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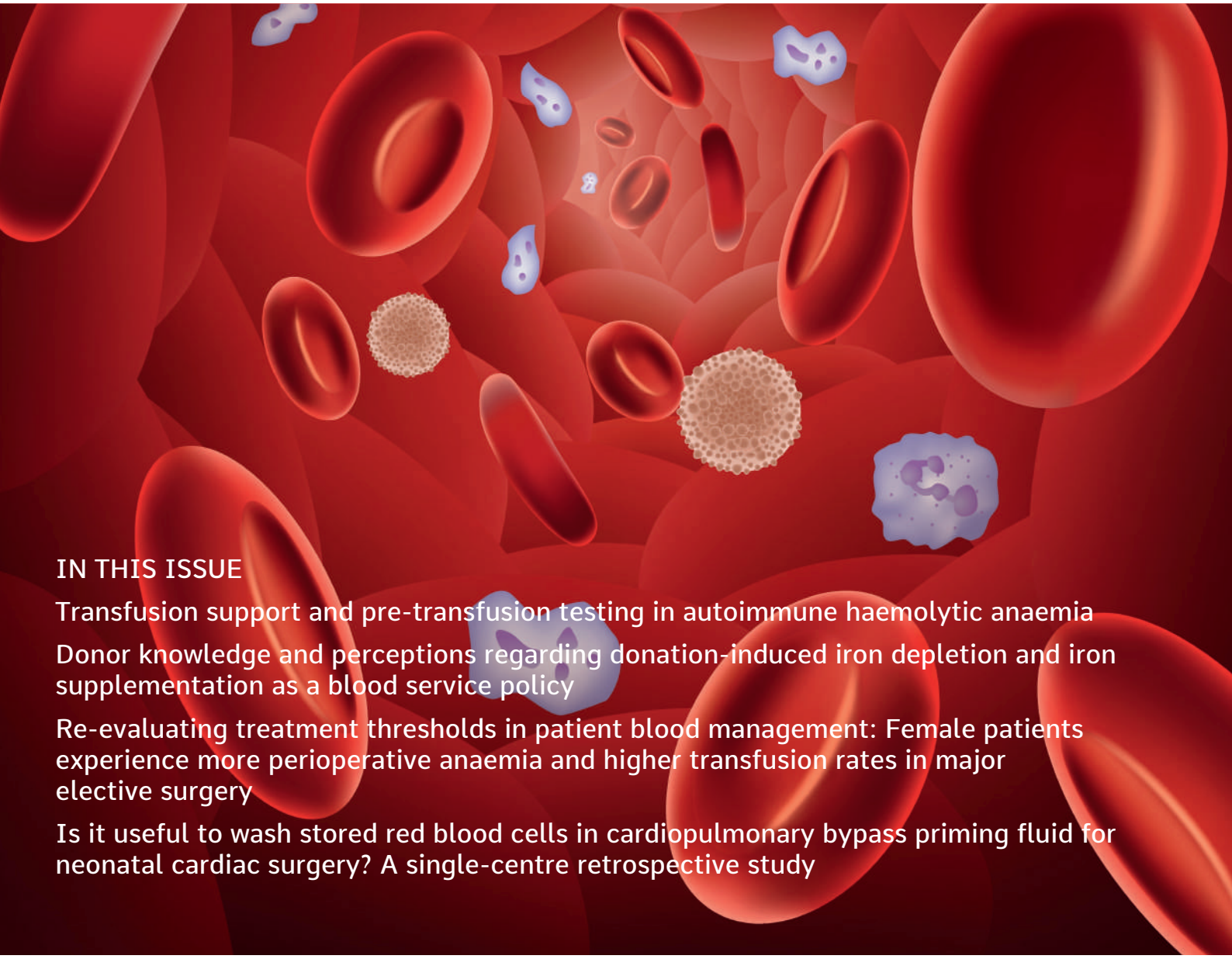

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Aims and Scope

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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.

Vox Sanguinis also publishes the abstracts associated with international and regional congresses of the ISBT. (Abstracts from meetings other than those held by the ISBT are not accepted.)

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REVIEW

Vox Sanguinis ISBT International Society of Blood Transfusion

Transfusion support and pre-transfusion testing in autoimmune haemolytic anaemia

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Abstract

Autoimmune haemolytic anaemia (AIHA) is characterized by an increased destruction of red blood cells due to immune dysfunction and auto-antibody production. Clinical manifestations are mainly related to anaemia, which can become life-threatening in case of acute haemolysis. Aiming at counterbalancing severe anaemia, supportive treatments for these patients frequently include transfusions. Unfortunately, free serum auto-antibodies greatly interfere in pre-transfusion testing, and the identification of compatible red blood cell units for AIHA patients can be challenging or even impossible. Problems faced in pre-transfusion testing often lead to delay or abandonment of transfusions for AIHA patients. In this review, we discuss publications concerning global transfusion management in AIHA, with a focus on pre-transfusion testing, and practical clues to manage the selection of transfusion units for these patients. Depending on the degree of transfusion emergency, we propose an algorithm for the selection and laboratory testing of units to be transfused to AIHA patients.

Keywords

autoimmune haemolytic anaemia, pre-transfusion testing, transfusion, transfusion algorithm, transfusion laboratory issues

Highlights

- Recent publications and recommendations suggest that, in adult patients presenting severe autoimmune haemolytic anaemia, transfusion support should not be delayed in life-threatening clinical situations.
- Depending on the degree of emergency, only minimum or more specific laboratory testing should be performed to avoid delay in transfusion support.
- Recommendations for transfusion management in the laboratory and blood bank are summarized in the proposed algorithm.

INTRODUCTION TO AUTOIMMUNE HAEMOLYTIC ANAEMIA

Autoimmune haemolytic anaemia (AIHA) designates different types of acquired pathologies that are characterized by an increased destruction of red blood cells (RBCs), caused by auto-antibodies directed against RBC membrane antigens. Primary and secondary AIHA forms can be distinguished, based on the absence or presence of another pre-existing condition that causes immune dysfunction [1, 2]. From a serological point of view, warm-type, cold-type and mixed-type AIHA can be distinguished.

Warm-type AIHA is the most frequent form, representing 60%–70% of AIHAs and generally involving polyclonal IgG-class antibodies that present maximum antigen binding at 37°C and frequently

recognize antigens of the RH system. As the complement system is only moderately activated, RBC destruction mainly occurs through extravascular haemolysis. Clinical manifestations are mainly related to anaemia, with a possible silent disease onset as long as anaemia is counterbalanced by physiological compensatory mechanisms. In case of severe anaemia, symptoms can go up to heart failure [3, 4].

Among cold-type AIHAs (20%–25% of AIHAs), the most frequent presentations are cold agglutinin disease (CAD) and cold agglutinin syndrome (CAS). These clinical conditions involve mono- or oligoclonal IgM-class antibodies that optimally bind to RBC antigens in vitro at 3–4°C. Intravascular haemolysis occurs as IgM molecules directly fix and activate the complement system. To a lesser extent, extravascular haemolysis occurs through opsonization. Haemolysis/anaemia can be chronic if auto-antibodies present high thermal amplitudes (20–37°C) or occur as acute haemolytic episodes after cold exposure if auto-antibodies present low thermal amplitudes (<20°C).

CAD is defined as a primary cold-type AIHA, but is associated in almost all cases with a silent B-cell lymphoproliferative disorder which causes monoclonal IgM production. CAS designates secondary forms that present similar characteristics but occur as a complication of another disease (infections, malignancies, etc.) or vaccination. Especially, infection-related CAS can occur in young people/children, leading to a transient haemolytic episode and, in the vast majority of cases, a spontaneous resolution [5–8].

Paroxysmal cold haemoglobinuria (PCH) is a rare cold-type AIHA involving an auto-antibody called Donath–Landsteiner biphasic haemolysin [9]. This cold-reactive IgG-type antibody presents a high complement-activating potential, leading to intravascular haemolysis. Most PCH cases are infection-related secondary forms [10, 11].

Mixed-type AIHA (5%–10% of AIHAs) is characterized by the simultaneous presence of warm-reactive and cold-reactive auto-antibodies. Cold-reactive antibodies in mixed type generally display higher thermal amplitudes (≥30°C) and higher antibody titres compared to CAD/CAS. Some cases present very severe and fulminating haemolytic anaemia [12–14].

Particular management is required in forms secondary to allogeneic haematopoietic stem cell transplantation (HSCT), which has a relatively high incidence of secondary AIHA, frequently presenting severe and treatment-refractory profiles [15, 16].

In this article, we review the transfusion support in AIHA and challenges faced in laboratories that perform pre-transfusion testing. We will discuss answers as to 'when' and 'how' to transfuse AIHA patients, corroborated with our own experience in the transfusion laboratory of Liège University Hospital in Belgium.

DIAGNOSTIC APPROACH AND LABORATORY TESTS IN AIHA

When a patient presents with anaemia in the absence of recent/active bleeding, investigations need to identify whether the cause is related to a production deficit or to an increased RBC destruction (i.e., haemolysis), which can be of non-immune or immune origin.

If immune-mediated RBC destruction is suspected, biological assessment first needs to confirm that haemolysis is occurring [17, 18]. Antibodies bound to the RBC surface can be detected by a direct antiglobulin test (DAT), which allows the in vitro identification of RBC sensitization occurring in vivo. RBC membranes can be sensitized by non-agglutinating antibodies (mostly IgG) that bind to RBC surface antigens and/or by molecules resulting from complement activation (C3c/C3d fractions). In a positive test, polyspecific (anti-IgG/C3) or monospecific anti-human globulin leads to RBC agglutination [19, 20]. Binding of IgM molecules to RBC surface is not very stable; thus in the vast majority of cases, a C3-positive DAT implies prior IgM binding and complement activation, while the monospecific IgM DAT remains negative.

AIHA is suspected when immune-mediated haemolysis is confirmed while the presence of allogeneic antibodies and RBCs (i.e., stem cell transplantation, transfusion, organ transplantation or foeto-maternal incompatibility) can be excluded. The most common DAT results in different AIHA presentations are summarized in Table 1 [1, 17].

Some AIHA patients (5%–10%) display a negative DAT result. Possible explanations include low-titre auto-antibodies, low-affinity IgG that detach in vitro from RBCs or atypical AIHA involving immunoglobulin types that are not detected by first-line anti-IgG/C3 reagents [21, 22]. Conversely, other patients can present a positive DAT and no haemolysis. COVID-19 patients present a higher prevalence of positive DAT results, associated with more severe forms of infection and anaemia [23, 24], but the association of autoimmune haemolysis in these patients remains more controversial [23–29].

Indirect antiglobulin test (IAT) reveals the presence of free antibodies after incubating a patient's serum with test RBCs of known phenotypes, followed by the addition of anti-human globulin. After a first-line IAT screening, larger panels are required to identify antibody specificity. In AIHA patients, free serum auto-antibodies can strongly interfere in these tests.

An elution of the RBC-sensitizing antibody can be performed in order to identify its specificity or panreactive profile. In AIHA patients presenting a 'negative' DAT result, elution might confirm the presence of an auto-antibody and is thus recommended in front of an unexplained haemolysis [2].

TABLE 1 Types of autoimmune haemolytic anaemia and most frequent direct antiglobulin test results.

AIHA type	Antibody type	DAT result	Eluate reactivity
Warm-type AIHA	IgG or IgA (rare)	IgG ± C3 or IgA (rare)	Panreactive
Cold-type AIHA	IgM	C3	Non-reactive
Mixed-type AIHA	IgG + IgM	IgG + C3	Panreactive
PCH	IgG (cold-reactive)	C3	Non-reactive

Abbreviations: AIHA, autoimmune haemolytic anaemia; DAT, direct antiglobulin test; PCH, paroxysmal cold haemoglobinuria.

DAT, IAT and elution tests that allow AIHA diagnosis are illustrated in Figure 1.

If DAT result is C3-positive, cold-type AIHA diagnosis is confirmed if the cold agglutinin titre at 4°C is greater than or equal to 1/64, below which cold agglutinins have usually no clinical relevance. This threshold represents a compromise for eliminating the majority of patients who present cold agglutinins without clinical relevance. In addition, thermal amplitude and antigen specificity should be determined. In most cases,

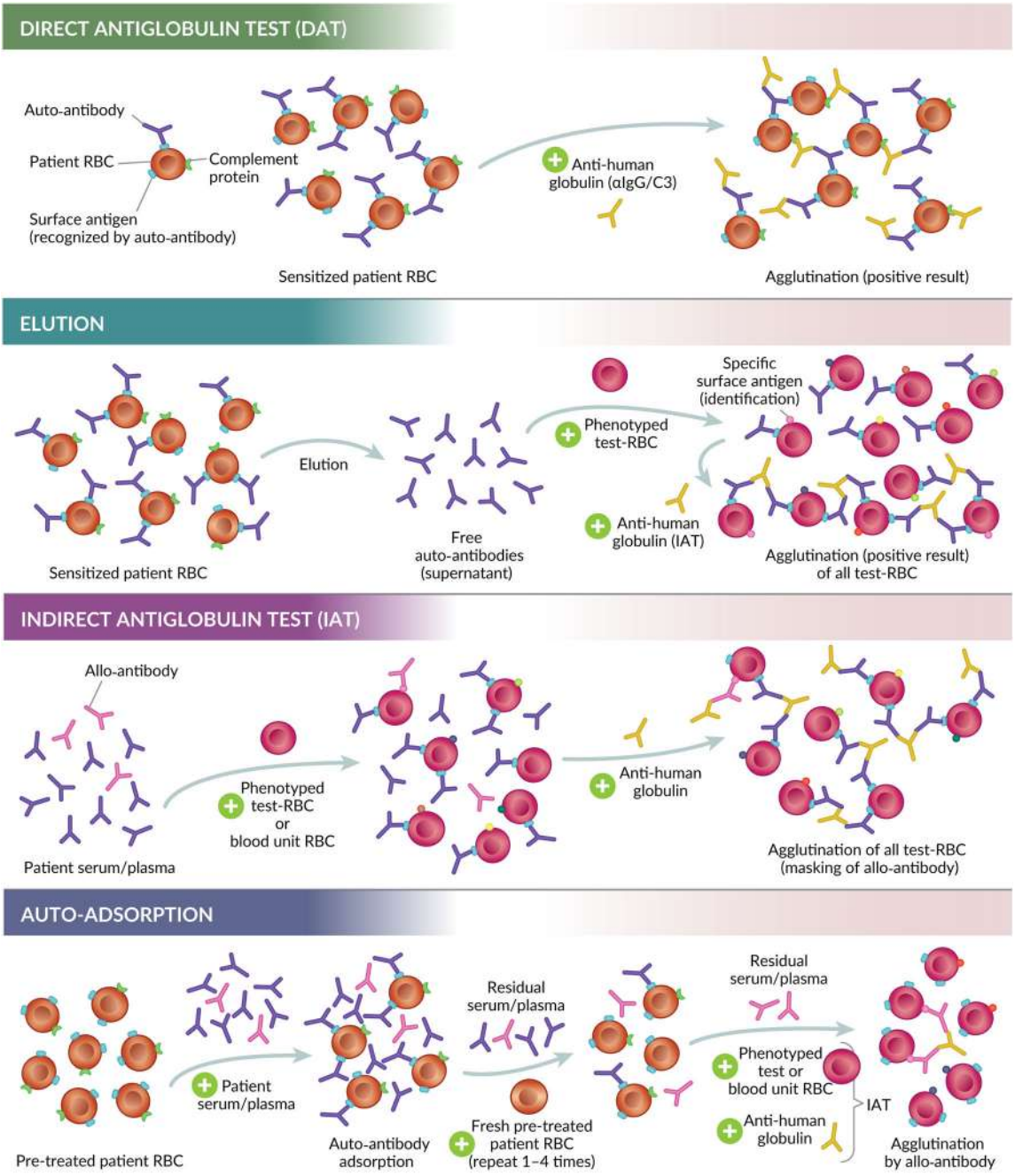

FIGURE 1 Laboratory tests performed in autoimmune haemolytic anaemia (AIHA). Direct antiglobulin test (DAT) reveals the in vivo sensitization of patient's red blood cells (RBCs) by the auto-antibody and/or complement proteins (C3c/d). In the figure, a polyspecific anti-IgG/C3 anti-human globulin is represented. Elution of antibodies from sensitized patient RBC allows the identification of a panreactive auto-antibody, which causes agglutination of all test RBC regardless of their specific phenotypes. Indirect antiglobulin test (IAT) reveals the presence of free antibodies present in patient serum/plasma through the agglutination of specific phenotyped test RBC or RBC from specific blood units. In AIHA, all test RBCs are agglutinated by the panreactive auto-antibody, with the risk of masking the presence of a specific allo-antibody. Auto-adsorption technique reduces interferences by adsorbing auto-antibodies on autologous, pre-treated patient RBCs throughout several adsorption cycles (2–5). Residual serum/plasma can be used for IAT on phenotyped test RBC and blood unit RBC, allowing the identification of an underlying allo-antibody.

clinically significant antibodies present higher thermal amplitudes (25–30°C) than non-relevant cold antibodies [2, 17, 30], and the vast majority of primary AIHA and secondary forms related to *Mycoplasma pneumoniae* infection present anti-I specificity [31].

PCH diagnosis can be complex and needs to be confirmed by a Donath–Landsteiner test through sequential incubations at different temperatures [10, 32].

If AIHA diagnosis is confirmed, international consensus recommendations [17] suggest further investigations in order to exclude the presence of certain diseases that cause secondary AIHA. For these forms, it is essential to treat rapidly the underlying disease in order to obtain AIHA regression [15].

TRANSFUSION SUPPORT AND PRE-TRANSFUSION TESTING IN AIHA

RBC transfusions are often required as supportive therapy in order to counterbalance severe anaemia and avoid hypoxia in all forms of AIHA, possibly in combination with other supportive therapies such as plasma exchange, erythropoiesis-stimulating agents and/or intravenous immunoglobulin (IVIg) administration [17]. In pre-transfusion testing, the final step is an in vitro compatibility test. A negative result indicates the absence of recipient reactivity against the RBCs (= compatible units), whereas a positive result indicates reactivity towards the tested RBC unit, qualified as 'incompatible' for this patient. When an allo-antibody has been identified, the units selected for transfusion must be negative for the corresponding antigen.

Unfortunately, in a patient presenting with AIHA it can be extremely difficult or even impossible to obtain compatible RBC units because of the various interferences in pre-transfusion tests.

Problems with pre-transfusion testing and recommendations

Auto-antibodies from AIHA patients also react with allogeneic RBCs if these display the corresponding antigens, usually high-frequency or 'public' antigens present in at least 99% of the random population. Thus, both the tests involving phenotyped test RBCs and RBCs to be transfused may present interferences or even all positive results, with the major risk of masking the presence of clinically significant allo-antibodies. Among patients with auto-antibodies, approximately 30%–50% have been reported to have an underlying allo-antibody. In subjects with no history of pregnancy or transfusion, this risk is very low [2, 33–35].

Thus, transfusions for AIHA patients often present an analytical challenge. Specific attempts to reduce interferences can take several hours or days and are not easily feasible during on-call periods. When it is not possible to obtain compatible RBC units and/or exclude the presence of underlying allo-antibodies, transfusions for these patients are often delayed or even abandoned for fear that the patients will suffer from haemolytic reactions. A common practice in routine transfusion services is to retain the so-called 'least incompatible' RBC units. This term refers to units that are at least ABO-compatible and present the weakest positive results in compatibility tests. Although commonly used in transfusion practice, this is controversial and discouraged due to the residual risk of undetected allo-antibodies [36]. However, several recent studies evaluating the impact of 'least incompatible' transfusions in AIHA have concluded that they were safe and effective [37–39].

Previous recommendations discouraged RBC transfusions in AIHA, stating that transfused RBCs would be destroyed similar to autologous RBCs and that transfusions would therefore be illogical and ineffective [40]. Conversely, recent guidelines recommend that transfusions should not be delayed in life-threatening clinical situations even if pre-transfusion compatibility tests are not fully completed. Nevertheless, the presence of allo-antibodies should be previously excluded, if possible. Patients with no history of pregnancy or transfusion present virtually no risk of allo-immunization, and for these subjects recommendations suggest that ABO-RHD/K matching may be sufficient in an emergency situation [2, 17]. For other subjects, more extended phenotyping is recommended using monoclonal reagents or genotyping [17, 41]. For patients with cold-reactive antibodies, RBC units should be preheated before transfusion using blood warmer devices [2, 17, 40].

Recent paediatric recommendations still suggest, however, to limit the transfusions only to very severe cases of anaemia, associated with a deterioration of vital parameters. In cases where transfusion is absolutely necessary, it is recommended to match the patients' extensive phenotype (RHCE; K; Jka/Jkb; Fya/Fyb; S/s) and to transfuse only the minimum amount required to improve symptomatology [2, 18].

In the particular case of AIHA secondary to allogeneic HSCT, in which AIHA presentation may be severe and life-threatening, it is also recommended that transfusions should not be delayed [16]. RBC units should ideally be compatible with the blood groups of both the HSCT donor and the recipient, but if this not possible, the donor's RBC phenotype should be chosen or, in the particular case of a mixed RBC chimerism, the predominant phenotype.

'In vivo' compatibility tests

A practice known as 'in vivo compatibility testing' has been described in order to determine whether the patient's RBC antibodies are clinically significant [42, 43]. Depending on the methodology, small amounts of incompatible RBCs labelled with a radioisotope (^{51}Cr) can be injected to the patient and haemolysis assessed by quantifying the radioactivity release in plasma after a few minutes and 1 h post injection. An easier alternative is the rapid injection of a small quantity of a standard RBC unit, followed by a period of observation and optionally a blood sampling in order to quantify the level of free plasma haemoglobin, translating intravascular haemolysis. In the absence of transfusion reaction at the end of the observation period, the test result would be interpreted as 'compatible' and the transfusion could be continued.

The international consensus recommendations suggest that an in vivo compatibility test should be performed for each transfusion in AIHA patients, that is, a rapid transfusion of 20 mL from the RBC unit followed by 20 min of observation [17].

Available techniques for limiting auto-antibody interferences

Various techniques have been described to limit auto-antibody interference in pre-transfusion testing, aiming at detecting possible underlying allo-antibodies. In this section, we will detail the most frequently described techniques.

Serum dilution

A simple and rapid technique that has been described is the dilution of patient's serum, aiming at the dilution of auto-antibodies in order to reduce interferences and improve detection of underlying allo-antibodies, with the assumption that the titre/reactivity of allo-antibodies would be higher than that of the auto-antibodies. Although the effectiveness of this technique is very limited because the majority of sera from patients with AIHA remain panreactive after dilution, the procedure may be easily and rapidly performed in laboratories with limited technical and human resources [44]. Nevertheless, this technique carries the risk of false negative results in the presence of low-titre allo-antibodies, which may become undetectable after dilution [45].

Adsorption techniques

The ideal technique for removing auto-antibodies from the patient's serum is by adsorption onto RBCs prior to the testing [46]. The adsorbing cells can either be the patient's autologous RBCs (i.e., auto-adsorptions) or allogeneic RBCs (i.e., allo-adsorptions).

Auto-adsorption, which is illustrated in Figure 1, should be preferred because it removes only auto-antibodies and not allo-antibodies. Unfortunately, auto-adsorptions present some limitations, such as the requirement of a certain amount of RBCs from patients with severe anaemia. Furthermore, this technique cannot be used in case of a recent transfusion history (less than 3–4 months) because circulating allogeneic RBCs might still be present in the patient's blood and adsorb allo-antibodies [47]. Another limitation is that RBCs from AIHA patients are often saturated with auto-antibodies that bind in vivo. In order to allow free serum auto-antibodies to adsorb in vitro, the RBCs first need to be treated. Various techniques have been described to this purpose while preserving cellular integrity [48–52]. ZZAP, a reagent composed of dithiothreitol (DTT) and papain, is effective for dissociating IgG molecules bound to the RBC surface [48]. Proteolytic enzymes and ZZAP treatments denature some antigens of the Duffy, Kell and MNSs systems, making these treatments inappropriate for adsorptions when auto-antibodies are directed against antigens of these systems [53].

