional consequences over the platelet lifespan and the potential for elution and possible transfer of the label to other blood cells. CAM-labelled platelets have been traced in mice for up to 96 h [8], but in vivo elution is not well-characterized. Immunogenicity, a concern raised by studies with biotinylated red cells [3], is unknown for biotinylated and CAM-labelled platelets. Although calcein is routinely used in preclinical animal models [8, 12], and its parent compound, fluorescein sodium, has FDA approval, further work is needed to evaluate calcein dyes for safety in humans and develop methods to estimate recovery, survival and function of CAM platelets in post-transfusion samples.

ACKNOWLEDGEMENTS

This study was conducted and funded by the American Red Cross.

T.P.F. designed the research, performed analysis and wrote the first draft of the manuscript; B.L.B. supervised the research and reviewed and edited the manuscript; illustrations in Figure 1a were created with BioRender.com.

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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How to cite this article: Feldman TP, Brown BL. Evaluation of a method to fluorescently label platelets for in-human recovery and survival studies. Vox Sang. 2024;119:764–8.

DOI: 10.1111/vox.13711

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