When it is impossible to perform auto-adsorption, differential allo-adsorption should be considered. To this end, a minimum of three RBC samples with different phenotypes should be carefully selected in order to avoid adsorption ('loss') of clinically significant allo-antibodies during the adsorption cycles. Ideally, RBCs with the most similar phenotype to patient's RBCs should be included, and antigen-negative phenotypes for each of the main antigens of the RH, Kell, Duffy, Kidd and MNSs systems must be represented among these RBCs [52, 54]. The implementation of allo-adsorptions is more laborious than auto-adsorptions, as a minimum of three different RBC samples, whose extended phenotypes must be determined, have to be available. Allo-adsorptions as well as residual serum analyses must be performed on different series. An alternative to some phenotypic constraints is the treatment of allogeneic RBCs with proteolytic enzymes, as these treatments denature the antigens of certain systems. Otherwise, RBCs used for allo-adsorptions can remain untreated [33, 45, 52]. Another major disadvantage of allo-adsorptions is the risk that allo-antibodies to high-frequency antigens may be adsorbed on all the allogeneic RBC samples and pre-transfusion test results may become false negatives.

In order to achieve efficient adsorption more rapidly, the use of potentiating media that promote antigen–antibody reactions has been proposed, notably polyethylene glycol (PEG) or a low ionic strength solution (LISS). The presence of PEG during the adsorption cycles eliminates auto-antibody interference quite efficiently without prior RBC treatment. However, a reduced or even lost detection of allo-antibodies when using PEG has been described, possibly caused by protein precipitation, especially for allo-antibodies displaying weak initial reactivity [55]. LISS is also known to promote antigen–antibody interactions and can also be used for adsorption. The main advantage of LISS is that it allows the use of the gel-filtration technique for residual serum testing, unlike PEG which may cause aspecific agglutination of all RBCs [56–58].

In vitro compatibility tests

The most common classical compatibility test is based on the use of anti-human globulin (IAT), and like other agglutination techniques, this test can be performed using different types of solid substrates, for example, tubes, columns/gel-filtration or microplates. In AIHA, interferences in IAT crossmatches and screening/panels can vary depending on the solid supports and potentiating media that are used. In the current practice of many laboratories, a tube/saline agglutination technique followed by anti-IgG IAT is frequently used for testing in AIHA patients, as it presents less interferences than other solid supports and poly-specific antiglobulins [59].

If interferences persist in compatibility tests, caution is advised especially when reactivity against donor RBC units is more important than the agglutination strength of autologous crossmatch control, potentially indicating the presence of allo-antibody/ies in addition to the auto-antibody.

Some authors question the usefulness of IAT compatibility tests in AIHA compared to a simple 'immediate-spin' crossmatch whose

only usefulness is to show ABO group incompatibilities [60]. Other authors suggest a prophylactic approach in these patients by respecting the erythrocyte phenotype or genotype [41, 61], an approach that may also call into question the usefulness of compatibility testing. The optimal method would be to perform auto- or allo-adsorptions before making the IAT crossmatch with residual serum [52].

Cold-reactive antibodies

Interferences due to cold-reactive auto-antibodies can often be eliminated by working at warm temperatures (37°C) eventually combined with the saline/tube + anti-IgG IAT technique. When it is necessary to work with RBCs from these patients, which usually agglutinate spontaneously at room temperature, washes of the RBCs in saline at 37°C should be performed prior to testing.

A method for removing specifically cold-reactive IgM auto-antibodies is the denaturation with 2-mercaptoethanol or DTT. This treatment eliminates IgM interferences and thus allows the detection of possible underlying allo-antibodies, which are usually IgG-type and not affected by these treatments. In some cases, auto-antibody adsorptions at cold temperatures may be necessary, especially when cold-reactive IgG antibodies are involved [62].

DISCUSSION

Transfusion support in AIHA

Although various national guidelines had been published, in the beginning of 2019 there was still a lack of international standardization regarding AIHA terminology, definitions, diagnosis and treatment [1]. The first international recommendations aiming at an harmonization of these different concerns have only recently been published [17]. Since then, the COVID-19 global pandemic made AIHA management a topical issue, as several reports described the occurrence of AIHA associated with SARS-CoV-2 infection [25–27, 29] or vaccination [63, 64].

Except for paediatric cases [2, 18], the various recent guidelines in adults tend to agree that transfusions should not be abandoned or delayed in critical cases of AIHA [2, 17]. Indeed, there is little evidence in the literature for transfusion-induced exacerbation of haemolysis in AIHA patients. Conversely, some studies and case reports rather alert about the risk of clinical decompensation and death when transfusions are too much delayed or abandoned [39, 65]. Lee et al. [60] and Chen et al. [37] observed that transfusions with 'incompatible' RBC units in 222 and 450 AIHA patients, respectively, did not increase the risk of haemolytic transfusion reactions and that they were effective. Park et al. [38] published similar results.

The best approach for choosing RBC units is to prophylactically select the most similar phenotype, as this reduces both the risk of haemolytic transfusion reactions and new allo-immunizations. This requires knowledge of the patient's extensive RBC phenotype, which is not always technically feasible for AIHA patients who present highly positive DATs and/or recent transfusion history [61]. An alternative to circumvent this problem is to perform genotyping of these patients [17, 18, 41]. For urgent transfusion needs, however, it is not always possible to wait for genotyping results.

In future trials, it could be interesting to evaluate the benefit of IVIg administration concomitantly with RBC transfusions, with the purpose of increasing transfusion efficacy by reducing the destruction of RBC. IVIg could neutralize auto-antibodies, thanks to a small proportion of anti-idiotype antibodies [66], which might explain why higher doses of IVIg are necessary for therapeutic effects in autoimmune diseases compared to immunodeficiencies. Unfortunately, the lack of raw materials for IVIg production represents a major limitation. High doses of IVIg might also increase the risk of side effects (allergic reactions, renal failure, thromboembolic reactions, etc.) including the risk of IVIg-related haemolysis [67], especially in patients with blood groups A/AB. Thus, evaluation of purified anti-idiotype antibodies in AIHA and/or transfusion conflicts will be of interest.

Pre-transfusion testing in AIHA

The auto-adsorption technique with ZZAP pre-treatment of RBCs appears to be the optimal serological method for detecting allo-antibodies underlying auto-antibodies [46, 48, 49, 52]. The main limitation of this technique is that it is time consuming, as RBC treatment and adsorption cycles can already take several hours. Other important limitations arise from the amount of autologous RBCs required for patients with low haematocrit value, and the prerequisite of no recent transfusion history. When auto-adsorption cannot be performed, the best alternative is allo-adsorption, although this does not allow the detection of allo-antibodies directed against high-frequency antigens. Their constraining and time-consuming implementation represents another major disadvantage, limiting the interest of this technique to patients presenting a risk of allo-immunization.

According to several authors [33, 45, 57, 68], adsorption in the presence of PEG would give comparable results to reference adsorption techniques while requiring less time and fewer adsorbing RBCs. In contrast, other publications have shown a reduced detectability or even disappearance of some clinically significant allo-antibodies in the presence of PEG [55, 69, 70].

One hypothesis that could explain these discrepancies in the literature is the storage of the serum–PEG mixture, as PEG is known to cause protein precipitation. Leger et al. [71] confirmed the hypothesis that some allo-antibodies may become less detectable due to precipitation after short-term storage, and therefore they recommend performing IAT on residual serum/PEG on the day of adsorption. Another study concluded that significant precipitation of immunoglobulins already occurs during the adsorption step [70]. The use of LISS in adsorptions could also lead to a decrease in allo-antibody reactivity, probably resulting from a simple dilution effect [56].

Another highly controversial technique is serum dilution. According to Leger and Garratty [45], it displays poor efficiency, as over 70%

TABLE 2 Algorithm for laboratory transfusion management in autoimmune haemolytic anaemia.

A: Transfusion emergency (<4 h)	B: Temporarily stable (transfusion in 4–12 h)	C: Non-urgent transfusion (>12 h)
A1. Selection of RBC units • ABO-compatible and RHDCE/K-compatible when available • Compatible with an eventual allo-antibody • Kidd-, Duffy- and Ss-compatible ONLY IF patient pheno/genotype and RBC units available in time	**B1. Selection of RBC units** • ABO-compatible and RHDCE/K-compatible when available • Compatible with an eventual allo-antibody • Kidd-, Duffy- and Ss-compatible ONLY IF patient pheno/genotype and RBC units available in time	**C1. Selection of RBC units** • ABO-compatible and RHDCE/K-compatible when available • Compatible with an eventual allo-antibody • Kidd-, Duffy- and Ss-compatible (if possible)
A2. Minimal pre-transfusion tests • 'Least incompatible' units in compatibility tests (IAT gel-filtration technique)	**B2. Minimal pre-transfusion tests** • Warm-type AIHA: serum dilution → IAT screen/panel • Cold-type AIHA: DTT serum-treatment → IAT • Tube technique (saline + anti-IgG IAT) → compatibility tests + RBC panel (if risk of allo-immunization)	**C2. Minimal pre-transfusion tests** • Warm-type AIHA: serum dilution → IAT screen/panel • Cold-type AIHA: DTT serum-treatment → IAT • Tube technique (saline + anti-IgG IAT) → compatibility tests + RBC panel (if risk of allo-immunization) And in addition • Patient RBC elution (possible identification of auto- or allo-antibodies bound to patient RBCs)
A3. Optional pre-transfusion tests (if time) • Warm-type AIHA: serum dilution → IAT screen/panel • Cold-type AIHA: DTT serum-treatment → IAT • Tube technique (saline + anti-IgG IAT) → compatibility tests + RBC panel (if risk of allo-immunization)	**B3. Optional pre-transfusion tests (if time)** • Patient RBC elution (possible identification of auto- or allo-antibodies bound to patient RBCs)	**C3. Optional pre-transfusion tests (if risk of allo-immunization)** • Auto-adsorptions on pre-treated RBCs (if possible) Or • Differential allo-adsorptions on native RBCs
+ 'In vivo' *crossmatch*	+ 'In vivo' *crossmatch*	+ 'In vivo' *crossmatch*

Note: Depending on the degree of transfusion emergency (A, B or C), a standardized approach is proposed for the selection of RBC units (A1 → C1), minimal pre-transfusion tests that should be performed (A2 → C2) and optional pre-transfusion tests, depending on time for analyses and/or patient history (A3 → C3).

Abbreviations: AIHA, autoimmune haemolytic anaemia; DAT, direct antiglobulin test; DTT, dithiothreitol; IAT, indirect antiglobulin test (refers to gel-filtration IAT tests, except for tube technique cited in A3, B2 and C2); RBC, red blood cell.

of sera with auto-antibodies retained panreactivity and 27% of samples containing allo-antibodies gave false negative results. Only in 19% of samples containing allo-antibodies, dilution was able to reveal their presence. Øyen and Angeles [44], who described serum dilution, found a similar proportion of auto-antibody-free samples after dilution, but they did not observe false negative reactions in the presence of allo-antibodies. This technique may be of interest as a first-line test in an urgent transfusion setting, as it is a simple and rapid way of detecting an underlying allo-antibody in approximately 20% of patients. However, the presence of an allo-antibody cannot be excluded based on negative results obtained with this technique.

An important notion to keep in mind is the increased prevalence of allo-antibodies in AIHA subjects, as described in various studies [33, 38, 46, 57, 61, 68], especially in case of transfusion or pregnancy history.

CONCLUSIONS

The general answer to the question 'when to transfuse patients with AIHA?' could be that they should be transfused as soon as possible in cases of severe and life-threatening anaemia. Except for paediatric cases where transfusions are recommended only if vital signs are impaired, the most recent data for adults show a benefit of prompt transfusion management. It is difficult to define universal transfusion thresholds for these patients, as the decision must be assessed on a case-by-case basis depending on possible co-morbidities and the evolution of the disease/AIHA. Furthermore, given the risk of rapid clinical decompensation on increased haemolysis when auto-antibodies reach very high tires, haemoglobin monitoring needs repeated measurements because its concentration can decrease very rapidly.

The answer to the question 'how to transfuse patients with AIHA?' is more complex, as all pre-analytical techniques have their limitations, whether through the risk of non-detection of an allo-antibody or their laborious and time-consuming implementation. An important factor that needs to be considered is the degree of transfusion emergency. When facing an urgent situation, the best approach seems to be the preventive selection of the most compatible RBC phenotype available for transfusion units, which can be combined with the results of minimum laboratory analyses by choosing 'least incompatible' units and/or performing antibody screening on diluted serum. Although highly controversial, these approaches are easily and quickly performed in all immuno-haematology laboratories, and as a

matter of common sense it seems preferable to include these results in the selection process rather than perform no laboratory testing at all when transfusion should not be delayed. In situations where transfusion can be delayed by a few hours/days, more thorough immunohaematological testing should be performed, especially for patients presenting a risk of allo-immunization. An algorithm for transfusion management is proposed in Table 2. The prerequisite is close communication between laboratory and medical staff. In all cases, in vivo compatibility assessments as recommended [17] should be performed at the patient's bed for each unit transfused.

A decisional algorithm for the selection of RBC units in patients with warm AIHA was recently published [35]. In the algorithm for transfusion management that we describe (Table 2), in addition to RBC selection we also focus on pre-transfusion laboratory tests that should be performed, depending on the degree of emergency required for transfusion support and on type of AIHA. The proposed algorithm in this review further takes into account the recommendations for transfusion management from the international consensus meeting [17].

In conclusion, it appears that transfusion management of patients with AIHA is often severely delayed or avoided due to concern about possible haemolytic transfusion reactions. However, recent data and guidelines suggest that the main risk for AIHA patients arises from delayed transfusion management in life-threatening situations. Although it is difficult to define a standardized approach given the diversity of presentations, there should be awareness about the risk represented by a transfusion delay in adult AIHA patients.

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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 analyzed in this study.

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ORIGINAL ARTICLE

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Screening, genotyping and haematological analysis of glucose-6-phosphate dehydrogenase deficiency in the blood donors of Wuxi City, China

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Abstract

Background and Objectives: To investigate the prevalence, genotype and haematological characteristics of glucose-6-phosphate dehydrogenase (G6PD) deficiency in the blood donor population of Wuxi area (Jiangsu Province, China) and to assess the impact of their red blood cell (RBC) units on clinical transfusion.

Materials and Methods: We conducted genotyping and large-scale screening for G6PD enzyme activity in the blood donors of Wuxi City. In addition, we assessed the haematological parameters of G6PD-deficient and non-deficient blood donors, and investigated the adverse transfusion reactions in patients transfused with G6PD-deficient blood.

Results: We investigated 17,113 blood donors, among whom 44 (0.26%) were tested positive for G6PD deficiency. We identified 40 G6PD gene variants, among which *c.1388G>A*, *c.1376G>T*, *c.1024C>T* and *c.95A>G* were common. In addition, we identified two novel *G6PD* gene variants, *c.1312G>A* and *c.1316G>A*. The G6PD-deficient and non-deficient blood samples showed a significant difference in the RBC, mean corpuscular volume (MCV), mean corpuscular Hb (MCH), RBC distribution width, total bilirubin (TBIL), direct bilirubin (DBIL) and indirect bilirubin (IBIL) values. However, the two samples showed no significant difference in the haemolysis rate at the end of the storage period. Finally, transfusion with G6PD-deficient RBC units did not lead to any adverse transfusion reactions.

Conclusion: The positive rate of G6PD deficiency in the blood donor population of Wuxi City is 0.26%, and the genetic variants identified in this population are consistent with the common genetic variants observed in the Chinese population. Blood centres can establish a database on G6PD-deficient blood donors and mark their RBC units to avoid their use for special clinical patients.

Keywords

blood donors, genotyping, glucose-6-phosphate dehydrogenase (G6PD) deficiency, haemolysis

Highlights

- Two novel glucose-6-phosphate dehydrogenase (G6PD) gene variants, *c.1312G>A* and *c.1316G>A*, were identified.
- Haematological parameters varied significantly between G6PD-deficient and non-deficient groups.
- Transfusion with G6PD-deficient blood did not cause adverse transfusion reactions.

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is a common metabolic disorder of the red blood cells (RBCs), affecting approximately 400 million people worldwide. It is prevalent in Latin America, Africa, the Mediterranean and Southeast Asia, especially southern China [1]. The G6PD gene is located on the long arm of the X chromosome (Xq28) and consists of 13 exons of 18 kb. G6PD deficiency is an X-linked incomplete dominant disorder, with a higher prevalence in males [2]. G6PD genotype varies widely among ethnic groups and regions, with over 200 G6PD gene variants identified worldwide. Among these, *c.487G>A*, *c.383G>T* and *c.202G>A* are common in the Thai [3], Indonesian, and African [4] and African-American [5] populations, respectively. The *c.202G>A* variant (*rs1050828*, *Val68Met*) is associated with higher oxidative haemolysis after RBC cold storage [5].

Individuals with G6PD deficiency are mostly asymptomatic and are often allowed to donate blood. In addition, most national laws and regulations do not require blood centres to screen donors for G6PD deficiency. However, the G6PD-deficient RBCs are less responsive to oxidative stress, due to their altered antioxidant mechanism, which can be aggravated by certain medications, infections or ingestion of fava beans. Therefore, transfusion of G6PD-deficient RBCs into a susceptible host, especially during an infection or treatment with oxidizing drugs, can lead to haemolysis. A few drugs that have been identified to cause haemolysis include antimalarials (primaquine), sulfonamides (sulfamethoxazole and sulfapyridine), antipyretics and analgesics (acetanilide), and berberine-containing herbal preparations [6]. Some early studies reported that transfusion of G6PD-deficient RBCs had potential negative effects on the blood recipients. For instance, Mimouni et al. reported haemolytic reactions in two preterm infants, with normal G6PD, who were transfused with G6PD-deficient blood [7]. In addition, Samanta et al. reported that exchange transfusion with G6PD-deficient RBCs causes haemolytic reactions, decreases post-treatment total serum bilirubin reduction, prolongs light therapy duration and increases the likelihood of repeated exchange transfusions in preterm infants [8]. Therefore, the safety of G6PD-deficient RBC transfusion and G6PD-deficiency screening of blood donors has gained increasing attention in the World.

Screening for G6PD deficiency in blood donors has been reported worldwide. The prevalence rate of G6PD deficiency, among blood donors, varies in different regions, with 8.27% in northern Brazil, 5.7%–14.2% in Thailand, 16.3% in Iran and 6.97% in Guangdong Province, China [9–14]. However, data on G6PD deficiency in the blood donor population of Jiangsu Province (China) have not yet been reported. Wuxi City, located in the Yangtze River Delta (Jiangsu Province, China), has a large non-native population, whose G6PD status is unknown. Therefore, in this study, we conducted a large-scale screening for G6PD deficiency among blood donors of Wuxi City to analyse their haematological characteristics and provide a secure blood supply for clinical use. In addition, this study provides the scientific basis for the correlation between G6PD deficiency and transfusion safety.

MATERIALS AND METHODS

Sample collection

In this study, we screened a total of 17,113 blood samples deposited in the central blood centres of Wuxi City (Jiangsu Province, China) from February 2023 to November 2023. The blood donors consisted of 10,227 males and 6886 females, aged 18–55 years. The blood samples were collected in blood collection tubes containing EDTA-K2 anticoagulant or inert separator gel-containing pro-coagulant; the former was used for G6PD enzyme activity detection, haematology detection and gene sequencing analysis, while the latter was used for serum bilirubin detection. The blood donors with G6PD deficiency were considered the Test group, while the donors with normal G6PD levels were considered the Control group. Only the donors aged 18–55 years, meeting the whole blood (WB) and blood component donor selection requirements [15], no haemolytic disease, normal G6PD enzyme activity and belonging to the Han Chinese population were included in the Control group. Meanwhile, donors with haemolysis, lipaemia or jaundice, and donors who underwent adverse reactions during blood donation were excluded from the Control group. After the screening, 33 (27 males and 6 females, aged 32.0 ± 8.0 years), 100 (73 males and 27 females, aged 26.6 ± 11.2 years) and 48 (43 males and 5 females, aged 30.8 ± 9.3 years) donors were selected as the Control groups for haematological, bilirubin detection and haemolytic analyses, respectively. There were no statistically significant differences in the sex, age or ethnicity of the Control and the Test groups. This experimental study was approved by the Institutional Ethics Committee of Wuxi Blood Center, and informed consent was obtained from the blood donors for sample collection and testing.

Instruments and reagents

The AU480 Chemistry Analyser was purchased from Beckman Coulter Co. The STAR sample-adding instrument was purchased

from Switzerland Hamilton Co. The ultraviolet–visible spectrophotometer was obtained from Shanghai MAPADA instruments Co. Ltd. The XS-500i automated haematology analyser was purchased from Sysmex Co. The SLAN-96S fluorescence quantitative polymerase chain reaction (PCR) instrument was obtained from Fosun Diagnostic Technology (Shanghai) Co. The ABI 3730XL automated DNA sequencer was obtained from Thermo Fisher Scientific Inc. The G6PD screening kit was obtained from Shenzhen Myriad Biomedical Electronics Co. The bilirubin assay kit was purchased from Beijing Wantai Diagnostics Co. Ltd., and the G6PD gene mutation detection kits were procured from Xiamen ZhiShan Bio-technology Co. Ltd. The free haemoglobin (Hb) detection kits were purchased from Ruerda (Beijing). All the instruments were within the validity period and calibrated. All the experiments were conducted in strict accordance with the instructions provided by the kit manufacturers.

G6PD enzyme activity detection

The blood samples were centrifuged at 3000 rpm for 5 min, after which 20 μL of the precipitated RBCs was aspirated and mixed thoroughly with 1 mL of deionized water for haemolysis. The G6PD enzyme activity of the samples was analysed within 30 min using the AU480 Chemistry Analyser. The reference range for G6PD enzyme activity was 1300–3600 U/L. Samples with ≤1300 U/L G6PD activity in two consecutive tests were considered G6PD-deficient.

G6PD genotyping analysis

The G6PD-deficient samples were analysed using the fluorescence polymerase chain reaction melting curve method by Hangzhou Bosheng Medical Laboratory Co. Ltd. (Zhejiang, China) to qualitatively detect 12 common G6PD genetic variants in the Chinese population: *c.95A>G*, *c.383T>C*, *c.392G>T*, *c.487G>A*, *c.517T>C*, *c.592C>T*, *c.871G>A*, *c.1004C>A*, *c.1024C>T*, *c.1360C>T*, *c.1376G>T* and *c.1388G>A*. The samples that tested negative for these genetic variants were subjected to Sanger sequencing, and the obtained DNA sequences were compared with the human reference genome hg19 retrieved from the University of California Santa Cruz genome browser database. The identified G6PD variant sites were analysed for pathogenicity.

Haematological analysis

The Control and Test groups were subjected to haematological analysis using the Sysmex XS-500i automated haematology analyser. The RBC, Hb, haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular Hb (MCH), mean corpuscular Hb concentration (MCHC) and RBC distribution width (RDW) values of the two groups were recorded.

Serum bilirubin detection

The Control and Test group samples were analysed using the AU480 Chemistry Analyser, and the total bilirubin (TBIL), direct bilirubin (DBIL) and indirect bilirubin (IBIL) levels of the two groups were recorded.

Haemolysis detection

Isolation and storage of blood components

The RBC units are generally of two types in China. The WB samples were centrifuged and separated into concentrated RBCs (CRBCs) and platelet-rich plasma. The CRBCs were stored in mannitol–adenine–phosphate (MAP) solution and labelled as suspended RBCs (SRBCs). Alternatively, the WB samples were subjected to leukofiltration and labelled as pre-storage filtered blood (PFB), which was subsequently centrifuged to obtain packed red cells (PRCs). Finally, the PRCs were stored in the MAP solution and labelled as suspended leukocyte-reduced RBCs (SLRBCs). The SRBCs and SLRBCs were stored for 35 days at 4 ± 2°C. The RBC component was stored as SLRBCs in the Wuxi Blood Centre.

Free Hb estimation

The SLRBC samples of the Control and Test groups were mixed thoroughly, and 20 mL of the samples was added to the corresponding sample pouches and stored at 4 ± 2°C. Thereafter, 5 mL of the SLRBC sample was aspirated aseptically after 35 days for haematological and haemolytic assays. The complete blood count (CBC) was obtained using an automated haematology analyser, and the free plasma Hb was detected using the ultraviolet–visible spectrophotometer. The rate of haemolysis of the Control and Test groups was detected as follows: haemolysis (%) = (100 − HCT) × free plasma Hb (g/L)/total Hb (g/L) × 100%. According to international quality standards, the permissible rate of haemolysis at the end of the storage period was <0.8%.

Monitoring of adverse blood transfusion reactions

The G6PD-deficient blood was not used for intrauterine transfusions and neonatal exchange transfusions. All hospitals in Wuxi City are required to report adverse transfusion symptoms to the Haemovigilance Management System of the Chinese Society of Blood Transfusion every month according to the Guidelines for Haemovigilance (T/CSBT001-2019) [16]. The following information is recorded in case of adverse transfusion reaction, including patient ID, adverse transfusion symptom(s) and IDs of the transfused blood products. Data on reports of non-infectious transfusion reactions, such as allergic reactions, acute haemolytic

transfusion reactions, delayed haemolytic transfusion reactions, delayed serological transfusion reactions, non-haemolytic febrile reactions and post-transfusion purpura, were retrieved from the Haemovigilance Management System to identify any adverse transfusion reactions in the patients transfused with G6PD-deficient RBCs.

Statistical analysis

All the data were analysed using the SPSS v23.0 software. The normally distributed data were expressed as the mean ± standard deviation (SD), and the categorical data were expressed as percentage (%). The normality of each group of variables was examined using the one-sample Kolmogorov–Smirnov test. Comparisons between multiple groups were analysed using a one-way analysis of variance, comparisons between two groups were analysed using independent samples *t*-test, and count data were analysed using chi-square test. The *p*-value <0.05 was considered statistically significant.

RESULTS

Results of G6PD deficiency screening

In this study, we screened 17,113 blood donors (10,227 males and 6886 females, aged 33.1 ± 9.7 years). The A, B, O and AB blood types accounted for 32.26%, 26.54%, 32.11% and 9.99% of the assessed blood samples, respectively. Among the 17,113 blood donors, 44 (0.26%; 38 males and 6 females; aged 28.8 ± 8.5 years) were diagnosed with G6PD deficiency. The G6PD deficiency rate was significantly correlated with the sex but not the blood groups or ethnicities of the donors (Table 1). In addition, compared with the Control group, the enzyme activity varied significantly in the Test group based on the sex of the donors (Table 2).

Results of G6PD genotyping

Among the 44 G6PD-deficient donors, 40 were detected with nine single-point mutations and no compound mutations in the *G6PD* gene. The common genetic variants identified in the G6PD-deficient donor samples were *c.1388G>A* (30.0%; 12 males and 0 females), *c.1376G>T* (27.5%; 9 males and 2 females), *c.1024C>T* (17.5%; 4 males and 3 females), *c.95A>G* (12.5%; 4 males and 1 female), *c.1004C>A* (2.5%; 1 male), *c.392G>T* (2.5%; 1 male) and *c.487G>A* (2.5%; 1 male). In addition, two rare and unreported genetic variants, *c.1312G>A* and *c.1316G>A*, were identified in two male G6PD-deficient donors. The frequency distribution of the *G6PD* gene mutations is shown in Figure 1.

Correlation between G6PD genotypes and G6PD enzyme activity

Of the 40 G6PD-deficient donors with genetic variants, 34 were male hemizygotes and 6 were female heterozygotes. The G6PD enzyme activity varied significantly between the male donors containing the four major genetic variants and was the lowest in the male donors with the *c.1376G>T* mutation (Table 3). Due to the lower prevalence of G6PD deficiency in females in this study, G6PD enzyme activity was only compared between the male and female donors containing the *c.1024C>T* mutation. The results revealed that the average G6PD enzyme activity was 71.6 ± 134.34 U/L in males and 924.03 ± 271.02 U/L in females, indicating that the enzyme activity was significantly lower in males ($t = -2.954$, $p = 0.032$).

Haematological analysis

In the Test group, RBC and RDW values were reduced, MCV and MCH values were elevated, and the difference with the Control group was statistically significant. However, the Hb, HCT and MCHC values were not significantly different between the two groups (Table 4).

TABLE 1 G6PD deficiency based on the sex, blood group and ethnicities of the donors.

Parameters	*N*	Number of positive donors	Positivity rate (%)	Chi-square value	*p*-Value
Sex					
Male	10,227	38	0.37	12.982	<0.001
Female	6886	6	0.09		
Blood groups					
A	5521	13	0.24	5.322	0.150
B	4388	10	0.23		
O	5495	20	0.37		
AB	1709	1	0.06		
Ethnic groups					
Han people	16,672	41	0.25	1.694	0.193
Minority people	441	3	0.68		

Abbreviation: G6PD, glucose-6-phosphate dehydrogenase.

TABLE 2 Comparison of G6PD activity between the G6PD-deficient and Control blood donors based on sex.

Groups	Sex	G6PD activity (U/L)	*T*	*p*-Value
G6PD deficiency	Male ($N = 38$)	255.98 ± 172.21	−9.176	<0.001
	Female ($N = 6$)	958.82 ± 189.51		
G6PD non-deficiency	Male ($N = 10{,}189$)	2929.73 ± 430.77	−0.341	0.733
	Female ($N = 6880$)	2932.01 ± 420.57		

Abbreviation: G6PD, glucose-6-phosphate dehydrogenase.

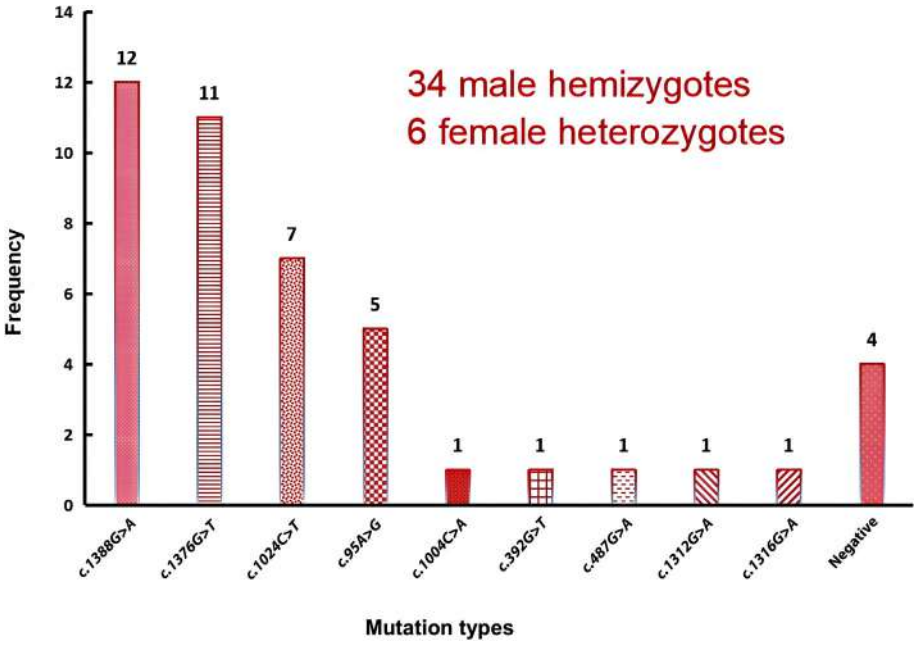

FIGURE 1 Distribution of glucose-6-phosphate dehydrogenase (G6PD) gene variants among the male and female blood donors of Wuxi City (China).

TABLE 3 Comparison of G6PD enzyme activity in males containing different G6PD gene variants.

Gene variants		*N*	Enzyme activity (U/L)	*F*	*p*-Value
Group A	*c.1388G>A*	12	218.16 ± 64.22	12.953	<0.001
Group B	*c.1376G>T*	9	163.14 ± 29.95		
Group C	*c.1024C>T*	4	471.6 ± 134.34		
Group D	*c.95A>G*	4	213.38 ± 42.34		

Note: One-way analysis of variance and post hoc multiple comparisons tests were used to analyse the data at $p < 0.05$ for Groups A and B, $p < 0.001$ for Groups A and C, $p < 0.001$ for Groups B and C, and $p < 0.05$ for Groups C and D.
Abbreviation: G6PD, glucose-6-phosphate dehydrogenase.

Serum bilirubin

The serum bilirubin level was significantly higher in the Test group compared with the Control group ($p < 0.05$, Table 5).

Haemolysis and adverse blood transfusion reactions

The haemolysis rate of SLRBC at the end of the storage period was <0.8% in both the Test and Control groups (Table 6). In 2023, a total of 230 adverse transfusion reactions, primarily allergic reactions and non-haemolytic febrile reactions, were reported in the Haemovigilance Management System in Wuxi City. However, no adverse transfusion reactions were recorded in the patients transfused with G6PD-deficient RBCs.

DISCUSSION

G6PD catalyses the first rate-limiting reaction of the pentose phosphate pathway that produces reduced nicotinamide adenine dinucleotide phosphate (NADPH), which acts as a hydrogen donor in a variety

TABLE 4 Comparison of haematological parameters between the G6PD-deficient and Control groups.

Haematological parameters	G6PD-deficient group ($N = 44$)	Control group ($N = 33$)	*T*	*p*-Value
Hb (g/L)	150.57 ± 12.81	148.76 ± 12.22	0.626	0.533
RBC (10^{12}/L)	4.82 ± 0.46	5.06 ± 0.38	−2.384	0.020
HCT (%)	0.47 ± 0.03	0.46 ± 0.03	0.181	0.418
MCV (fL)	97.31 ± 3.72	91.51 ± 3.50	6.950	<0.001
MCH (pg)	31.28 ± 1.45	29.42 ± 1.14	6.106	<0.001
MCHC (g/L)	321.57 ± 12.32	321.58 ± 8.24	−0.003	0.998
RDW (%)	12.08 ± 0.54	12.63 ± 0.49	−4.606	<0.001

Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; Hb, haemoglobin; HCT, haematocrit; MCH, mean corpuscular Hb; MCHC, mean corpuscular Hb concentration; MCV, mean corpuscular volume; RBC, red blood cell; RDW, RBC distribution width.

TABLE 5 Comparison of plasma bilirubin levels between the G6PD-deficient and Control groups.

	Groups	Serum bilirubin (μmol/L)	*T*	*p*-Value
TBIL	Control group ($N = 100$)	12.93 ± 6.10	−3.106	0.002
	G6PD-deficient group ($N = 44$)	16.70 ± 5.62		
DBIL	Control group ($N = 100$)	4.74 ± 1.90	−2.812	0.006
	G6PD-deficient group ($N = 44$)	5.80 ± 1.72		
IBIL	Control group ($N = 100$)	8.19 ± 4.41	−3.426	0.001
	G6PD-deficient group ($N = 44$)	10.92 ± 4.38		

Abbreviations: DBIL, direct bilirubin; G6PD, glucose-6-phosphate dehydrogenase; IBIL, indirect bilirubin; TBIL, total bilirubin.

TABLE 6 Comparison of plasma haemolysis rate between the G6PD-deficient and Control groups.

Groups	Haemolysis (%)	*T*	*p*-Value
Control group ($N = 48$)	0.29 ± 0.16	1.492	0.139
G6PD-deficient group ($N = 44$)	0.24 ± 0.11		

Abbreviation: G6PD, glucose-6-phosphate dehydrogenase.

of metabolic reactions in the body. Researchers believe that G6PD-deficient RBCs are susceptible to oxidative stress-induced haemolysis under low NADPH production, reduced glutathione (GSH) levels and reduced protection of the sulfhydryl-containing membrane proteins and enzymes.

G6PD deficiency is an X-linked incomplete dominant disorder. Because males have only one X chromosome, males with G6PD deficiency will always be hemizygous, whereas females with this disorder can be hemizygous or heterozygous. Therefore, G6PD gene mutation can lead to a significant lack of enzyme activity in males. However, *G6PD* gene mutation in females can lead to varying degrees of enzyme activity, ranging from normal to absent, based on the random X chromosome inactivation of hemizygous or heterozygous mutations. Therefore, it is possible that some female heterozygotes with G6PD deficiency were not identified by our enzymatic screening. In this study, we identified six heterozygous G6PD-deficient females; however, they all had significantly higher levels of G6PD enzyme activity compared with the G6PD-deficient males. In addition, the G6PD enzyme activity differed between different genotypes in males, consistent with other studies. Zhou et al. found that the G6PD enzyme activity of heterozygous and compound heterozygous mutations do not vary based on genotypes, but the enzyme activity of hemizygous *c.1024C>T* mutation is significantly higher than that of the other genetic variants, consistent with the results of our study [17].

G6PD deficiency is widespread in malaria-prone areas of Latin America, Africa, the Mediterranean and Southeast Asia. Some studies found that G6PD-deficient RBCs are resistant to *Plasmodium* infection; therefore, experts believe that G6PD mutation serves as a positive selection against malaria [18]. Thus far, over 35 G6PD variants have been identified in Chinese populations, among which *c.1376G>T*, *c.1388G>A*, *c.95A>G* and *c.1024C>T* are the most common [19]. In addition to these variants, we identified two new genetic variants, *c.1312G>A* and *c.1316G>A*, in this study. As the frequency of these two *G6PD* variants has not been provided in the Genome Aggregation Database, they were considered novel mutations in this study. However, the pathogenicity and pathogenesis of these two variants need to be further analysed. In this study, four donor samples with low enzyme activity were found to be negative for *G6PD* mutation. This may be attributed to the false-positive results obtained from enzyme activity screening or the presence of pathogenic variants in deep intronic regions or specific mutations, such as dynamic mutations, deletions or duplications, of large segments of gene sequences, which

were detected as false negatives due to methodological limitations. Therefore, these results need to be further verified by blood retesting and family lineage surveys of the four blood donors.

Previous studies have shown that G6PD-deficient blood is not suitable for intrauterine transfusion, neonatal blood exchange therapy or patients with G6PD deficiency. Nabavizadeh and Anushiravani screened 261 cases of paediatric and neonatal transfusions with G6PD-deficient RBCs and found that the patients transfused with G6PD-deficient RBCs had more haemolytic complications, such as low Hb, haemoglobinuria and elevated bilirubin, compared with those transfused with G6PD non-deficient RBCs; thus, the authors recommend screening for G6PD deficiency in donors in areas with a high prevalence of haemolysis [20]. Kumar et al. reported that a jaundiced neonate underwent massive intravascular haemolysis with abnormally elevated plasma bilirubin after an exchange transfusion with G6PD-deficient blood, requiring a threefold blood exchange [21]. Zekavat et al. studied Hb levels in G6PD-deficient patients after blood transfusion and found that patients transfused with G6PD non-deficient blood had higher Hb levels than those transfused with G6PD-deficient blood [22]. Therefore, in this study, we specifically labelled the G6PD-deficient RBC units and withheld their transport to the local children's hospitals and maternal and child health hospitals to prevent the occurrence of adverse transfusion reactions. However, no adverse transfusion reactions were reported due to the transfusion of G6PD-deficient RBCs in this study.

It is causally difficult to prove the risks of G6PD-deficient blood transfusion to the recipients due to the different experimental designs of the researchers. However, there may be some storage damage in the G6PD-deficient RBCs during refrigeration. In this study, the haemolysis rate was <0.8% in both the G6PD-deficient and non-deficient groups at the end of the storage period, consistent with the findings of Rojphoung et al. [23]. Swastika et al. showed that there was no significant difference in GSH levels between the G6PD-deficient and non-deficient RBCs [24]. Altogether, these reports suggest that haemolysis and GSH may not accurately reflect the storage damage of G6PD-deficient RBCs. Adu et al. compared RBCs, osmosis-induced haemolysis and plasma potassium levels of G6PD-deficiency and non-deficient blood samples after 0, 7, 14, 21 and 35 days of storage and concluded that the storability of citrate–phosphate–dextrose–adenine WB varied with the age, Hb type, G6PD status and blood group of the donor [25]. Francis et al. conducted metabolomics analysis to assess the 24-h post-transfusion RBC recovery after the refrigeration of G6PD-deficient and non-deficient blood and concluded that normal Hb and HCT and increased reticulocyte counts and decreased RBC in the G6PD deficiency group suggest that sub-clinical compensatory haemolysis may be present in G6PD-deficiency individuals. In addition, the study concluded that G6PD-deficient RBC units do not meet the current Food and Drug Administration (USA) requirements for storage quality [26]. Our study revealed that the G6PD-deficient samples had lower RBCs, increased MCV and higher bilirubin, suggesting that G6PD-deficient RBCs have relatively rapid destruction and metabolism.

The World Health Organization guidelines indicate that G6PD-deficient individuals without a history of haemolysis can donate blood, but blood from these donors should not be used for intrauterine transfusions, neonatal exchange transfusions or patients with G6PD deficiency [27]. Considering the cost of screening and the risk of transfusion, further analysis is required on the need for G6PD deficiency screening of blood donors. As the prevalence of G6PD deficiency varies among populations of different races and geographic regions, areas with higher prevalence should have policies for G6PD deficiency screening of blood donors. In addition, the blood centres can create a database of G6PD-deficient donors, marking their RBC units to avoid their use in specific patients, to encourage the donation of other blood components except RBCs by G6PD-deficient donors.

There are a few limitations to this study. First, we could only rely on the hospitals to report on any adverse transfusion reaction cases, without conducting an in-depth analysis of the haematological parameters of the patients after clinical transfusion. Second, we could not systematically assess the transfusion efficacy, as it varies based on the sex, age, medical history and history of transfusion among clinical patients.

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Jianhuai J. designed the research study and wrote the paper, Jianhuai J. and J.G. collected and analysed the data, W.S. and R.J. performed the research and wrote the first draft of the manuscript and Jian J., Y.X. and L.G. supervised the research and 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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ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Donor knowledge and perceptions regarding donation-induced iron depletion and iron supplementation as a blood service policy

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Abstract

Background and Objectives: Regular whole blood donations are associated with an increased risk of iron deficiency. Iron supplementation is an effective strategy to prevent donation-induced iron deficiency. However, research on donor perceptions towards such a policy is limited. Therefore, we aim to evaluate donors' knowledge on donation-induced iron depletion and their perceptions regarding iron supplementation as a blood service policy.

Materials and Methods: Three thousand Dutch whole blood donors were invited to complete a survey assessing their knowledge of donation-induced iron depletion and attitudes and perceptions towards iron supplementation as a policy. Linear regression modelling was used to evaluate associations between explanatory variables and perceptions.

Results: In total, 1093 (77.1%) donors were included in the analysis. Donors had poor knowledge of current iron management policies, but a better understanding of iron metabolism and supplementation. Iron supplementation as a policy was perceived mainly positive by donors, and the majority were willing to use iron supplements if provided. Iron supplementation was not perceived as invasive or negatively affecting donors' motivation to continue donating. Additional iron monitoring, information and donor physician involvement were regarded as important conditions for implementation. Male sex, trust in the blood service, prior experience with iron supplements and openness towards dietary supplements were strongly positively associated with willingness to use iron supplementation.

Conclusion: Donors' knowledge regarding donation-induced iron depletion is limited, but not associated with their perceptions regarding iron supplementation. Donors do

not consider iron supplementation as invasive, deterring or demotivating, and a majority are willing to take supplements if offered.

Keywords
donor knowledge, donor perceptions, iron depletion, iron supplementation, survey study, whole blood donation

Highlights

- Dutch whole blood donors have limited knowledge about donation-induced iron depletion.
- Iron supplementation as a blood service policy is not considered invasive or deterring by donors and will not result in demotivation to continue donating.
- Donors are generally willing to take iron supplements if provided by the blood service.

INTRODUCTION

Whole blood donors are prone to develop iron deficiency due to haemoglobin (Hb)-bound iron depletion during donation [1, 2]. Blood services worldwide use different strategies to manage the iron levels of their donors. In accordance with European legislation, the Dutch blood service (Sanquin) measures Hb levels before every donation. Donors with Hb levels below 125 g/L (female) and 135 g/L (male) are deferred from donating for 3 months. Moreover, as Hb levels do not reflect a donor's iron stores, Sanquin implemented ferritin-guided donation intervals. This policy includes post-donation venous serum ferritin measurements at new donor screening and at every fifth donation [3]. When ferritin levels are between 15 and 30 or <15 μg/L, donors are deferred from donating for 6 or 12 months, respectively. Although extended donation intervals have a beneficial effect on the iron status of a donor, it reduces donor availability [4]. It has been shown that after a 12-month deferral period, ∼75% of the female and ∼95% of male iron-deficient whole blood donors recovered from iron deficiency [5]. However, although Meulenbeld et al. showed that the implementation of ferritin-guided donation intervals improves Hb and ferritin levels, it also leads to lower donor return rates [4].

An alternative to ferritin-guided donation intervals is iron supplementation, which might prevent reduced donor availability. Post-donation iron supplementation is already used by blood services in, amongst others, Denmark, United States and Finland [6, 7]. While the beneficial effect of iron supplementation on donor health might attract or retain (new) donors, one could speculate that it could also deter subgroups of donors due to its interventional nature compared with extended deferral and potential gastrointestinal side effects. In a prior study, we assessed donor perceptions regarding iron supplementation policies through focus group interviews. We identified three main domains: willingness to use iron supplements, the perceived invasiveness of iron management policies and conditions necessary for implementation of an iron supplementation policy [8]. Donors generally held a positive perception of iron supplementation as a policy. However, although the qualitative nature of this study allowed for a comprehensive evaluation of donor perceptions, the limited size of the study population restricts the generalizability of the findings to the broader Dutch donor population. Moreover, prior research indicates that demographic characteristics such as sex, age and education level may influence an individual's perception regarding dietary supplements [9]. In addition, past studies have demonstrated associations between donors' knowledge levels regarding donation-induced iron depletion and their utilization of iron supplements [10].

The objective of this study is to evaluate whole blood donors' knowledge of donation-induced iron depletion. Furthermore, we aim to assess donor perceptions of post-donation iron supplementation in terms of willingness, perceived invasiveness and conditions for implementation. Subsequently, we will investigate associations of knowledge of iron metabolism, supplementation and management policies, demographic variables and donor characteristics, with the perceptions of donors regarding iron supplementation as an iron management policy.

METHODS

Setting

Sanquin Blood Bank is the only organization in the Netherlands authorized to collect, process and provide blood products for transfusion and for the development of plasma-derived medicinal products [11]. Blood donation in the Netherlands is voluntary and non-remunerated. Before every donation, a donor's eligibility to donate is evaluated through the donor health questionnaire and an onsite assessment [12]. Donors are allowed to donate when they are in good health, weigh at least 50 kg, are at least 18 years old and are not at risk for or have any transfusion-transmissible infectious disease. Newly registered donors do not make a full donation at their first visit to a blood collection centre but only have blood samples drawn for infectious disease and blood group testing. In accordance with European legislation, Hb cut-offs for donor eligibility for male and female whole blood donors are >135 and >125 g/L (measured using the HemoCue 201, Angelholm, Sweden), respectively. In the Netherlands, donors with Hb levels below these cut-offs are deferred from donating for 3 months. Additionally, starting November 2017, Sanquin has gradually

implemented a ferritin-guided donation interval policy. Donors with serum ferritin levels between 15 and 30 or <15 μg/L are deferred for 6 or 12 months, respectively [3].

Study population

Repeat whole blood donors, who donated at least once in the past 2 years ($N = 2500$), and newly registered donors ($n = 500$) were invited to participate in this survey study. Permanently deferred donors and participants in the ferritin-guided iron supplementation in whole blood donors: optimal odsage, donors resonse, return and eficacy study were excluded [13]. Based on power calculations and response rates from previous studies (Supporting Information Appendix A), this sample was expected to be sufficient for the inclusion of 1000 donors in total. We aimed for an equal distribution of donors above and below 50 years of age to account for pre- and post-menopausal differences [14, 15].

Study design

A cross-sectional online survey was used to assess donors' knowledge concerning donation-induced iron depletion and their perceptions regarding iron supplementation as a blood service policy (Supporting Information Appendix B). In addition, the survey assessed demographic information (e.g., age, sex, menstrual status and donor career), donor opinions (regarding, e.g., donation deferral, dietary [iron-rich] supplements), donor experiences (e.g., donation-related and iron deficiency-related symptoms, dietary supplement use) and donor behaviour (e.g., dietary adjustments, donor return). The survey contents were based on results from previously performed focus group research amongst donors, blood collection staff and donor physicians [8]. The survey was distributed on 4 January 2023, through e-mail. The invitational e-mail contained information on the study procedures and a URL to open the survey. The invitational mail did not specify the exact topic of the survey but indicated that it contained questions regarding donor deferral in general. It was indicated that donors provided informed consent by participating in the anonymous survey. The survey data were collected using Qualtrics© software (Qualtrics, Provo, UT). Donors were asked to complete the survey within 3 weeks after receiving the invitation. One week after the initial invitations were sent out, all donors received a reminder about the possibility to participate in the study. Participants were asked not to use any external information sources to answer the survey questions. To prevent missing data, the participants were reminded through a pop-up message to answer questions that were left unanswered. However, donors were still able to proceed with the survey when a question was left unanswered. A survey response was considered valid if it included at least demographic information for age and sex, along with data on the outcome variables.

Primary outcomes

Based on the results from previously performed focus group interviews [8], donor perceptions regarding iron supplementation as a blood service policy were evaluated by the level of agreement with nine statements, listed in Supporting Information Appendix C. These statements assessed three domains: (1) willingness: evaluating donors' willingness to use iron supplements if provided by the blood service, (2) invasiveness: assessing if and to what extent iron supplementation as a policy would be perceived as invasive and (3) conditions: evaluating the extent to which conditions identified in previous research would be desired if iron supplementation would be implemented as a blood service policy [8] For each statement, a 5-point Likert scale (1 = completely disagree, 2 = disagree, 3 = neutral, 4 = agree, 5 = completely agree) was used to assess the perceptions regarding iron supplementation.

Secondary outcomes

The knowledge level regarding donation-induced iron depletion was evaluated for three topics: current iron management policies, iron metabolism and iron supplementation. Knowledge was evaluated using a combination of true or false statements and multiple-choice questions with multiple answer options. The questions and statements were grouped per topic. All correctly answered true/false statements corresponded with 1 point, and all multiple-choice questions had a maximum score of 3 points. Based on the ratio of correct/incorrect answering, each correct answering option for the multiple-choice question concerning current iron management policies (comprising 2 correct and 3 incorrect options) earned 1.5 points, whereas each incorrect option incurred a deduction of 1 point. Conversely, for multiple-choice questions related to iron metabolism and iron supplementation (comprising 3 correct and 2 incorrect options), each correct option earned 1 point, whereas each incorrect option resulted in a deduction of 1.5 points. A sum score was calculated for each knowledge area, including iron management policies (maximum score: 7 points), iron metabolism (maximum score: 10 points) and iron supplementation (maximum score: 7 points).

The attitude towards iron supplementation as a blood service policy was assessed using two hypothetical scenarios. In the first scenario, participants were asked to rank four options for donation-related consequences of low iron levels. In the second scenario, participants ranked the importance of five prerequisites for receiving iron supplements from the blood service.

Determinants

In the analysis, multiple determinants were included in the model to evaluate their association with the perception outcomes (willingness, invasiveness and conditions). Demographic characteristics such as sex, age, education level, prior experience with iron supplements and

attitudes towards dietary supplements in general were included. Donor knowledge regarding donation-induced iron depletion encompassed knowledge of current iron management policies, iron metabolism and iron supplementation, with knowledge scores scaled to a 0–10 range for comparability. Additionally, donor characteristics including previous deferral, number of donations, trust in the blood service expertise and intentions and considerations regarding donation-induced iron depletion were incorporated into the models.

Statistical analysis

Statistical analyses were performed using RStudio (version 4.0.3. RStudio Team [2020]). Descriptive statistics are presented for the entire study population. For continuous variables, data are presented as mean ± standard deviation. For categorical variables, data are presented as proportions.

To evaluate to what extent the individual perception statements correspond with the a priori selected domains of perception (i.e., willingness, invasiveness and conditions), the individual scores were used for a confirmatory factor analysis. The performance of the structured model compared with an unstructured model is evaluated through the comparative fit index (CFI) and Tucker–Lewis index (TLI), where a better fit is indicated by a score closer to 1. In addition, the fit of the model is evaluated by the root mean square error of approximation (RMSEA) and standardized root mean square residual (SRMR), with a score closer to 0 indicating a better fit [16]. Furthermore, the factor loading is evaluated through statistical significance level, with $p < 0.05$ being statistically significant. Based on the results of the confirmatory factor analysis, a 15-point sum score was calculated for each perception domain, with a scale of 3–15 points, using the scores from the corresponding statements. These sum scores were used in linear regression models to assess associations of the described determinants with donors' knowledge and perceptions. A complete overview of the configuration of all explanatory variables is presented in Supporting Information Appendix D. The linear regression analyses comprised two steps. In the univariable analyses, each explanatory variable was analysed separately. In the multiple variable analyses, all explanatory variables were analysed simultaneously. Participants with missing data for covariates were excluded from the regression analyses to guarantee consistent datasets for the univariable and multivariable analyses. Regression coefficients and 95% confidence intervals (CIs) are presented, and a two-sided p-value <0.05 is considered statistically significant.

RESULTS

Of the 3000 invited donors, 1418 (47.3%) participated in the survey. After exclusion of participants with missing data for the outcome variable 'perception regarding iron supplementation' (20.7%) and sex (0.4%), data of 1093 donors were included in the analyses. Of the donors included for the analyses, 670 (61.3%) were female and 574 (52.5%) were 50 years or older (Table 1). The majority were experienced donors (80% made more than 10 donations) and were highly educated (61%).

TABLE 1 Participant characteristics.

Variables	
N	1093
Sex (%)	
Women	670 (61.3)
Age (mean [SD])	52.3 (8.9)
Donor experience (%)	
New (0 donations)	36 (3.4)
Beginner (1–10 donations)	171 (16.2)
Experienced (>10 donations)	851 (80.4)
Education level (%)	
Lower education (primary/middle school)	101 (9.3)
Mid-level education (college)	321 (29.4)
Higher education (applied sciences/university)	669 (61.3)
Previous ferritin/Hb deferral (%)	
Never	444 (42.0)
Once	337 (31.9)
More than once	276 (26.1)
Trust in the blood service[a] (mean [SD])	13.6 (2.0)
Prior iron supplement use (%)	
Yes	619 (56.6)
No	474 (43.4)
Accounts for donation-induced iron depletion (%)	
Yes	222 (20.3)
No	871 (79.7)
Open towards dietary supplements in general (%)	
Yes	789 (75.1)
No	261 (24.9)

Abbreviations: Hb, haemoglobin; SD, standard deviation.
[a]Scale 3–15.

Knowledge

Figure 1 shows the scaled sum scores (scaled from 0 to 10) for the knowledge areas current iron management policies, iron metabolism and iron supplementation. Knowledge of Sanquin's current iron management policies was poor (3.9 ± 1.6). Most donors knew that Hb is measured in donors (91.9%), that it is measured before every donation (94.0%) and that potential consequence of low Hb levels include a 3 month deferral period (73.0%) (Supporting Information Appendix E1). In contrast, fewer donors were aware that ferritin is also measured (50.1%), of the measurement frequency (every fifth donation) (47.3%) and of potential consequences of low ferritin levels (30.7%).

The mean scaled sum score for knowledge of iron metabolism was 5.7 ± 1.7 (scaled from 0 to 10). Most donors were aware that iron

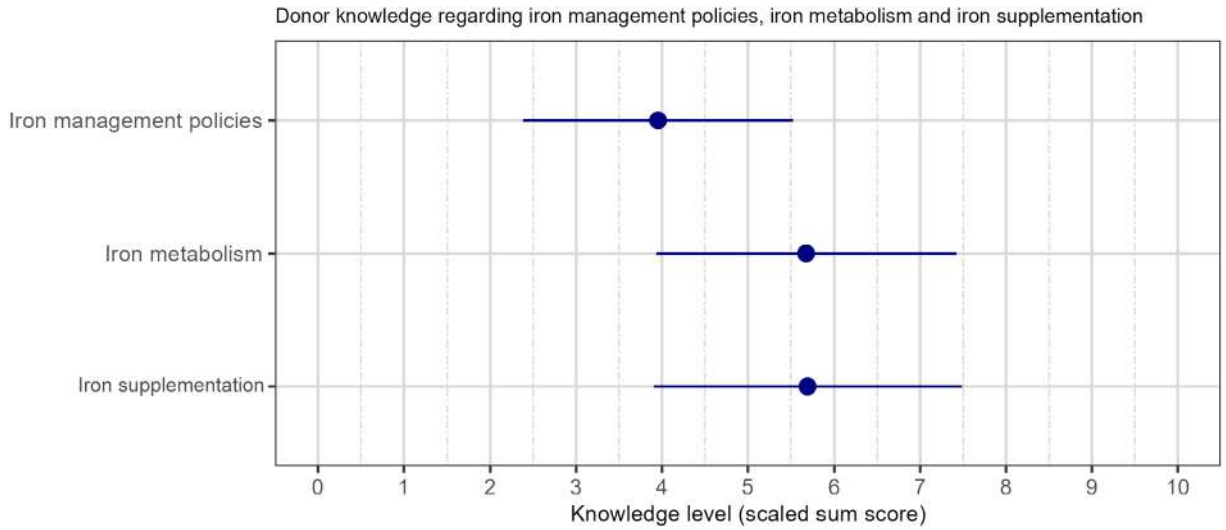

FIGURE 1 Sum scores for the knowledge domains current iron managements policies, iron metabolism and iron supplementation. Scores are presented as mean ± standard deviation.

is lost during every whole blood donation (77.0%), that ferritin is a marker for body iron stores (83.5%) and that Hb is involved in oxygen transport (78.6%) (Supporting Information Appendix E2). However, knowledge regarding potential symptoms of iron deficiency was lacking; for instance, only 18.8% of the donors replied 'yes' when asked if restless legs syndrome is a potential symptom.

The mean sum score for knowledge regarding iron supplementation was 5.7 ± 1.8 on a scale from 0 to 10. Most donors knew that iron supplementation can improve iron store recovery after donation (77.4%), that it can potentially cause side effects (80.9%), that a prescription for iron supplements is not necessary in the Netherlands (81.4%) and that Sanquin currently does not provide iron supplements (96.6%) (Supporting Information Appendix E3).

Attitudes

When asked about their preferred policy in case of low ferritin levels, the majority of donors indicated ferritin-guided deferral combined with dietary advice as the favoured option, with 38.1% ranking it as most preferred and 45.9% as second most preferred. This was followed by longer regular donation intervals (32.1% most preferred, 26.9% second most preferred) and post-donation iron supplementation without prolonged donation intervals (27.8% most preferred, 26.2% second most preferred). Only 2.0% of the donors would most prefer to stop donating completely when their ferritin levels are low, with 2.0% ranking it as the second preferred option. An overview of the responses is presented in Figure 2.

Donors were asked what they felt to be the most important reason to provide iron supplements (Figure 3). The majority of donors ranked 'health maintenance' as most important (38.8% most important, 37.2% second most import), followed by 'scientific evidence regarding safety and efficacy' (36.2% most important, 29.8% second most important). Next, 18.1% of the donors felt that prevention of extended donation intervals was the most important reason, with 23.9% ranking it as second most important. Only 5.8% of the donors ranked 'iron supplementation should never be considered as a policy' as most important and 1.2% as second most important. Lastly, 1.1% of the donors scored 'the implementation of iron supplementation by other international blood services' as the most important reason, with 8.9% ranking it second most important. A large majority of 82.1% of the donors ranked 'iron supplementation should never be considered as a policy' as least important.

Donor perception domains

Donors were asked to express their perceptions of iron supplementation provided by the blood service for donors with low ferritin levels using 5-point Likert scale statements. Donors exhibited a mainly positive attitude towards iron supplementation, with 55.5% expressing agreement (i.e., agree and completely agree combined) and 25.6% remaining neutral. Approximately half of the donors expressed willingness to utilize iron supplements if offered, with 49.0% indicating agreement and 28.0% remaining neutral. Furthermore, a third of the donors expressed a preference for iron supplementation over deferral, with 33.8% expressing agreement and 30.6% remaining neutral (Figure 4). Donors exhibited strong disagreement regarding the perception that iron supplementation as a policy would be too much to ask of donors, with 77.5% expressing disagreement and 14.1% remaining neutral. Similarly, a majority of donors disagreed with the notion that the policy would be perceived as pushy (76.8% disagreed, 15.7% neutral) and that it would negatively impact their motivation to donate (78.5% disagreed, 14.5% neutral). Donors expressed agreement with the importance of involving a donor physician (63.5% agreed, 22.1% neutral), more frequent measurement of iron levels (61.2% agreed, 28.5% neutral) and moderate agreement on the need for additional information

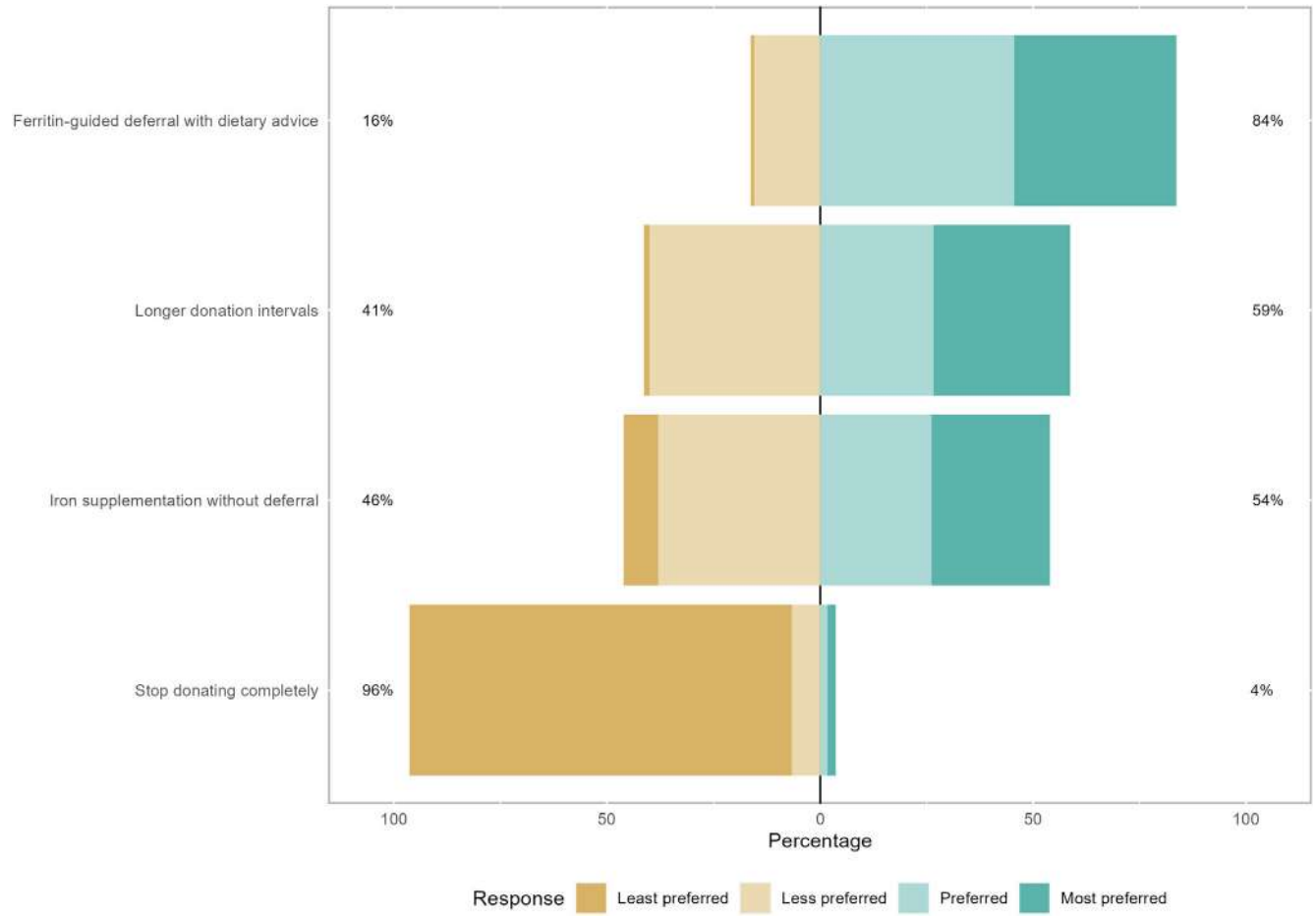

FIGURE 2 Percentages of the responses regarding the question 'Which of the following consequences would be your preferred option when your iron level would be low?'. Percentages on the left indicate the combined percentage of least and less preferred, and the right indicate the combined percentages of preferred and most preferred.

regarding iron supplementation before initiating supplementation (46.4% agreed, 24.5% neutral).

The results of the confirmatory factor analysis indicated that the factor loadings for each statement were significantly positively associated with their respective perception domains and that the structured model performed better compared with the unstructured model (Supporting Information Appendix F). This resulted in mean scores (on a scale of 3 to 15) of 9.8 ± 3.2 for willingness to utilize iron supplements if provided by the blood service, 5.1 ± 2.7 for the perceived invasiveness of iron supplementation as a policy and 10.4 ± 2.7 for the conditions required for the blood service to implement iron supplementation as a policy. A higher score reflects a greater level of willingness, perceived invasiveness and desired conditions regarding iron supplementation as a policy.

Factors associated with donor perceptions

In the univariable analyses, knowledge of iron management policies and iron metabolism showed associations with all perception domains (Figure 5). However, in the multivariable analyses, only a marginal negative association between knowledge of iron metabolism and willingness remained (−0.12, 95% CI: −0.24 to 0.01), indicating that a 1-point increase of knowledge of iron metabolism results in a decrease in willingness with 0.12 points. A higher level of trust in the expertise and intentions of the blood service was significantly associated with increased willingness towards iron supplementation (0.21, 95% CI: 0.12–0.31) and lower levels of perceived invasiveness (−0.24, 95% CI: −0.33 to −0.16) in the multivariable model, as well as the univariable models. In the multivariable models, females were less willing to take iron supplements (−1.18, 95% CI: −1.60 to −0.75) and expected more in terms of conditions (0.53, 95% CI: 0.15 to 0.92) compared with male donors. Higher levels of education were significantly associated with higher levels of perceived invasiveness in the univariable model, but not in the multivariable model. Only in the multivariable analysis, donor experience was significantly associated with lower levels of willingness for donors with more than 10 donations (0.78, 95% CI: 0.73–0.84) compared with new donors. In line with the univariable analyses, prior iron supplementation was significantly associated with a higher level of willingness (0.9, 95% CI: 0.55–1.40)

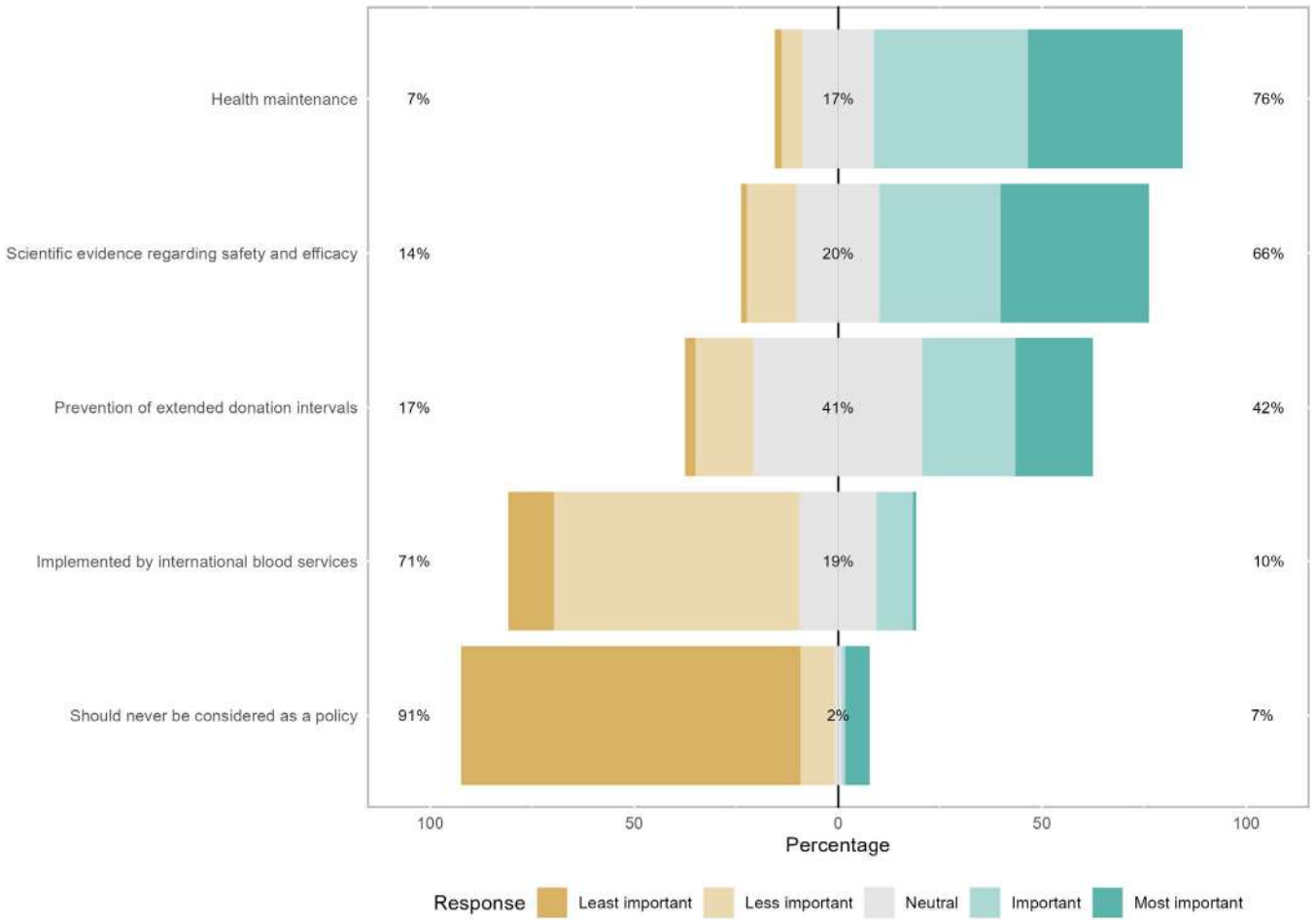

FIGURE 3 Percentages of the responses to the question 'Which of the following reasons to be provided with iron supplements by the blood service do you consider most important?'. Percentages on the left indicate the combined percentage of least and less important, the right indicate the combined percentages of important and most important and the middle indicate the percentage of neutral responses.

and lower levels of invasiveness (−0.58, 95% CI: −0.94 to −0.21) and expected conditions (−0.40, 95% CI: −0.79 to −0.02) in the multivariable analyses. Similarly, being open towards dietary supplement use in general was strongly significantly associated with a higher level of willingness (2.03, 95% CI: 1.58–2.48) and a lower level of perceived invasiveness (−0.97, 95% CI: −1.35 to −0.58) in the multivariable analyses. Previous deferral due to low Hb and/or ferritin levels, as well as active consideration of donation-induced iron depletion, was not found to be significantly associated with any of the perception domains. The coefficients and CIs for each individual explanatory variable in both the univariable and multivariable regression models are shown in Supporting Information Appendix G.

DISCUSSION

In this study, we demonstrate that donors at our blood service generally have a poor knowledge of the current iron management policies. They show a more comprehensive understanding of iron metabolism and iron supplementation. Donors are generally willing to take iron supplements if offered by the blood service, do not consider it as too invasive and consider physician involvement and monitoring important conditions for implementation. Male sex, trust in the expertise and intentions of the blood service, more donor experience, prior iron supplementation and openness towards dietary supplementation in general were significantly associated with an increased willingness to use iron supplements if provided by the blood service.

Donors exhibited a mainly positive perception towards iron supplementation as a blood service policy. However, consistent with findings from our previous focus group discussions, most donors concurred that involvement of a donor physician and more frequent monitoring of iron levels than is presently conducted would be appreciated [8]. These findings were in line with results from a study by Cable et al. who reported that approximately 80% of donors expressed a willingness to initiate iron supplementation if provided [17]. This was, however, dependent on the amount and type of information participants received regarding donation and iron depletion. Interestingly, although donors in our study only had moderate knowledge regarding iron supplements and current policies, they did not strongly agree that additional information was needed to decide

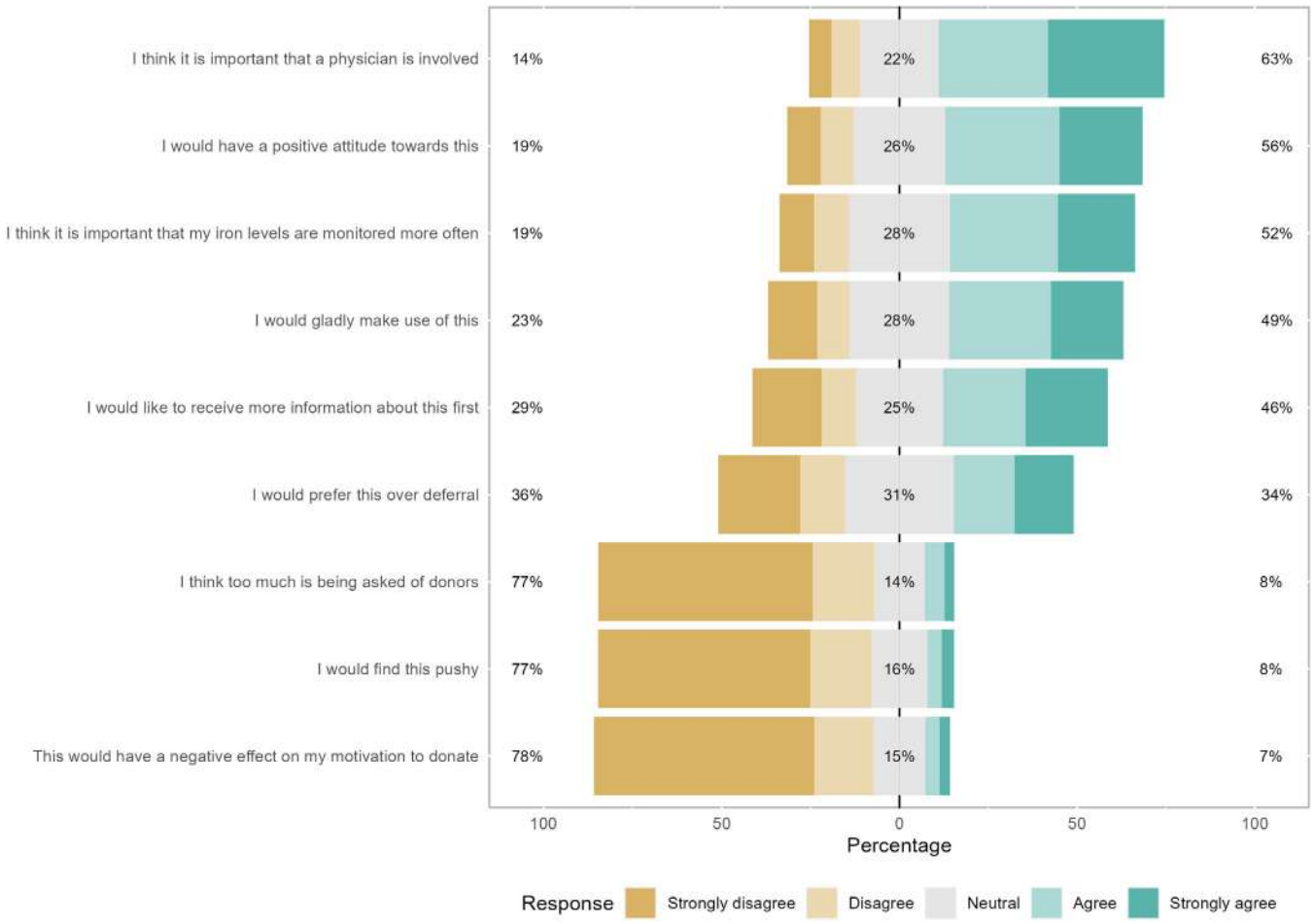

FIGURE 4 Likert scale responses (percentages) regarding donors' perceptions towards iron supplementation based on the statement 'If the blood service would provide iron supplements …'

on iron supplement use. This might be explained by their high level of trust in the expertise and intentions of the blood service, consistent with the results from our previous study [8]. A high level of trust in the health care system amongst the Dutch population has already been described, along with its impact on health-related behaviours [18, 19]. This could explain the association between higher levels of trust in the blood service and elevated willingness to use iron supplements and lower levels of perceived invasiveness. This further stresses the importance of involvement of medical experts, such as donor physicians, when implementing iron supplementation as a blood service policy. In addition, although not evaluated in this study, the donors' apparent lack of interest in receiving additional information about iron supplements might be attributed to their perceived overconfidence in their understanding of donation and iron supplementation. Overconfidence has been described in literature to be associated with a lower level of perceived risk and discounting information provided by experts [20–23]. Therefore, donors might underestimate the risk of developing iron deficiency and the need for preventative measures when informed by a donor physician. In turn, this could reduce the perceived need for post-donation iron supplementation.

Knowledge of iron metabolism only showed a marginal negative association with willingness, but no associations with invasiveness and conditions. These findings contradict previous research showing that a better knowledge of dietary supplements in general, including iron supplements, is significantly associated with an increased willingness to use dietary supplements. This would suggest that the willingness of donors to use iron supplements might be dependent on their awareness of the effects of donation on their iron status [10]. Furthermore, results from the strategies to reduce iron deficiency study showed that donors who received information on the effect of donation on iron stores were more likely to start using iron supplements [17]. Similarly, donors who were interviewed by Lynch et al. indicated to initiate iron supplementation after donation upon being informed of their low iron levels [24]. The contrast between our findings and those from previous studies might suggest that latent knowledge of iron metabolism could differently affect the willingness of donors to use iron supplements than being personally confronted with low iron levels. Furthermore, our findings align with a study by Pajor et al., demonstrating that the general Dutch population's use of dietary supplements is influenced more by beliefs and social environment than by an understanding of the

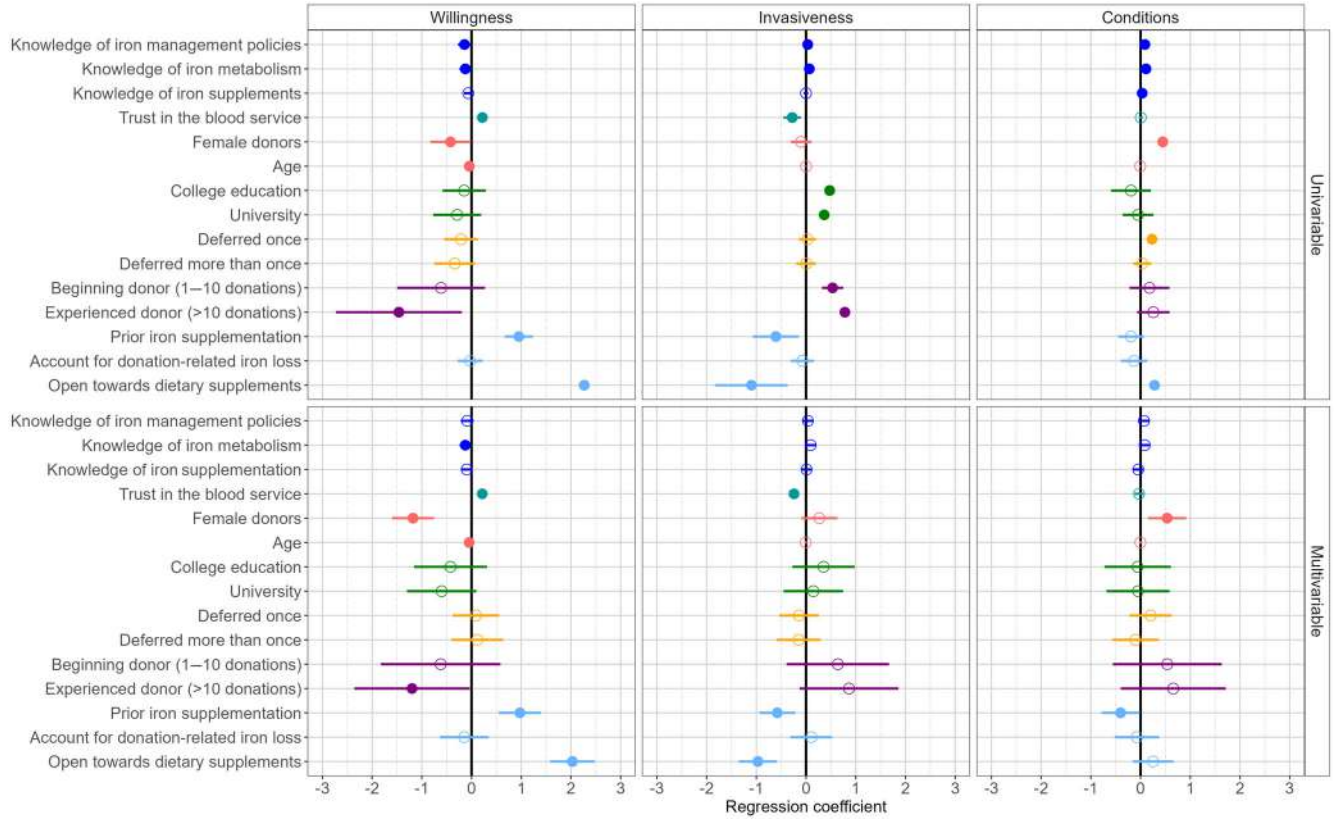

FIGURE 5 Univariate and multivariate associations between the explanatory variables and the perception domains willingness, invasiveness and conditions separately. Filled circles represent a significant association.

health effects of such supplements [9]. This could also explain why openness towards dietary supplements in general and prior experience with iron supplements was shown to be strongly associated with willingness and invasiveness. Furthermore, Pajor et al. also discussed that in the Netherlands, individuals are not inclined to start using dietary supplements in absence of specific health necessities. This supports our finding that donors feel that health maintenance should be the most important reason to be provided with iron supplements by the blood service. The contrast between international studies and those specific to the Netherlands suggest that perceptions regarding dietary supplement use might be country- and culture-specific [25].

Ferritin-guided deferral including dietary advice was most preferred by donors as a policy for donors with low ferritin levels. However, ferritin-guided deferral, although effective in certain aspects, may not facilitate complete iron store recovery and lowers donor availability, potentially impacting blood inventory levels [4, 5]. In addition, our findings are contradictory to results from our focus group meetings in which Dutch donors indicated to prefer iron supplementation over ferritin-guided deferral, whilst being able to continue to donate was important for these donors [8]. These different findings may be explained by the additional information provided to focus group participants on the amount of iron lost with a donation and how this relates to dietary iron intake.

In a previous study, blood collection staff raised concerns that donors might feel exploited if iron supplementation would be used to let donors donate more often [8]. These concerns were not mentioned by donors, nor by donor physicians. In the present study, donors themselves indicated that they did not perceive iron supplementation as a blood service policy as pushy or too much to ask. Furthermore, donors strongly concurred that iron supplementation as a policy would not negatively affect their motivation to continue to donate.

Female donors were less willing towards iron supplementation as a policy and expected more in terms of conditions compared with male donors. This is an important finding as women are more prone to developing iron deficiency due to pregnancy, childbirth and menstrual blood loss [26]. As a result, female donors are more often deferred due to low Hb levels than male donors, which can coincide with iron deficiency-related symptoms and donor loss [27, 28]. While additional research would be necessary to further investigate potential explanatory factors for the differences in perceptions between men and women, it should be taken into account by blood services when implementing iron supplementation as blood service policy.

The high response rate in combination with its representativeness of the Dutch whole blood donor population in terms of sex and age enables us to provide reliable evaluations of the perceptions of Dutch donors towards iron supplementation. Although women were slightly

overrepresented in our study population, this is also the case for the active Dutch whole blood donor population. A limitation is that we were not able to perform stratified regression analyses (e.g., stratified by sex), because this resulted in too small numbers for the categorical explanatory variables. However, univariate and multivariate regression models allowed for a comprehensive evaluation of the association between each explanatory variable and perception of iron supplementation as a policy in donors and to evaluate potentially confounding factors. Due to the nature of this research (i.e., survey), there is a risk of selection bias. Donors with a more explicit opinion on the topic of iron supplementation might be more inclined to participate. However, the risk of selection bias and voluntary response bias was minimized by not enclosing the exact contents of the survey (i.e., iron management) in the invitation. Quantitative research on the perceptions of donors regarding iron supplementation as an iron management strategy is limited, especially in association with their knowledge level on donation-induced iron depletion. However, this approach is important to help determine if iron supplementation should be considered as a blood service policy and what should be considered and addressed upon implementation. Our study provides evidence that knowledge concerning donation-induced iron depletion appears to be limited in whole blood donors, yet this limitation is not associated with their perception of iron supplementation. The implementation of iron supplementation as a blood service policy is anticipated to be generally well-perceived by donors in terms of willingness and is not expected to discourage them from continuing to donate.

To conclude, whole blood donors' knowledge regarding donation-induced iron depletion and iron management policies is limited, but not associated with their perceptions regarding iron supplementation. Donors do not consider iron supplementation as invasive, deterring or demotivating, and a majority are willing to take supplements if offered.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

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Plasma-derived product recipients' views on the acceptability of implementing a programme of plasma donation for fractionation from men who have sex with men

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Abstract

Background and Objectives: Over the past few years in Québec, Canada, exclusion criteria for blood donation and plasma donation for fractionation have been modified. Héma-Québec, the institution responsible for blood products, has made changes to allow more inclusive access to blood and plasma donation, in accordance with evolving scientific data concerning donation safety. The study, conducted before those changes were implemented, aimed to assess acceptability of recipients and parents of recipients of plasma-derived products for men who have sex with men (MSM) to become eligible to donate plasma for fractionation.

Materials and Methods: Eight qualitative interviews (4 focus groups, 4 individual) were conducted with a total of 17 plasma product recipients and parents of children needing plasma-derived products. Data were analysed using thematic analysis.

Results: Participants were rather favourable regarding acceptability of MSM as potential donors. Participants viewed this change as necessary and beneficial. They also felt they must rely on trust in Héma-Québec, conferred automatically or by default. However, some participants raised concerns about donation safety and reported feeling helpless regarding inclusion of MSM. The importance of being informed and that recipients' safety be prioritized first and foremost were also mentioned.

Conclusion: Despite their nuanced attitudes, recipients showed high levels of acceptability of including MSM in plasma donation for fractionation. Actions can be taken to reduce concerns regarding the safety of products received.

Keywords
acceptability, MSM, plasma donation for fractionation, plasma product recipients

Highlights

- Plasma product recipients had relatively favourable views on the acceptability of men who have sex with men as potential donors.
- Relying on trust in institutions responsible for blood products is necessary.
- Being informed on the risks for recipients could help overcome concerns about donation safety.

INTRODUCTION

Situation in Québec and Canada

Since the emergence of human immunodeficiency virus (HIV) in the early 1980s, significant efforts have been made to ensure the safety and quality of blood products [1]. In 1983, in the midst of the tainted blood scandal, men who have sex with men (MSM) were identified as a group at risk and were permanently deferred from blood donation. Since then, the lifelong deferral of MSM donors has been challenged, leading to many debates about equity [2]. In Québec and Canada since 2013, the criteria have gradually been relaxed from lifelong exclusion to 12 months and then 3 months from last sexual contact with another man.

To make blood donation more inclusive, institutions that distribute blood products looked into plasma donation for fractionation because pathogen inactivation inherent in the fractionation process ensures product safety. In Québec, plasma can be collected by whole blood donation, or by apheresis, after which it is quickly frozen to preserve its proteins, then tested for infection and disease. To produce plasma-derived medicines, the plasma is sent to high-tech factories, which extract proteins from the plasma to manufacture specialized medications that are then returned to Héma-Québec (the institution responsible for blood products in Québec) for distribution to hospitals across the province. All donations made in Québec are used to manufacture products for people living in Québec.

Around the same time, consideration was also given to the issue of individual screening based on risky sexual behaviours rather than on belonging to a risk group. In October 2022, Héma-Québec made it possible for MSM to donate plasma for fractionation by introducing an individual risk-based screening approach, which was soon followed by other blood donations. Thus, screening is now the same for everyone, regardless of gender and sexual orientation. Exclusion is based on having had anal sexual relations in the past 3 months, with multiple or new partners.

Acceptability among various populations of blood donation from MSM

Few Canadian studies have considered MSM's perceptions of access to plasma donation for fractionation [3–6]. These studies demonstrated that exclusion of MSM was perceived as an affront to equity and an issue of institutional homophobia [3, 4]. Blood donation is often characterized as a mark of altruism that has an impact on other people and elicits a sense of pride [3, 4]. Studies [3–6] highlighted the will of MSM communities to contribute to blood banks and their strong desire to help others. MSM's perceptions of changes in policy were nuanced by the measures to be implemented [3]: an approach based on sexual risk behaviours was more acceptable than one based on gender or sexual orientation [5]. Some MSM viewed the possibility that donations be limited to plasma as a positive and progressive alternative, whereas others were quite sceptical about it [3, 4]. For MSM, acceptability of plasma donation without inclusion in other types of donations made them feel like second-class donors and offered yet another proof of stigmatization and homophobia [3, 4].

A Canadian study [7] evaluated donor centre staff's perspectives regarding a programme of plasma donation for fractionation that included quarantine of donations for MSM. It concluded that staff supported the addition of inclusive eligibility criteria but were concerned that the new criteria remained discriminatory. Some interviewees perceived this change to be a step in the right direction, whereas others viewed it as insufficient and would have preferred a gender-neutral approach. Staff also wished to foster positive donor experiences, but mentioned the possibility of feeling uncomfortable about using stigmatizing criteria and involuntarily conveying nonverbal cues of discomfort [7].

A Québec study of active donors showed high acceptability of MSM donating blood and that implementation of new gender-neutral questionnaires had very little impact on intention to donate again [8]. Canadian donors reported being comfortable with new questions on sexual risk behaviours since the questions were consistent with their expectations of donor screening and did not deviate from social norms [9]. Still, a minority (6%) of donors reported feeling uncomfortable answering these types of questions, which felt too personal and their safety benefits, unclear [10].

To our knowledge, no study has evaluated perceptions of plasma product recipients, even though this type of product is intended mostly for these individuals. The goal of this study is to assess the acceptability of plasma product recipients and parents of children needing plasma-derived products regarding the possibility that MSM be eligible to donate plasma for fractionation. At the time of the study (2021), the deferral of men who had had sex with another man in the past 3 months was still in force in Québec.

MATERIALS AND METHODS

Acceptability was measured by analysing recipients' beliefs regarding the inclusion of MSM in plasma donation for fractionation. According to Fishbein and Azjen [11], a person may hold both favourable and unfavourable beliefs about an issue. The outcome of those beliefs qualifies the person's attitude towards the given issue. Interviews for the study took place in November 2021: 8 interviews (4 focus groups, 4 individual) were conducted with a total of 17 recipients—10 plasma product recipients and 7 parents of child recipients. Because the interviews took place before the change in eligibility criteria was announced, results reflect participants' opinions of a potential change, in a context where sexually active MSM were still excluded from all types of blood donation. Québec groups and organizations working with recipients and parents of child recipients were asked to share a recruitment poster on their social networks, in their newsletters and with their contact lists. Participants had to meet the following criteria: be a recipient or the parent of a child recipient of products derived from human plasma; be at least 18 years old and understand and speak French. Recruitment continued until data saturation.

Before the interviews, participants were asked to fill out a consent form and a short questionnaire composed of three sections: (1) sociodemographic profile; (2) experiences with plasma-derived products and level of health care empowerment (adapted from the Health Care Empowerment Inventory [12]; 8 items on a 5-point Likert scale from 1: 'Strongly disagree' to 5: 'Strongly agree', where the higher the score, the greater the empowerment level) and (3) acceptability of the donation programme (Likert scale from 1: 'Not at all acceptable' to 10: 'Completely acceptable').

Participants were then asked to do a 90-min Zoom interview conducted by the research team. They were asked about their acceptability of the idea that MSM be allowed to donate plasma for fractionation (e.g., At first glance, what do you think about MSM being eligible to donate plasma for fractionation? What do you think are the advantages/ disadvantages? In your opinion, what would be the consequences of including MSM in a programme of plasma donation for fractionation?). Participants were paid $50 as compensation. Interviews were recorded, transcribed and analysed in NVivo using thematic analysis, which consists in systematically coding relevant data and sorting them into themes [13]. Co-researchers validated thematic coding to ensure the internal validity of results [14]. The authors translated all quotes included in this article.

RESULTS

Sample characteristics

The average age of participants was 35.5. A majority of participants reported having a university degree (53.0%), an annual income of over $30,000 (64.7%), a full-time job (64.7%) and being born in Québec (82.4%). Most participants identified as heterosexual (70.6%)

TABLE 1 Sociodemographic characteristics.

Variables	*N* (%)/mean + standard deviation *N* = 17
Average age	
Varies from 19 to 50 years	35.54 ± 9.66
Gender	
Female	14 (82.4)
Male	3 (17.6)
Highest level of education completed	
No diploma	1 (5.9)
High school diploma or professional degree	5 (29.4)
College or technical diploma	2 (11.8)
Bachelor's degree	7 (41.2)
Master's degree or PhD	2 (11.8)
Personal income	
Under $30,000	1 (5.9)
$30,000 or more	11 (64.7)
Rather not answer	5 (29.4)
Main occupation	
Full-time job	11 (64.7)
Part-time job	3 (17.6)
Full-time student	1 (5.9)
Retired or unemployed	2 (11.8)
Place of birth	
Québec	14 (82.4)
Canada (Ontario)	1 (5.9)
Another country (France, Qatar)	2 (11.8)
Number of years in Canada (people born outside Canada)	
Varies from 2 to 32 years	17.00 ± 21.21
Ethnic origin	
White	16 (94.1)
African, Caribbean	1 (5.9)
Relationship status	
Single	5 (29.4)
Couple relationship/living common law	9 (52.9)
Civil union/Married	3 (17.6)
Sexual orientation	
Heterosexual	12 (70.6)
Homosexual/lesbian/gay	1 (5.9)
Bisexual	3 (17.6)
Rather not answer	1 (5.9)
Friends and acquaintances from sexual and gender diversity communities	
None	3 (17.6)
A few	6 (35.3)
Many	8 (47.1)

TABLE 2 Experience with plasma-derived treatments.

Variables	*N* (%)/mean ± standard deviation	
	Recipients *n* = 10	Parents *n* = 7
Average age of children		
Varies from 2 to 17 years		8.36 ± 6.74
Health condition requiring use of plasma-derived treatments (not mutually exclusive)		
Haemophilia, rare bleeding disorder or von Willebrand disease	2 (20.0)	2 (28.6)
Hereditary angioedema	2 (20.0)	
Primary or secondary immunodeficiency	5 (50.0)	5 (71.4)
Autoimmune disease	1 (10.0)	
Years of plasma-derived treatment use		
1–4 years		4 (57.1)
5–9 years	5 (50.0)	1 (14.3)
10–19 years	1 (10.0)	2 (28.6)
20 years and over	4 (40.0)	
Frequency of plasma-derived treatment use		
At least once a day		1 (14.3)
At least once a week	4 (40.0)	4 (57.1)
At least once a month	5 (50.0)	2 (28.6)
A few times a year	1 (10.0)	
Level of empowerment for health care related to a health condition requiring use of human plasma-derived treatments		
From 1 'Strongly disagree' (low empowerment) to 5 'Strongly agree' (high empowerment), mean of 8 statements	3.91 ± 0.31	4.16 ± 0.58

(Table 1). In all, half of plasma product recipients and 71.4% of parents of child recipients said they had been using plasma-derived products for at least 10 years, and 40.0% of recipients and 71.4% of parents did so at least once a week. In terms of health care empowerment regarding plasma-derived treatments, the mean score for recipients was 3.91/5 and 4.16/5 for parents (Table 2). As for acceptability of MSM donating plasma for fractionation, the mean was 9.20/10 for recipients and 7.17/10 for parents of child recipients (Table 3).

Described below are the different attitudes of plasma product recipients and parents of child recipients towards MSM being eligible to donate plasma for fractionation.

'Stop putting MSM in a class apart': A beneficial and necessary change

Most participants said it was important that MSM finally be recognized as potential donors and that such inclusion would help reduce discrimination against MSM: 'The advantage of such a change isn't necessarily related to plasma, but rather to public perception. At some point, stop putting them in a class apart'. (F, 39 years old [yo], recipient). A few participants stated they did not understand why MSM were still excluded from donating and some expressed outrage. Excluding MSM was no longer considered viable given the evolution of science and product processing, and participants said they would be pleased to see them be able to donate plasma. Certain participants expressed a desire that sexual orientation no longer be an eligibility criteria for plasma donation, echoing a desire for change. For them, a person's sexual orientation did not necessarily suggest risk behaviours; rather, individual risk behaviours should be considered for eligibility criteria. 'I'm sure that there are people who are homosexual and in very stable couple relationships and there's no danger, and there are people who identify as heterosexual and have lots of risky behaviours too'. (F, age not available [n/a], parent).

Including MSM as potential donors was also determined to be beneficial to plasma supply and quality in Québec. Many participants stated that it could increase the availability of plasma-derived products and so reduce the risks of shortages: 'Plasma shortages, yes, it's important that our banks be full, for myself and for other patients. I don't know the percentage [of MSM] that could help us, but I'm pretty sure that it's not just a tiny drop. It's measurable and it's worth it, I think'. (F, 45 yo, recipient). Participants also considered that the quality of plasma-derived products would improve because of a broader diversity of antibodies in donations.

'I trust Héma-Québec': Trust conferred automatically or by default

Participants' attitudes towards changes to eligibility criteria are influenced in part by trust in the organization and processes currently in place that ensure plasma-derived products are protected. Many participants said they trusted Héma-Québec to ensure donations for recipients are safe. Such trust is built around three axes: (1) the belief that the protection of blood products is paramount to a competent organization: 'They have really good expertise. I imagine they have good training and knowledge. I trust them. I find them competent. Nothing makes me think they're not competent or that they make mistakes'. (F, 45 yo, recipient); (2) the perception that the organization would never jeopardize the safety of recipients: 'It isn't advantageous for them to open donations and have us lose out in the end or give us something that isn't safe. So I tell myself that I trust them'. (F, n/a, parent) and (3) the fact that all donations are screened and tested: 'I trust that Héma-Québec will do a quality control check, and they won't give me just anything. They'll run tests'. (F, 45 yo, recipient).

Other participants also reported feeling confident about the changes because of the science and technological advances that ensure donation safety, even with the inclusion of MSM. A few participants indicated they felt sure the fractionation process eliminates impurities, and the tests used on the donations are satisfactory and effective. Some participants mentioned having the impression that MSM do not represent an additional risk when compared with other donors, and that zero risk is simply impossible: 'It is statistically

TABLE 3 Acceptability of the fact that men who have sex with men (MSM) could donate plasma for fractionation.

Variables	Mean ± standard deviation		
	Recipients $n = 10$	Parents $n = 6$	Total $N = 16$
Acceptability that MSM could donate plasma for fractionation			
From 1 'Not at all acceptable' to 10 'Totally acceptable'	9.20 ± 1.62	7.17 ± 3.71	8.44 ± 2.68

impossible that 100% of plasma donors' behaviours have been perfect, whatever those may be'. (M, n/a, recipient).

However, a small number of participants said they felt they had no choice but to trust Héma-Québec because it has a monopoly on the distribution of plasma-derived products: 'Well, I kind of have to trust Héma-Québec. It's not like we have a lot of choice … It's them and that's it. Their mandate is to make sure the blood is safe'. (F, n/a, parent).

'We have no other options': Feelings of concern and helplessness with regard to inclusion of MSM

Although participants' attitudes towards inclusion of MSM were positive overall, some participants expressed feeling concerned and helpless about the situation: 'We have no other options. We're like hostages'. (M, 43 yo, recipient). Some indicated fearing for their safety if exclusion of MSM was lifted; they considered their lives would be at stake since their health depended on plasma-derived products: 'You can't say, 'I'm going to stop the treatment' or 'I'm going to take a different molecule or medication'. I have no other option. There's one manufacturer, one medication and I suffer all the consequences. I have a wife and two kids. If I'm contaminated, it's … This isn't a game'. (M, n/a, recipient). Some recipients voiced fears that inclusion of MSM was motivated by politics rather than science: 'There are concerns that politics regarding Canada's image eclipses science and that they let some things slip'. (F, 44 yo, parent). Some participants reported fearing that MSM are more likely to have sexually transmitted and blood borne infections (STBBI). Other participants said this fear is amplified by being the parent of a child recipient: 'In the situation we're in with our kids, we don't want to add on other stuff. It's clear that if the child is given something, it's not to make them sicker'. (F, 44 yo, parent).

'We want to know what's in those things': Access to information to feel reassured

Many participants stated that one reason behind their concerns, fears and anxieties is a lack of information. Some said the source of their insecurities is a lack of information about the safety of products they will receive: 'I think there's not enough data to determine if we can be sure it's safe'. (F, 36 yo, parent). A few participants reported that lack of information can cause stress and concerns related to changes in eligibility criteria: 'Can you please tell [Héma-Québec] that we're stressed out and all worked up, and that we want to know what's in those things? We want numbers, transparency and openness. Only scientific facts'. (F, n/a, parent). One participant noted that removing a measure such as the exclusion of MSM, put in place to protect donations, could create feelings of mistrust among recipients, who would then question the reasons that lead to changes.

To know if they are at risk, a majority of recipients said they wanted additional information, in particular about the statistics, risks for recipients and Héma-Québec's reasoning behind such a change, so they could clearly understand what it involves. Others indicated a desire to be reassured concerning the safety of products used: 'What I want to be told is this: "We know we have to screen and we do it; the way we do it and what we know today guarantees that what we're giving you is good."' (F, n/a, parent). A few recipients suggested this could be achieved through access to information in more accessible language. Others said they would feel more reassured knowing that the changes would be implemented progressively, which would lessen the perception that Héma-Québec simply wants to enhance their image by being more inclusive.

'Receiving blood products isn't a choice': The importance of safety as the priority

A majority of participants said it is important that products remain safe despite the inclusion of MSM as potential donors. Moreover, despite agreeing with including MSM as new donors, a few of them remarked that they were not ready to increase risks for recipients just to be more inclusive: '[…] but when rights and freedoms can result in destroying a life, the life of someone who has no choice … Giving blood is a choice, whereas receiving blood products isn't a choice. It's essential, a necessity to stay alive. I find that if you weigh the pros and cons, if the risk is much higher, of course I'd be against it'. (M, 43 yo, recipient). One participant spoke about the importance that products remain safe, especially because recipients trust the safety measures currently in place: 'Right now we trust it because the product's been safe for years. Now, there's a change. It's like they're going back on the decision of a ban made years ago. We just have to make sure that it's done well'. (F, 26 yo, parent).

DISCUSSION

The goal of this study was to focus on plasma-derived product recipients' and parents of recipients' acceptability that MSM be eligible to

donate plasma for fractionation. Earlier studies have looked at the attitudes of MSM, donor centre staff and current donors towards changes to plasma donation eligibility criteria. This study fills a gap in understanding the attitudes of plasma product recipients and parents of child recipients. Even though in Québec changes in eligibility requirements for blood and plasma donation have already been implemented, our study results are relevant for countries where exclusion criteria for MSM are still in place. Nonetheless, this study has limitations. First, data do not necessarily reflect this population's opinions now that the changes in eligibility criteria have been implemented. Also, the number of participants in the study could be an issue as regards to representativeness and generalizability of the findings. Attitudes held could have influenced motivation to participate, although our results revealed wide-ranging attitudes. Although efforts were made during the interviews to reduce the effect of social desirability, it could have dissuaded some participants from voicing their fears about the changes. In addition, participants might have already been interested and involved in this issue, since they were recruited through organizations and groups working with recipients. Additionally, the participants' initial level of knowledge about the transfusion system had not been questioned, which could have allowed to provide a more nuanced picture of participants' beliefs. To avoid gaps in knowledge between participants, a presentation was given at the beginning of interviews to present security measures that are in place for plasma-derived products.

In sum, our study results suggest that as a group, recipients' attitudes were rather favourable to including MSM in plasma donation for fractionation. Still, their attitudes were marked by beliefs both favourable and unfavourable to such a change. Most recipients were in favour of including MSM as they perceived this change as necessary and beneficial. However, their attitudes could have been influenced by trust in the processes currently in place. Even so, many recipients had unfavourable attitudes due to their concerns and felt helpless regarding this inclusion. Recipients indicated that having access to more information about its impact on donation safety could reassure them. Despite nuanced attitudes towards acceptability of including MSM, recipients emphasized that safety must be prioritized.

The literature on the various populations involved in or affected by the potential inclusion of MSM in plasma donation for fractionation shows similar results with respect to acceptability. Studies show that MSM wish to be treated equally, to participate in blood and plasma donation and to help others [3–6]. Donor centre staff support eligibility criteria that are inclusive and not stigmatizing or discriminatory and want to foster positive experiences for all donors [7], whereas current donors show high acceptability of including MSM as potential donors [8]. Although studies of donors mostly focus on their perceptions of gender-neutral questionnaires, donors appear to be ready to adapt to new questions that allow for inclusion of MSM [9, 10].

Our findings suggest that acceptability levels of parents of child recipients are lower than those of recipients (average of 9.20/10 for recipients and 7.17/10 for parents). The difference can be explained by the fact that parents in our sample said they did not want an additional stress—that is, increasing the risk for contamination of plasma-derived products—when they were already very worried about their child's diagnosis. For parents, uncertainty regarding treatments, the child's survival and quality of life over the long term is perceived as a threat and a loss of control because the diagnosis affects a loved one rather than themselves [15, 16]. Since treatment frequency is higher for child recipients in our sample, parents perceive the risk that these children come into contact with contaminated plasma-derived products to be greater, which could explain why their level of acceptability of potential changes is lower. Also, studies have shown how making decisions for someone else is a responsibility that triggers emotional responses that cause these individuals to analyse decisions differently. In particular, because parents are looking for the best outcome possible for their child, such a responsibility may trigger reactions like anxiety or worry linked to understanding the risk, underlying differences in decision-making [17, 18]. As parents, there is an emphasis on being good parents, which frames parental perspective on the child's best interest, shapes parental role priorities and influences perceived parental duties [19].

Although the scientific literature in Canada demonstrates that donation safety is not affected by changes in eligibility criteria [20], safety does not appear to be guaranteed for recipients, who spoke of the need for access to concrete scientific information that presents the advantages and disadvantages of this change. Indeed, individuals tend to change or update their beliefs more easily when the evidence is scientific, as well as when the evidence is portrayed as normative [21]. The effects are mediated by individuals' subjective evaluation of the convincingness of the evidence. A more favourable attitude towards acceptability of including MSM as potential donors could be encouraged by altering some of the more unfavourable beliefs (e.g., MSM are at greater risk of STBBI) through access to convincing scientific information [11].

In short, results show that like other populations involved in the donation process, recipients hold both positive and negative beliefs that coexist to form an overall positive attitude towards inclusion of MSM as potential donors. A number of factors, such as the safety procedures in place, can influence acceptability depending not only on the group to which a person belongs but also on some individual factors. Therefore, it can be suggested that, for example, in a country where trust in institutions is low, attitudes would not be as favourable. Because the study was conducted when changes had not been implemented or planned, there is a possibility that some beliefs would now be reinforced and others minimized. Consequently, it could be fitting to look at the beliefs and attitudes of the various actors involved once changes to eligibility criteria have been implemented as well as periodically over time. In addition, further research on levels of knowledge and trust in the public health care system in recipients would be relevant because this could influence their beliefs towards changes to make donations more accessible.

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J.B. conducted focus groups and individual interviews; J.B., J.C. and J.O. analysed the data and wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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ORIGINAL ARTICLE

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Quantification of the contribution of individual coagulation factors to haemostasis using a microchip flow chamber system and reconstituted blood from deficient plasma

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Abstract

Background and Objectives: Quantifying the contribution of individual coagulation factors to haemostasis may aid our understanding of the haemostatic function in patients with rare coagulation deficiencies (RCDs) and the exploration of suitable treatments.

Materials and Methods: Reconstituted blood prepared from specific coagulation factor-deficient plasma (factor [F]II; prothrombin, FV, FVII, FVIII, FIX, FX, FXI or FXII) and red blood cell/platelet products were used to simulate the whole blood of patients with RCD. We prepared in vitro treatment models for patients with prothrombin deficiency using coagulation factor agents and fresh frozen plasma. Haemostatic function was measured using a microchip flow chamber system at 600 s^{-1}.

Results: The haemostatic function was low, especially in blood samples reconstituted with prothrombin- and FX-deficient plasma. In a plasma transfusion model of prothrombin deficiency, haemostatic function recovered after 10% replacement with normal plasma and reached a plateau at ≧60% replacement. A treatment model of prothrombin deficiency with prothrombin complex concentrates revealed dose-dependent therapeutic effects in the range of 0–50 IU/kg.

Conclusion: Microchip flow chamber system-based quantification of haemostatic function using reconstituted blood could predict haemostasis and therapeutic effects of treatments in patients with prothrombin deficiency.

Keywords

coagulation disorders, haemostasis, plasma, prothrombin, prothrombin complex concentrates

Highlights

- Prothrombin was the most important factor in haemostatic function in vitro.
- We showed that the contribution of factor X is second only to prothrombin.
- The total thrombus-formation analysis-based in vitro system can help predict haemostasis and therapeutic effects of treatments in patients with prothrombin deficiency.

INTRODUCTION

Coagulation factors exist in the blood in an inactive form and are converted to the active form when coagulation reaction is initiated, which further activates the next inactive blood coagulation factor, which eventually turns into insoluble fibrin to form a strong haemostatic thrombus (coagulation cascade) [1]. Defective or improper functioning of any of the coagulation factors results in the block of the coagulation cascade and poor haemostasis (rare coagulation deficiency [RCD]). The clinical symptoms include deep bleeding, such as bleeding within the joints and muscles, nosebleeds and skin haemorrhage, and vary according to the deficient coagulation factor. FVIII and FIX deficiencies are known as haemophilia A and B, respectively, and the general prevalence is estimated to be approximately 10 per 100,000 males [2]. Other RCDs have prevalence rates in the general population ranging from 1:500,000 to 1:2,000,000 [3]. RCDs are diagnosed by measuring each coagulation factor after considering prothrombin time and/or activated partial thromboplastin time as coagulation screening tests, and their bleeding symptoms depend on the type and activity of the deficient coagulation factor [4]. However, because the tests use platelet-poor plasma as the sample, they are useful as an indicator of secondary haemostasis but do not reflect platelet function, which is the primary haemostatic factor. Therefore, recently, methods for comprehensive evaluation of primary haemostasis and subsequent secondary haemostasis, not only evaluation of single coagulation factor, have attracted research attention, and when combined with conventional methods, these can provide a deeper understanding of the haemostatic function of individual patients.

PFA-100 (Siemens Healthcare Diagnostics GmbH, Marburg, Germany) is widely used as a system for analysing haemostasis under flow conditions; the capillary coated with collagen/epinephrine or collagen/adenosine diphosphate can evaluate the occlusion time associated with platelets aggregate formation; however, the haemostasis test is less sensitive to secondary haemostasis [5]. Recently, a device called total thrombus-formation analysis system (T-TAS; Fujimori Kogyo, Tokyo, Japan), which comprehensively evaluates primary and secondary haemostasis, has been developed [6]. In this system, $CaCl_2$ and corn trypsin inhibitor (CTI) are added to citrated blood samples to restore the coagulation system. They are injected to a microchip coated with collagen and tissue thromboplastin to promote the adhesion of platelets to collagen and the extrinsic pathway initiated by tissue thromboplastin. As a result, a haemostatic thrombus comprising activated platelets and fibrin can be quantitatively measured under flow conditions (600 or 1500 s^{-1}). We recently established a novel T-TAS-based haemostatic test in which whole blood was reconstituted by three blood components—red blood cell (RBC) products, standard human plasma (SHP; Siemens Healthcare Diagnostic Products GmbH, Marburg, Germany) and platelet products [7]. Our system can quantitatively evaluate the haemostatic function based on the single component using the other two of the three blood components as samples of the same quality origin.

In this study, we aimed to assess whether our new T-TAS test was useful for quantitative analysis in haemostasis of plasma derived from patients with RCDs and those on various therapeutic agents. We therefore tested the haemostatic function of specific coagulation factor-deficient plasma in reconstituted blood and the effect of adding plasma or prothrombin concentrates to mimic the treatment of RCDs.

MATERIALS AND METHODS

Ethics statement

The Institutional Review Board of the Ethics Committee of the Japanese Red Cross Society, Blood Service Headquarters, approved this study (ethical review number: 2019-016).

Thrombus-formation analysis system

We introduced the T-TAS, which enables the measurement of thrombus formation as a numerical value representing the change in flow pressure on a microchip (AR-chip; Fujimori Kogyo) with type I collagen plus tissue thromboplastin coated on the inside of a capillary channel (Figure S1A). Briefly, the citrated blood sample (480 μL) was mixed with 20 μL of 0.3 M $CaCl_2$ containing 1.25 mg/mL of CTI (Fujimori Kogyo) immediately before analysis to restore the coagulation system. Upon adding the test sample onto the microchip, adhesion of platelets to collagen and the coagulation cascade with tissue thromboplastin as the initiation factor is promoted at 37°C under shear conditions (600 s^{-1}), resulting in a thrombus composed of platelets and fibrin. The thrombus gradually grows in size while repeating the collapse and reformation processes and subsequently occludes the flow path within the chip. The test time does not exceed 30 min. A typical analysis pattern and measured parameters are presented in Figure S1B.

Sources of plasma, RBC and platelet samples

A single lot of SHP or coagulation factor-deficient plasma (prothrombin, FV, FVII, FVIII, FIX, FX, FXI or FXII), purchased from Siemens Healthcare Diagnostic Products GmbH (Marburg, Germany), was used as plasma samples. SHP contains 0.86–1.06 IU/mL of coagulation factors and 2.57 g/L of fibrinogen. Coagulation factor-deficient plasma is manufactured by immunoadsorption from normal plasma and contains <1% of the target coagulation factor, >40% of other coagulation factors and >1 g/L of fibrinogen. This composition contains sufficient coagulation-related factors other than the deficient factors necessary for haemostasis. SHP and coagulation factor-deficient plasma were lyophilized human plasma produced with citric acid as an anticoagulant and dissolved in 1 mL water at the time of use.

RBC products were prepared by collecting 400 mL of whole blood using citrate phosphate dextrose solution as an anticoagulant, followed by leukocyte reduction, RBC separated by Terumo

Automated Centrifuge & Separator Integration (Terumo BCT, Lakewood, CO, USA), suspended in 95 mL of mannitol–adenine–phosphate solution, and stored at 4°C for 3 days [8]. The RBC products were washed with equal volumes of phosphate-buffered saline (PBS) followed by centrifugation at 500*g* for 15 min. The resultant RBC pellets were used as RBC samples.

The platelet products were apheresis-derived platelet-rich plasma collected by Trima Accel (Terumo BCT, Lakewood, USA) or component collection system (Haemonetics Co., Boston, USA) using acid–citrate–dextrose solution as an anticoagulant and stored with agitation at 22°C for 2 days. Platelet products were mixed with PBS and acid–citrate–dextrose solution in a ratio of 1:2:0.3 and centrifuged at 800*g* for 5 min. The supernatant was removed, and the remaining pellet was suspended in plasma deficient in the coagulation factor to be evaluated and used as platelet samples. In vitro properties of platelet samples prepared using the same method were separately assessed and demonstrated that platelets were slightly activated by centrifugation but still possessed good aggregability (Table S1).

T-TAS analysis of reconstituted blood deficient in a specific coagulation factor

Reconstituted blood deficient in specific coagulation factors was prepared by combining plasma, RBC and platelet samples. Platelet and RBC samples were prepared from the same products in a series of tests to evaluate the effect of each coagulation factor. The test samples were reconstituted so that the platelet counts and haematocrit (Hct) values, the other two components of the three blood components, were $300 \times 10^3/\mu L$ and 40% constant, respectively, to evaluate the haemostatic function attributable to the plasma component. Ten reconstructed blood samples were prepared from different platelet and RBC samples ($N = 10$). Haemostatic function was analysed using T-TAS. To confirm whether the blood samples reconstituted with RBCs, platelets and prothrombin-deficient plasma were really lacking prothrombin activity with the other coagulation factors retaining their activities, the supernatant of the samples, but not prothrombin-deficient plasma itself, was tested for the concentration of each coagulation factor (Table S2).

T-TAS analysis of the plasma transfusion model for prothrombin deficiency

Reconstituted blood was prepared by adding RBC samples to platelet and plasma samples with adjusted mixing ratios of prothrombin-deficient plasma and SHP. Specifically, the mixing ratio of prothrombin-deficient plasma to SHP was set at seven concentration series of 0%–100% (SHP ratios: 0%, 10%, 20%, 40%, 60%, 80% and 100%). A plasma transfusion model with platelet counts of $300 \times 10^3/\mu L$ and Hct values of 40% was measured using T-TAS ($N = 10$). Prothrombin activity in the test samples prepared using the same method was separately analysed (Table S3).

T-TAS analysis of the prothrombin complex concentrate dosing model for prothrombin deficiency

Reconstituted blood, prepared from prothrombin-deficient plasma, platelet samples and RBC samples, was used as models of prothrombin deficiency. Kcentra (intravenous injection, 500 units; CSL Behring K.K., Tokyo, Japan) was used as the prothrombin complex concentrate (PCC) containing 500 IU/vial of prothrombin, FVII, FIX and FX [9]. The product was prepared by lyophilization and dissolved in 20 mL of water at the time of use. PCCs were added to the reconstituted blood at 0, 5 and 25 IU/kg based on 50 IU/kg, which is the dose when the prothrombin time-international normalized ratio surpasses 6. PCC dosing models with platelet counts of $300 \times 10^3/\mu L$ and Hct values of 40% were assessed using T-TAS ($N = 10$). Prothrombin activity in the test samples prepared using the same method was separately examined (Table S4).

Statistical analyses

Statistical analyses were performed using Prism 8 (Graph Pad, Inc., La Jolla, USA). Differences between the groups were analysed using one-way analysis of variance when data were assumed to be normally distributed. To compare the haemostatic function of reconstituted blood from SHP and coagulation factor-deficient plasma, the Tukey–Kramer multiple comparison test was performed for post hoc analysis. To compare the treatment effects in the patient model with prothrombin deficiency, Dunnett's multiple comparison post hoc test was performed for PCC dosing at 50 IU/kg dose and other conditions, as well as for plasma transfusion at 100% SHP and other conditions. Statistical significance was set at p values <0.05. When samples were over the limit of detection, the numbers were compared.

RESULTS

T-TAS showed reduction in haemostatic function in reconstituted blood from coagulation factor-deficient plasma

The relationships between reconstituted blood deficient in specific coagulation factors and haemostatic function are shown in Figure 1. In addition, a representative result of this study showing the relationship between the time to occlusion and pressure curve is shown in Figure 2. The test samples reconstituted with SHP showed the highest haemostatic function. In all test samples without prothrombin, time to 80 kPa (T_{80}) exceeded the detection limit of 30 min despite the presence of sufficient amounts of platelets, RBCs and the other coagulation factors. In the test samples reconstituted with FX-deficient plasma, T_{80} exceeded the detection limit of 30 min in 3 of 10 samples. Test samples reconstituted without FV showed significantly higher area under the curve for 30 min (AUC_{30}) than those reconstituted without FXI, FIX, FVIII, FX or prothrombin. In the test samples reconstituted without FXII, FXI, FIX, FVIII or FVII, no significant differences were detected in the AUC_{30} between the groups.

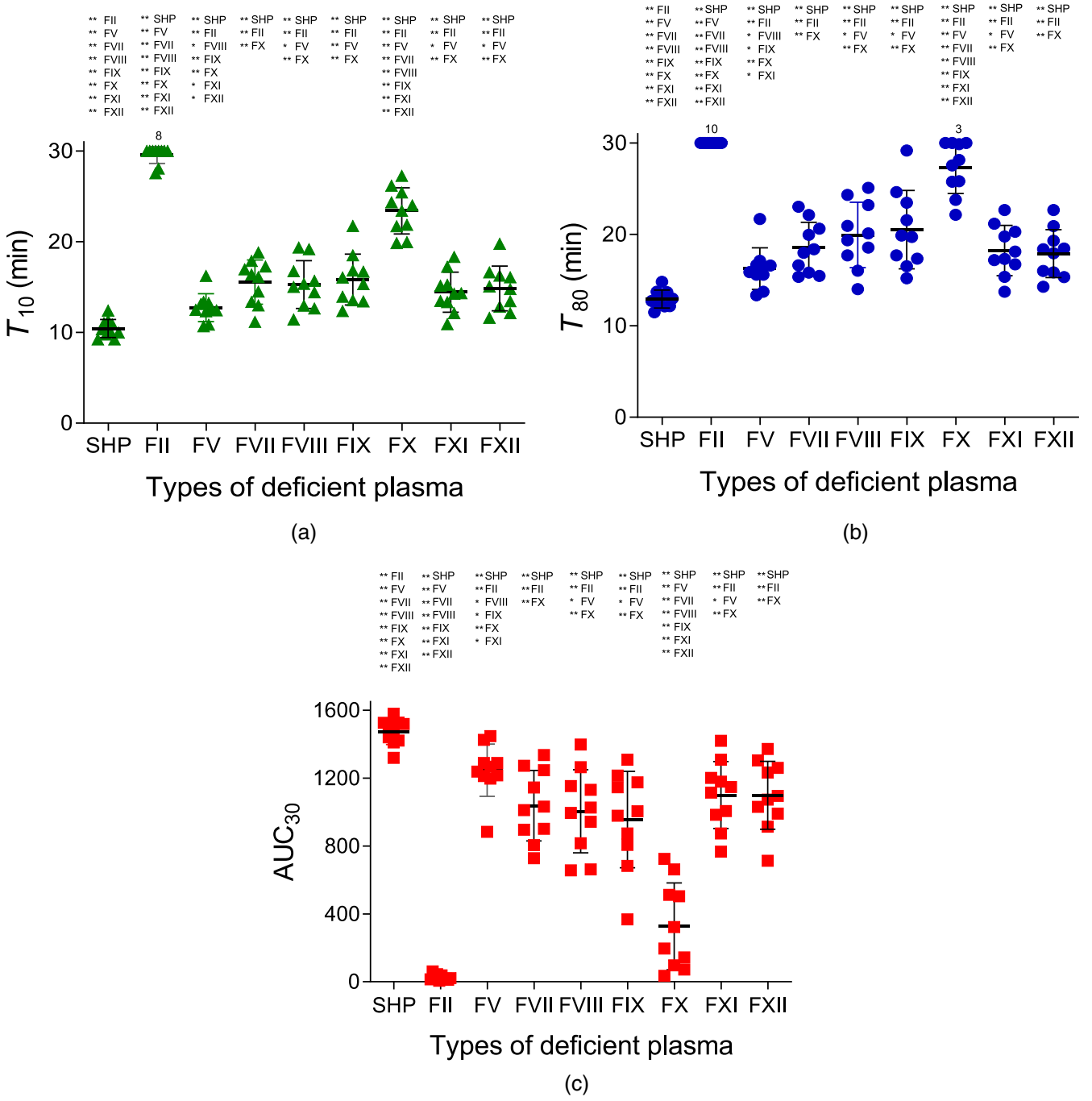

FIGURE 1 Haemostatic function of reconstituted blood that is deficient in a specific coagulation factor. The relationships between reconstituted blood from deficient plasma and each parameter, T_{10} (time to 10 kPa), T_{80} (time to 80 kPa) and AUC_{30} (area under the curve for 30 min), are presented in (a), (b) and (c), respectively ($N = 10$). Numbers above the dot plots indicate the number of samples exceeding the 30-min measurement range. Significance was set at $^{*}p < 0.05$ (one-way analysis of variance [ANOVA], Tukey–Kramer post-hoc test) and $^{**}p < 0.01$ (one-way ANOVA, Tukey–Kramer post hoc test). When samples were over the limit of detection, the numbers were compared ($^{**}p < 0.01$). SHP, standard human plasma.

T-TAS is sensitive to replacement with normal plasma in a model for treatment of prothrombin deficiency

The relationships between the SHP to prothrombin-deficient plasma ratio in reconstituted blood plasma samples and haemostatic function are shown in Figure 3. In all test samples without prothrombin (0% SHP), T_{80} exceeded the detection limit of 30 min, while the haemostatic function was observed in all samples with ≥10% SHP. As the SHP to prothrombin-deficient plasma ratios in reconstituted blood plasma samples increased to 10%, 40% and 100%, T_{10} (time in minutes for the flow pressure to reach 10 kPa) shortened to 14.4 ± 2.6, 13.3 ± 2.0 (0.92-fold) and 11.9 ± 1.5 min (0.83-fold),

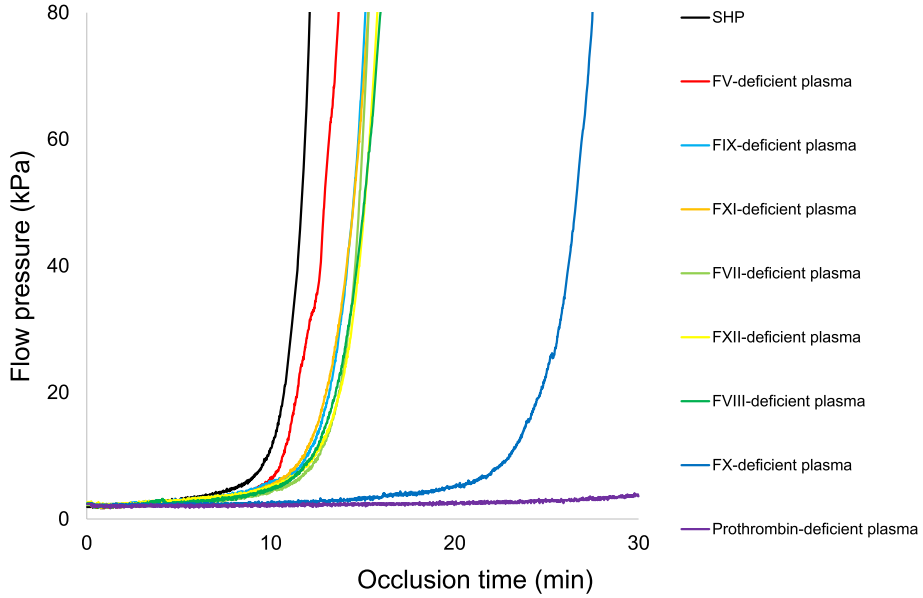

FIGURE 2 Representative data (sample no. 8). Representative time-dependent pressure changes of reconstituted blood using standard human plasma (SHP) (black line), plasma deficient in factor (F) V (red line), plasma deficient in FIX (light blue line), plasma deficient in FXI (orange line), plasma deficient in FVII (light green line), plasma deficient in FXII (yellow line), plasma deficient in FVIII (green line), plasma deficient in FX (blue line) and plasma deficient in prothrombin (purple line).

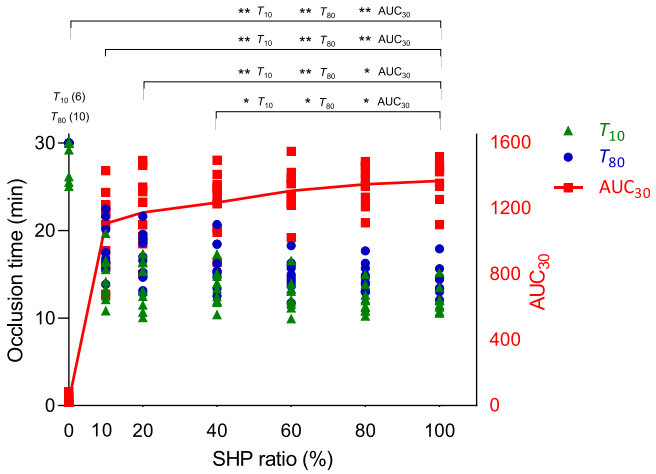

FIGURE 3 Relationship between plasma transfusion and haemostatic function in a model of prothrombin deficiency. Plasma transfusion models were measured using the total thrombus-formation analysis system ($N = 10$). Numbers above the dot plots indicate the number of samples exceeding the 30-min measurement range. Significance was set at $^{*}p < 0.05$ (vs. standard human plasma [SHP] ratio 100%) and $^{**}p < 0.01$ (vs. SHP ratio 100%). When samples were over the limit of detection, the numbers were compared ($^{**}p < 0.01$). AUC_{30}, area under the curve for 30 min; T_{10}, time to 10 kPa; T_{80}, time to 80 kPa.

respectively; T_{80} (time in min for the flow pressure to reach 80 kPa) shortened to 17.7 ± 2.8, 16.0 ± 2.5 (0.90-fold) and 14.2 ± 1.6 min (0.80-fold), respectively; and consequently, the AUC_{30} values increased to 1107 ± 225, 1235 ± 185 (1.12-fold) and 1366 ± 121 (1.23-fold), respectively. No significant difference was observed in AUC_{30} when the ratio of SHP to prothrombin-deficient plasma in reconstituted blood plasma samples was 60%–100%.

PCC administration model for prothrombin deficiency restored the haemostatic function in a dose-dependent manner

The relationships between the administration of PCCs to prothrombin-deficient plasma in reconstituted blood and haemostatic function are shown in Figure 4. In all test samples without prothrombin (PCC dose 0 IU/kg), T_{80} exceeded the detection limit of 30 min, while the haemostatic function was observed in all samples with a PCC dose of ≥5 IU/kg. As the PCC dose to prothrombin-deficient plasma in the reconstituted blood was increased to 5, 25 and 50 IU/kg, T_{10} shortened to 13.9 ± 2.2, 12.2 ± 2.4 (0.88-fold) and 11.4 ± 2.1 min (0.82-fold), respectively; T_{80} shortened to 17.6 ± 2.9, 15.5 ± 2.8 (0.88-fold) and 14.3 ± 2.4 min (0.81-fold), respectively; and consequently, the AUC_{30} values increased to 1141 ± 206, 1280 ± 221 (1.12-fold) and 1375 ± 177 (1.21-fold), respectively.

DISCUSSION

In this study, we believed that the results of the T-TAS-based in vitro test would mimic the in vivo haemostatic function of patients with RCD who did or did not receive coagulation factor treatments, and consequently, this would help optimize real-world therapies for RCD. Production of even a small amount of thrombin from prothrombin feeds back to the formation of Xase, and prothrombinase complexes

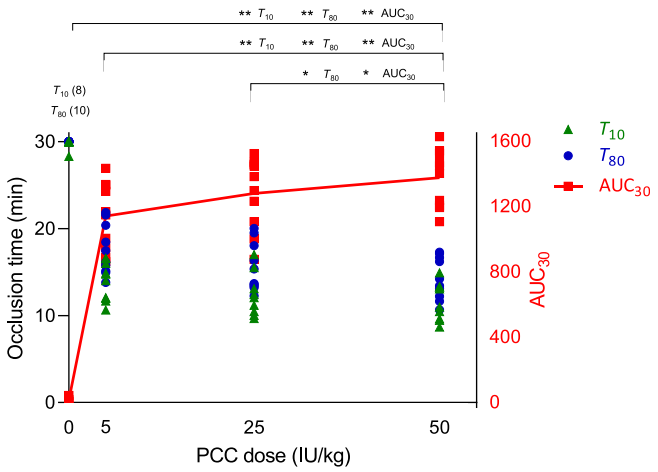

FIGURE 4 Relationship between prothrombin complex concentrate dosage and haemostatic function in a model of prothrombin deficiency. Prothrombin complex concentrate dosing models were measured using the total thrombus-formation analysis system ($N = 10$). Numbers above the dot plots indicate the number of samples exceeding the 30-min measurement range. Significance was set at $^{*}p < 0.05$ (vs. prothrombin complex concentrate [PCC] dose of 50 IU/kg) and $^{**}p < 0.01$ (vs. PCC dose of 50 IU/kg). When samples were over the limit of detection, the numbers were compared ($^{**}p < 0.01$). AUC_{30}, area under the curve for 30 min; T_{10}, time to 10 kPa; T_{80}, time to 80 kPa.

are repeated instantly, resulting in the production of enormous amounts of thrombin (thrombin burst) [10, 11]. Peyvandi et al. reported that there had been no living patient with undetectable plasma prothrombin, consistent with the evidence in mice that complete prothrombin deficiency obtained by gene knockout was incompatible with life [12]. Our system also demonstrated that prothrombin is the most important factor in haemostatic function in vitro (Figure 1c). FX is activated by intrinsic or extrinsic Xase to generate FXa, which initiates the common pathway [13]. FXa forms a prothrombinase complex with FVa, a cofactor, to play a major role in immediate thrombin generation [14]. We showed that the contribution of FX is second only to prothrombin (Figure 1c). In contrast to other coagulation factors, FV has no enzymatic activity and functions as a cofactor for FXa. The most common bleeding phenomenon in FV deficiency is mucosal haemorrhage, which is often mild and rarely life-threatening [15]. In our study, the effect of FV deficiency on haemostatic function was the smallest compared with that of other coagulation factors (Figure 1c). Conversely, there was no difference in haemostatic function between samples mimicking FVIII and FIX deficiency, in which the activity value is <1%, and the bleeding is classified as severe, and FXI and FXII deficiency, which show almost no bleeding tendency (Figure 1c) [16, 17]. Ogawa et al. assessed the contribution of FVIII and FIX to haemostatic function at two different shear rates and reported that at a high shear rate (1100 s^{-1}), the relative platelet contribution is higher, and at a low shear rate (110 s^{-1}), the contribution of FVIII and FIX to haemostatic function is increased [18]. As the T-TAS has arterial blood flow conditions (600 s^{-1}), it is assumed that the contribution of FVIII and FIX deficiencies to haemostatic function was reduced in a shear stress-dependent manner and was not different from that of FXII and FXI deficiencies.

Patients with RCD are treated with replacement therapy of fresh frozen plasma or coagulation factor products to correct deficient coagulation factor activity [19]. Plasma prothrombin activity clinically reaches haemostatic levels at approximately 0.2 IU/mL, and haemostatic function was observed at similarly low prothrombin activities when assessed using T-TAS-based in vitro tests, regardless of the pharmaceutical dosage form (10% SHP: 0.09 ± 0.00 IU/mL, 5 IU/kg PCC: 0.09 ± 0.01 IU/mL) (Figures 3 and 4) [3]. The plasma prothrombin activity, at which haemorrhagic symptoms rarely occur clinically, was approximately 0.5 IU/mL, similar to the level at which haemostatic function reached a plateau in our system (≧60% SHP: 0.47 ± 0.02 IU/mL) (Figure 3). These results suggest that our system can mimic physiologically haemostatic function.

Our study has two limitations. First, the reconstituted blood contains few leukocytes, and it does not reflect coagulation-promoting events such as neutrophil extracellular traps [20]. Second, FI, fibrinogen, and FXIII, fibrin stabilizing factor, are important factors that work downstream in the coagulation cascade, apart from the coagulation factors evaluated in our study [21, 22]. However, fibrinogen- and FXIII-deficient plasma derived from dried plasma, using the same preparation method as other deficient plasma, could not be obtained; therefore, their contribution to haemostatic function could not be quantified. Thus, due to some drawbacks, our study did not necessarily match haemostatic function in all patients with RCD in the real world. However, the T-TAS-based in vitro test, which quantified the haemostatic function of mimicked patients with RCD in reconstituted blood that relies only on plasma factors and not on platelets or RBCs, could mimic the in vivo haemostatic function of patients with RCD who did or did not receive coagulation factor treatments, and when combined with conventional coagulation tests, the test could provide a deeper understanding of the haemostatic function of individual patients. In the future, the new system is expected to be used as an in vitro bedside test that can rapidly determine the haemostatic function of patients with RCD and predict the effects of therapeutic agents.

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A.F. performed the research and wrote the first draft of the manuscript; A.F., K.Y., T.H. and K.S. designed the research study; C.O., T.O.-W. and K.H. participated in T-TAS analysis; Y.F., T.K., F.H. and Y.T. supervised the research and reviewed and edited the manuscript; all authors discussed the results and commented on the manuscript and approved the publication of the manuscript.

CONFLICT OF INTEREST STATEMENT

The total thrombus-formation analysis system is a product of Fujimori Kogyo Co., Ltd., where C.O., T.O.-W. and K.H. are employed.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

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Is it useful to wash stored red blood cells in cardiopulmonary bypass priming fluid for neonatal cardiac surgery? A single-centre retrospective study

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Abstract

Background and Objectives: Neonatal cardiac surgery requires careful consideration of cardiopulmonary bypass (CPB) priming fluid composition due to small blood volume and immature physiology. This study investigated the impact of allogeneic stored red blood cells (RBCs) processed using an autotransfusion system in CPB priming fluid for neonates.

Materials and Methods: We compared perioperative parameters, inflammatory mediators, coagulation indicators, vasoactive-inotropic score (VIS) and clinical outcomes between neonates receiving unwashed ($n = 56$) and washed ($n = 45$) RBCs in CPB priming fluid. Regression models were used to assess the independent association between RBC washing and patient outcomes.

Results: The autotransfusion system improved stored RBC quality. The washed group showed higher peak haematocrit ($p < 0.01$) and haemoglobin levels ($p = 0.04$) during CPB, an increased oxygen delivery index during rewarming ($p < 0.05$) and lower postoperative lactate levels and VIS ($p < 0.05$). Inflammatory (IL-6, IL-8 and IL-10) and coagulation parameters (D-dimer, fibrinogen and fibrin degradation product) fluctuated compared with baseline but did not significantly differ between groups. The washed group had a lower incidence of hyperlactacidaemia and delayed sternal closure at CPB weaning.

Conclusions: Adding washed allogeneic stored RBCs to neonatal CPB priming fluid reduced postoperative lactate elevation and VIS without early improvement in the inflammatory and coagulation systems.

Keywords

blood washing technique, cardiac surgery, cardiopulmonary bypass, neonate, red blood cell storage

He Wang and Yu Jin contributed equally to this study.

Highlights

- Improved blood quality was seen in the washed red blood cell (RBC) group.
- The washed RBC group had lower vasoactive-inotropic score and reduced lactate levels and complications.
- Adding washed allogeneic stored RBCs to cardiopulmonary bypass priming fluid had limited impact on inflammatory and coagulation systems.

INTRODUCTION

The minimal priming volume of current cardiopulmonary bypass (CPB) circuits in neonates approaches or exceeds their circulating blood volume (approximately 80–85 mL/kg). Excessive priming dilutes blood, lowers colloid osmotic pressure (COP) below target levels and may lead to coagulation disorders [1]. To maintain an adequate COP, CPB specialists often prefill CPB circuits with red blood cells (RBCs) during neonatal cardiac surgery. This aims to stabilize the COP and microcirculatory perfusion, and improve tissue oxygenation via increased haemoglobin (Hb) content [2].

Due to limited blood resources, neonates often receive allogeneic stored RBCs during CPB. However, storage duration alters RBC composition, leading to increased potassium and lactate levels, decreased pH and bicarbonate levels [3], and potentially inducing metabolic imbalances and electrolyte disturbances in recipients [1]. Furthermore, stored RBCs exhibit diminished energy reserves and compromised membrane integrity [4], which can exacerbate the inflammatory response during CPB, contribute to postoperative organ dysfunction [5, 6], prolong hospitalization and increase postoperative complications [7]. Therefore, minimizing the adverse effects of stored blood on neonates is a critical concern for doctors.

RBC washing technology has been shown to reduce the levels of lactate, potassium, glucose and other metabolites in stored RBCs [8]. This technique improves RBC deformability [9], mitigates storage-induced damage [10], decreases inflammatory mediators [11], and increases Adenosine Triphosphate content [12]. Despite these benefits, its application in neonatal CPB priming fluids remains uncommon. Furthermore, no studies have assessed the impact of adding washed RBCs to CPB priming fluid on post-cardiac surgery circulatory function, inflammatory response or coagulation parameters in patients. This study aimed to investigate whether CPB priming with washed RBCs improves outcomes in neonates undergoing cardiac surgery by evaluating perioperative laboratory and clinical parameters.

MATERIALS AND METHODS

Patients

This study followed the Declaration of Helsinki (2013) and was approved by Fuwai Hospital's ethics board (No: 2022-1830). All the participants' parents/legal guardians provided written informed consent. Neonates undergoing elective cardiac CPB surgery at Fuwai Hospital between September 2020 and November 2023 were included ($N = 101$) and divided into washed ($N = 45$) and unwashed ($N = 56$) groups based on RBC treatment. Inclusion criteria were elective cardiac CPB surgery, age ≤28 days and CPB duration of 1–3 h. The exclusion criteria were previous cardiac surgery, prematurity, severe preoperative complications, deep hypothermic circulatory arrest during CPB and preoperative glucocorticoid use.

Cardiopulmonary bypass technique

All children underwent CPB using a roller pump (Stockert S5, Sorin, Italy). The CPB circuit consisted of an oxygenator (Fx05, Terumo, Japan), an ultrafiltration device (Maquet BC20, Hirlingen) and neonatal tubing packages (1/4 inch pump tubing, Tianjin, China). The circuit was primed with compound electrolyte injection and then drained. The priming fluid contained processed/unprocessed allogeneic stored RBCs (30–50 mL/kg, depending on preoperative haematocrit [Hct]), 1000 U unfractionated heparin, 25% magnesium sulphate (0.6 mL/kg), 5% carbonic acid, 10–15-mL sodium bicarbonate, 50-mL 20% albumin and 5-mg furosemide. The total prefill volume was approximately 250–350 mL.

All patients received 1 U (200 mL) of stored RBCs from a hospital blood bank. The number of RBCs added to the priming fluid was calculated based on the individual differences in the patients to maintain the predicted Hct during CPB at >25%. The remaining RBCs were infused intermittently during the CPB. All patients received 1 U of RBCs before weaning from CPB. Anticoagulation treatment with 400 U/kg heparin was administered before CPB transfer, which was initiated once activated clotting time (ACT+) (HEMOCHRON Jr. Signature+, USA) exceeded 410 s. All patients underwent modified ultrafiltration after weaning from CPB. The heparin was neutralized with protamine (10 IU/kg) after CPB.

Priming fluid products

In the washed group, stored RBCs were processed using the Sorin Xtra® Autotransfusion System (ATS) (Italy) before CPB prefill. This process employed a 55 mL washing cup (X55) and washed the RBCs with 25 IU/mL heparin-0.9% sodium chloride solution in Popt mode for approximately 5 min. Blood samples (3 mL) were drawn for pre- and post-wash analysis of blood gases and free Hb levels using the HemoCue® Plasma/Low Hb Photometer, HemoCue AB (Ängelholm,

Sweden) analyser. Both groups utilized cell salvage devices for intraoperative blood recovery.

Definition of outcome indicators

The primary outcome measures were changes in the patients' internal environment, circulatory system and inflammatory mediators during the perioperative period.

The secondary outcome measures were perioperative blood laboratory and coagulation parameters, oxygen delivery index (DO_2i), vasoactive-inotropic score (VIS), hyperlactatemia [13], delayed chest closure and in-hospital mortality.

Outcome definitions

Hyperkalaemia [14] and hyperglycaemia [15] were defined as the relevant neonatal criteria. AKI was defined using the Kidney Disease Improving Global Outcomes diagnostic criteria [16] using elevated creatinine as a surrogate marker within 7 days of surgery. DO_2i ($mL/min/m^2$): (CI $[L/min/m^2] \times [1.36 \times Hb \{g/L\} \times SaO_2 \{\%\} + 0.031 \times PaO_2 \{mmHg\}]$). VIS was defined according to the methodology described by Gaies et al. [17]. Coagulation indexes and inflammatory mediators were examined on the day before the operation and upon the patients' admission to the Pediatric Intensive Care Unit (PICU) postoperatively (usually within 2–4 h after the CPB). D-dimer (DD): <0.5 μg/mL; fibrinogen (FIB): 2.0–4.0 g/L; fibrin degradation products (FDP): 0–5 μg/mL; thrombin time (TT): 14–21 s. Fluid balance was defined as total fluid intake minus fluid output. Delayed chest closure was defined as failure to achieve routine chest closure in the operating room after surgery. Postoperative infection was confirmed via bacterial/fungal cultures or viral nucleic acid testing within 30 days of surgery. Blood samples were collected from all patients within 6 h before surgery to establish baseline data. Samples were taken at 4 h after the weaning from CPB.

Blood products management and usage in this study

According to the blood products management regulations in our region, all blood products are collected, prepared, and distributed to medical institutions by the city's blood bank. Our centre's blood bank is solely responsible for storing and dispensing these blood products. In this study, only RBCs in additive solution (citrate–phosphate-dextrose-adenine [CPDA-1]) were used for neonatal patients.

Per national quality standards, the volume of RBCs-AS must reach the labelled volume (mL) ±10%, and the Hct should be between 0.50 and 0.65. Each unit of RBCs-AS is derived from 200 mL of whole blood and contains ≥20 g of Hb. The storage temperature for RBCs-AS is maintained at 2–6°C. The additive solution used in our centre is CPDA-1, and the RBCs stored in this solution have a shelf life of 35 days from the date of collection.

Statistical analysis

Quantitative data were presented as medians and interquartile ranges, whereas qualitative data were reported as frequencies and percentages. The *t*-test or Mann–Whitney U test was used for continuous variables, and the Chi-square or Fisher's exact test was used for categorical variables. Logistic regression analysis was applied for categorical outcome variables, and multivariate linear regression was applied for continuous outcomes, considering clinically relevant baseline variables or those showing univariate relationships with the outcomes. The variables for inclusion were carefully chosen for parsimony and accuracy. Analyses were conducted using SPSS 26.0 (SPSS, Inc., Chicago, IL, USA), with statistical significance set at $p < 0.05$. The data were visualized using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). The sample size was estimated using PASS software 2020 (NCSS LLC., Kaysville, UT, USA).

RESULTS

Characteristics of the patients

We enrolled 114 neonates, 13 of whom were subsequently excluded based on the exclusion criteria. The remaining 101 patients who completed the study and were included in the final analysis were divided into washed ($n = 45$) and unwashed ($n = 56$) groups. Apart from weight (3.30 vs. 3.00 kg, $p < 0.05$), baseline demographic characteristics showed no significant differences between groups. Similarly, preoperative blood tests, except for total protein (46.40 vs. 49.60 g/L, $p = 0.02$), did not differ significantly (Table 1). Further details of the primary surgical procedures and patient distribution are shown in Table S1.

Evaluation of Sorin Xtra® ATS on priming RBCs

This study evaluated the effect of an autologous blood salvage system on the preprocessing of stored RBCs in our hospital. Twenty consecutive units (200 mL each) of stored RBCs were analysed. Haematological parameters were measured before and after washing using the same blood units.

Table 2 shows that compared to pre-washing values, the washing process significantly reduced the volume of stored RBCs. Additionally, washing significantly lowered levels of PCO_2, BE value, HCO_3^-, lactate, blood glucose, and serum potassium (all $p < 0.001$). Conversely, washing significantly increased Hb, Hct, PO_2 and pH values. Notably, no significant difference was observed in free Hb levels before and after washing.

Intraoperative data and clinical outcomes

Table 3 demonstrates significant reductions in intraoperative sodium bicarbonate usage (both additional and total) in the washed group

TABLE 1 Patients' characteristics and preoperative laboratory values.

Variable	Unwashed RBCs group ($N = 56$)	Washed RBCs group ($N = 45$)	p value
Demographic feature			
Ages (day)	15.50 (9.00,22.75)	10.00 (6.50,16.00)	0.06
Weight (kg)	3.30 (3.0,3.70)	3.00 (2.70,3.50)	<0.05
BSA (m^2)	0.20 (0.18,0.21)	0.19 (0.17,0.20)	0.10
Male (%)	37 (66.1)	30 (66.7)	0.95
STAT category	4.00 (4.00,4.00)	4.00 (4.00,4.00)	0.10
Preoperative laboratory values			
Hct (%)	37.15 (31.78,42.83)	38.20 (32.40,41.95)	0.99
Hb (g/L)	134.00 (113.25,152.75)	133.00 (115.50,149.00)	0.97
RBC (10^{12}/L)	3.26 (2.95,3.59)	3.23 (3.06,3.84)	0.87
WBC (10^9/L)	9.27 (7.00,13.13)	9.98 (8.23, 12.40)	0.43
ALB (g/L)	32.80 (29.78,35.18)	31.50 (28.40,34.20)	0.09
Total Protein (g/L)	49.60 (45.82,52.35)	46.40 (42.25,51.40)	0.02
Creatinine (mg/dL)	30.33 (19.13,44.20)	38.05 (28.40,54.62)	0.09
Lactate (mmol/L)	1.10 (0.80, 1.50)	1.00 (0.73, 1.20)	0.22
Glucose (mmol/L)	4.90 (4.31,5.84)	5.02 (4.39,6.15)	0.79
K (mmol/L)	3.83 (3.58, 4.15)	3.89 (3.53, 4.21)	0.81
Na (mmol/L)	138.00 (136.50,139.65)	137.30 (135.60,139.05)	0.46
Hs-CRP (mg/L)	0.41 (0.15,1.27)	0.86 (0.22,1.87)	0.27
TBil (mg/dL)	104.87 (66.57,153.30)	128.13 (82.59,163.35)	0.53

Note: Values are expressed as *n* (%) or medians and interquartile ranges.
Abbreviations: ALB, albumin; BSA, body surface area; Hb, haemoglobin; Hct, haematocrit; Hs-CRP, hypersensitive C-reactive protein; K, potassium; Na, sodium; RBC, red blood cell; STAT, Society of Thoracic Surgeons-European Association for Cardio-Thoracic Surgery; TBil, total bilirubin; WBC, white blood cell.

TABLE 2 Comparison of RBCs before and after washing.

Variable	Before washing	After washing	p value
RBC volume (mL)	222.50 (216.25,230.00)	195.50 (180.25,205.00)	0.001
Hb (g/L)	127.50 (121.25,132.75)	139.50 (129.75,142.75)	0.019
Hct (%)	41.00 (39.00,42.75)	48.50 (41.50,51.50)	0.005
PH	6.57 (6.53,6.72)	6.70 (6.62,6.75)	0.004
PCO_2 (mmHg)	126.40 (124.56,130.95)	15.25 (13.68,16.25)	<0.001
PO_2 (mmHg)	51.05 (41.28,57.90)	72.95 (60.18,77.08)	<0.001
Lactate (mmol/L)	14.55 (13.53,16.05)	4.20 (2.95,5.83)	<0.001
Glucose (mmol/L)	22.32 (21.44,23.47)	3.45 (2.96,5.39)	<0.001
Na (mmol/L)	106.60 (104.63,112.30)	142.2 (140.53,143.68)	<0.001
K (mmol/L)	20.65 (16.88,23.08)	1.26 (0.95,1.94)	<0.001
BE (mmol/L)	−27.60 (−30.00,−25.08)	−33.70 (−35.98,−32.85)	<0.001
HCO_3^- (mmol/L)	11.60 (10.325,12.575)	1.80 (1.60,2.20)	<0.001
pFHb (mg/dL)	55.00 (42.40,68.60)	55.00 (45.40,79.60)	0.432

Note: Values are expressed as medians and interquartile ranges.
Abbreviations: BE, base excess; BUN, blood urea nitrogen; Hb, haemoglobin; HCO_3^-, carbonic acid hydrogen radical; Hct, haematocrit; K, potassium; Na, sodium; PCO_2, partial pressure of carbon dioxide; pFHb, plasma free haemoglobin; PH, potential of hydrogen; PO_2, partial pressure of oxygen; RBC, red blood cell.

TABLE 3 Intraoperative data.

Variables	Unwashed group ($N = 56$)	Washed group ($N = 45$)	p value
RBC storage time (day)	20.50 (17.25,23.75)	19.00 (15.00,23.50)	0.39
CPB prime volume (mL)	315.00 (270.00,340.00)	290.00 (252.50,322.50)	0.08
CPB time (min)	125.50 (101.00,161.75)	136.00 (119.50,152.00)	0.86
MHCA (%)	18 (32)	12 (27)	0.55
MHCA duration (min)	32.00 (27.00,37.25)	32.50 (28.50,36.00)	0.95
Aorta cross clamp time (min)	79.00 (59.00,106.00)	78.00 (68.50,91.00)	0.48
Lowest temperature CPB (°C)	29.75 (28.38,31.00)	29.45 (28.08,30.20)	0.21
RBCs during CPB (mL/m^2)	1002.51 (943.42,1176.47)	1039.81 (920.31,1130.05)	0.88
5% $NaHco_2$ in the prime (mL)	10.00 (5.00,12.50)	10.00 (1.25,15.00)	0.95
Additional amount of 5% $NaHco_2$ (mL)	20.00 (10.00,30.00)	10.00 (5.00,20.00)	<0.01
Total 5% $NaHco_2$ (mL)	28.00 (20.00,41.50)	21.50 (15.00,29.50)	0.02
Peak Hb (g/L)	91.00 (84.00,104.50)	100.00 (91.00,106.00)	0.04
Peak Hct (%)	27.00 (25.00,30.00)	30.00 (28.50,32.00)	<0.01
Hypothermic nadir DO_2i (mL/min/m^2)	213.56 (184.57,245.45)	211.38 (192.66,246.48)	0.76
Rewarming nadir DO_2i (mL/min/m^2)	244.00 (201.42,317.56)	265.86 (240.51,309.80)	0.04
CUF volume (mL)	200.00 (150.00,300.00)	300.00 (200.00,400.00)	<0.01
MUF volume (mL)	100.00 (100.00,100.00)	100.00 (100.00,100.00)	0.13
Total ultrafiltration volume (mL)	450.00 (350.00,550.00)	600.00 (495.00,800.00)	<0.001
Fluid balance (mL)	387.38 (282.75,511.25)	441.00 (299.50,629.50)	0.34
Operative time (min)	230.00 (193.50,178.75)	237.50 (214.50,268.00)	0.40
Used glucocorticoids (%)	33 (59)	29 (64)	0.57

Note: Values are expressed as *n* (%) or medians and interquartile ranges.
Abbreviations: CPB, cardiopulmonary bypass; CUF, conventional ultrafiltration; DO_2i, indexed oxygen delivery; Hb, haemoglobin; Hct, haematocrit; MHCA, moderate hypothermic circulatory arrest; MUF, modified ultrafiltration; RBC, red blood cell.

compared to the unwashed group ($p < 0.05$). While no significant difference was observed in total RBCs transfused or maximum flow rate, peak Hct and peak Hb (100.00 vs. 91.00 g/L, $p = 0.04$) were elevated in the washed group. During the CPB decreasing temperature period, the DO_2i showed no significant difference between the groups. However, during rewarming, the DO_2i of the washed group was significantly higher ($p < 0.05$). Notably, the washed group exhibited higher conventional and total ultrafiltration volumes than the unwashed group ($p < 0.01$). While lactate levels and peak VIS remained similar between groups throughout CPB, the washed group demonstrated significantly lower lactate values at the end of CPB (2.30 vs. 3.15 mmol/L, $p < 0.01$) and upon transfer to the PICU (2.30 vs. 3.50 mmol/L, $p < 0.05$). The washed group also displayed lower VIS scores, both upon admission to the PICU (14.00 vs. 16.00, $p < 0.05$) and 6 h later (11.00 vs. 14.00, $p = 0.001$), as shown in Figure 1. Additionally, the incidence of hyperlactatemia at the end of CPB was significantly lower in the washed group ($p = 0.04$) (Table 4).

The rate of delayed chest closure was significantly lower in the washed group (16% vs. 2%, $p = 0.02$). There were no significant differences in the other adverse prognostic outcomes (Table 4).

Table S2 shows the multivariate linear regression results assessing the effect of washed priming RBCs on neonatal perioperative outcomes. After adjusting for confounding factors (age, body surface area, CPB time, hypothermic circulatory arrest, RBC storage time and intraoperative fluid balance), statistically significant associations were found. Washed RBCs in priming fluid correlated with decreased $NaHCO_3$ usage during CPB ($\beta = -0.23$, $p = 0.03$), increased peak Hct ($\beta = 0.36$, $p < 0.001$), elevated DO_2i during rewarming ($\beta = 0.33$, $p < 0.01$) and higher conventional ultrafiltration (CUF) volume ($\beta = 0.31$, $p < 0.01$). Post-CPB ($\beta = -0.31$, $p = 0.01$) and PICU admission ($\beta = -0.26$, $p < 0.001$) and washed RBCs were associated with reduced lactate levels. Additionally, washed RBCs correlated with decreased VIS scores ($\beta = -0.28$, $p = 0.01$) in the early postoperative period.

Inflammatory and coagulation markers

Twelve inflammatory mediators and nine coagulation indicators were measured in both groups before surgery and upon postoperative admission to the PICU. Data were summarized and analysed within each group, with significant differences between the groups highlighted in Figures 2 and 3.

Preoperatively, only Interferon-γ (IFN-γ) differed significantly between groups (2.22 vs. 1.45 pg/mL, $p < 0.01$). Postoperatively, IL-6, IL-8 and IL-10 levels increased significantly in both groups compared

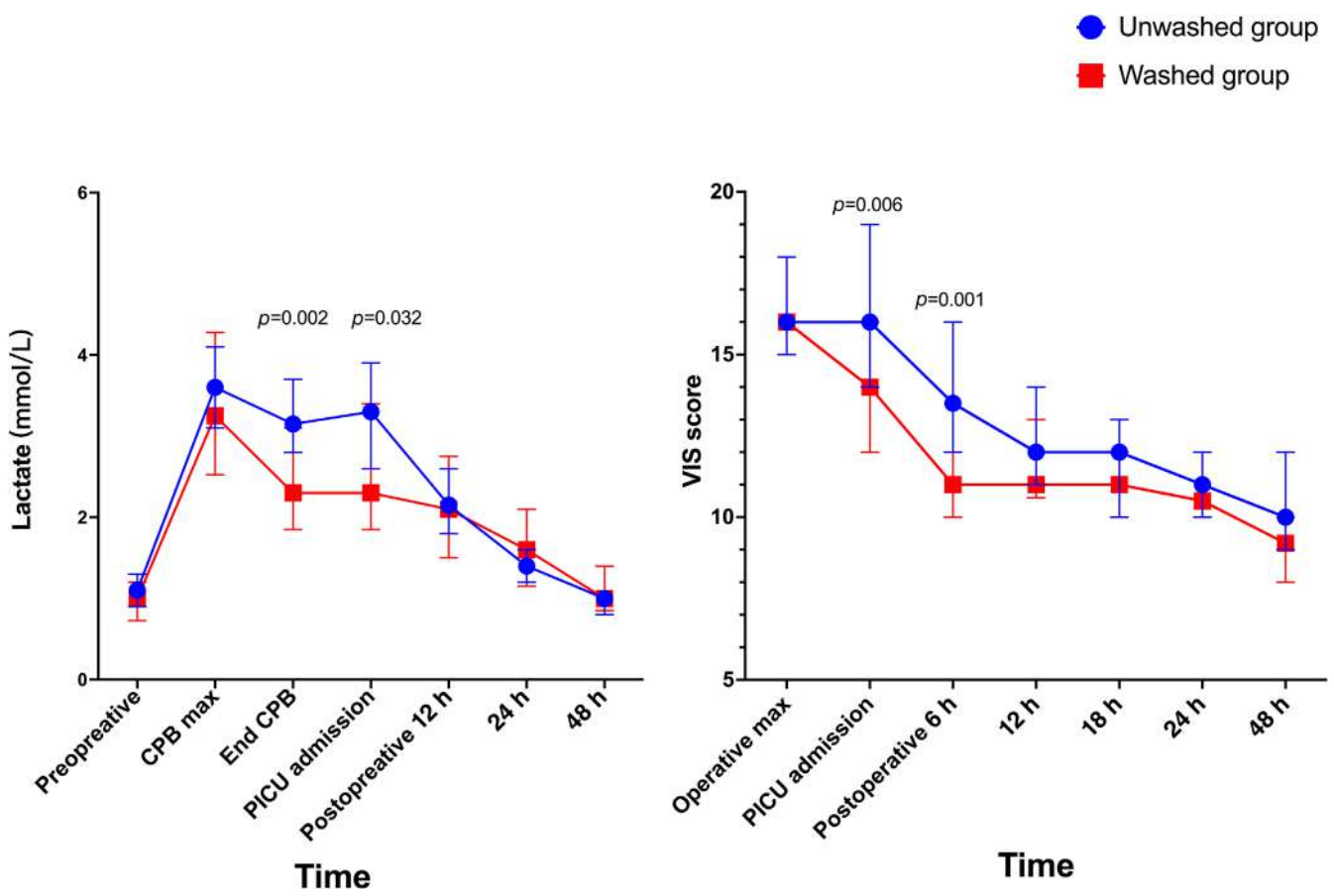

FIGURE 1 Changes in levels of lactate and VIS in two groups at different time points. Data are presented as medians and interquartile range values. CPB, cardiopulmonary bypass; VIS, vasoactive-inotropic score.

with baseline ($p < 0.001$). Interestingly, IFN-γ in the washed RBC group decreased compared to baseline ($p < 0.05$), while Tumor necrosis factor-α (TNF-α) was significantly higher in the washed group compared with the unwashed group (2.28 vs. 1.64 pg/mL, $p < 0.05$).

The coagulation parameters remained similar between the groups before and after surgery. However, DD, FIB and FDP increased significantly in both groups compared with baseline. Notably, the unwashed RBC group exhibited a significant decrease in TT compared with the baseline (15.26 vs. 18.61 s, $p < 0.001$).

Multiple linear regression analysis adjusted for preoperative values and confounding factors revealed that CPB priming fluid with washed RBCs did not significantly influence changes in inflammatory or coagulation markers (Table S3).

DISCUSSION

The RBC washing technology demonstrably reduces lactate and other metabolites in stored RBCs [8] and mitigates storage-related lesions [10]. However, limited research is available on the effects of CPB on the circulatory, coagulation and inflammatory systems in patients undergoing cardiac surgery, especially neonates, who are at a unique physiological stage of development. Our findings reveal that using the Sorin Xtra® ATS device to process stored RBCs in CPB priming fluid was associated with significant improvements in Hb and Hct levels, as well as a reduction in cellular products such as lactate, potassium and glucose. There was no significant effect on the free Hb levels. In the early postoperative period, the patients who received washed RBCs had lower blood lactate levels and reduced vasoactive drug use. However, no significant improvements were observed in coagulation system parameters or inflammatory responses.

This study demonstrates that Sorin Xtra® ATS significantly improves blood acid–base balance and increases Hb content and Hct, consistent with findings by Overdevest [18] and Seyfried [19] who recommended the Popt mode for this device. Immediate assessment of free Hb in RBCs post-ATS treatment showed no significant change compared with pre-treatment levels, suggesting no haemolytic destruction of blood at this stage. Various processing devices and post-processing storage times have different effects on the haemolysis of stored RBCs, as confirmed by O'Leary et al. [20]. Therefore, we recommend performing RBC washing in the operating room and reducing the post-processing RBC standing time.

Although Rabinowitz's study underscores the beneficial role of lactate [21], elevated lactate levels remain a potent indicator of

TABLE 4 Postoperative laboratory test and outcomes.

Variables	Unwashed group ($N = 56$)	Washed group ($N = 45$)	p value
End CPB			
Hb (g/L)	104.50 (98.00,113.75)	106.00 (100.00,118.50)	0.11
HCT (%)	32.00 (29.00,34.00)	32.00 (30.00,35.50)	0.12
Glucose (mmol/L)	9.98 (7.80,12.75)	10.15 (8.75,12.34)	0.72
K (mmol/L)	4.25 (3.90,4.66)	4.33 (3.79,4.61)	0.99
Hyperlactacidaemia (%)	19 (34)	7 (16)	0.04
Hyperglycaemia (%)	37 (66)	37 (82)	0.09
Hyperkalaemia (%)	9 (16)	5 (11)	0.47
Admission time to PICU			
RBC (10^{12}/L)	3.26 (2.95,3.60)	3.23 (3.06,3.84)	0.23
Creatinine (mg/dL)	50.78 (41.02,64.43)	46.31 (39.42,57.79)	0.32
ALB (g/L)	40.20 (37.96,42.90)	41.90 (38.90,44.90)	0.02
Leukocyte (10^9/L)	10.39 (8.34,13.24)	10.38 (7.71,12.39)	0.75
Hs-CRP (mg/L)	12.09 (10.47,12.80)	11.29 (9.70,12.02)	0.02
PCT (ng/mL)	1.80 (0.16,8.90)	0.27 (0.13,1.38)	<0.01
NT-proBNP (pg/mL)	10,432 (7311,17,833)	10,402 (5256,17,171)	0.51
Postoperative outcomes			
Delayed sternal closure (%)	9 (16)	1 (2)	0.02
Mechanical ventilation (h)	55.00 (34.50,149.50)	49.00 (27.00,91.00)	0.18
PICU LOS (day)	15.50 (12.00,25.00)	18.00 (13.25,25.75)	0.47
Hospital LOS (day)	23.50 (17.25,34.75)	24.50 (19.00,31.00)	0.93
Chest-tube time (day)	6.00 (4.00,7.00)	5.00 (5.00,6.00)	0.79
Postoperative 72 h RBC transfusion (mL/m^2/day)	44.80 (24.98,68.81)	48.08 (27.44,68.45)	0.63
AKI (%)	44 (79)	33 (73)	0.54
Peritoneal dialysis (%)	19 (34)	16 (36)	0.86
Infection (%)	40 (71)	29 (64)	0.45
In-hospital mortality (%)	2 (3)	0 (0)	0.50

Note: Values are expressed as *n* (%) or medians and interquartile ranges.
Abbreviations: AKI, acute kidney injury; ALB, albumin; CPB, cardiopulmonary bypass; Hb, haemoglobin; Hct, haematocrit; Hospital LOS, length of hospital stay; Hs-CRP, hypersensitive C-reactive protein; K, potassium; Na, sodium; NT-proBNP, N-terminal pro-brain natriuretic peptide; PCT, procalcitonin; PICU LOS, length of PICU stay; RBC, red blood cell.

mortality and poor prognosis in neonates and critically ill patients [22, 23]. Therefore, minimizing exogenous RBC transfusion-induced lactate increase has significant clinical value. The anaerobic glycolysis of erythrocytes during storage, in which pyruvate is metabolized to lactate, serves as the primary energy source for RBCs. This elucidates the reason for the elevated lactate levels in stored RBCs after prolonged storage. Deep hypothermic circulatory arrest (DHCA) during CPB is a significant factor affecting lactate levels. Therefore, to minimize bias from this factor, we excluded patients undergoing DHCA procedures from this study. Our study demonstrated that washed RBCs required less sodium bicarbonate during CPB and exhibited lower early lactate levels at the end of CPB than unwashed RBCs. This can be attributed to two key factors: (1) the lower baseline lactate of the washed group's CPB priming fluid and (2) the levels of 2,3--diphosphoglycerate in stored RBCs decrease over time [24], resulting in reduced oxygen delivery [25].

Post-washing RBCs showed higher Hb and Hct levels, suggesting improved oxygen-carrying capacity. Our findings indicated a higher DO_2i in the washed group during rewarming. Multivariate linear regression analysis revealed a correlation between the washed priming fluid stored in the RBCs and decreased postoperative lactate levels. Discrepancies from the findings of Boks et al. [26] may be due to the large age disparity between the study populations, with neonates being more susceptible to exogenous acid–base balance interference. Additionally, our stored RBCs had a longer storage time (20.00 ± 5.13 vs. 7.6 ± 2.7 days). Ranucci et al. noted the risks of pulmonary complications, AKI and infections with RBCs stored for over 4 days in CPB primes during infant cardiac surgery [27]. Hence, implementing a washing process for the stored RBCs in our inventory is recommended.

Paediatric patients often require perioperative vasoactive drugs due to their immature physiology and reduced myocardial

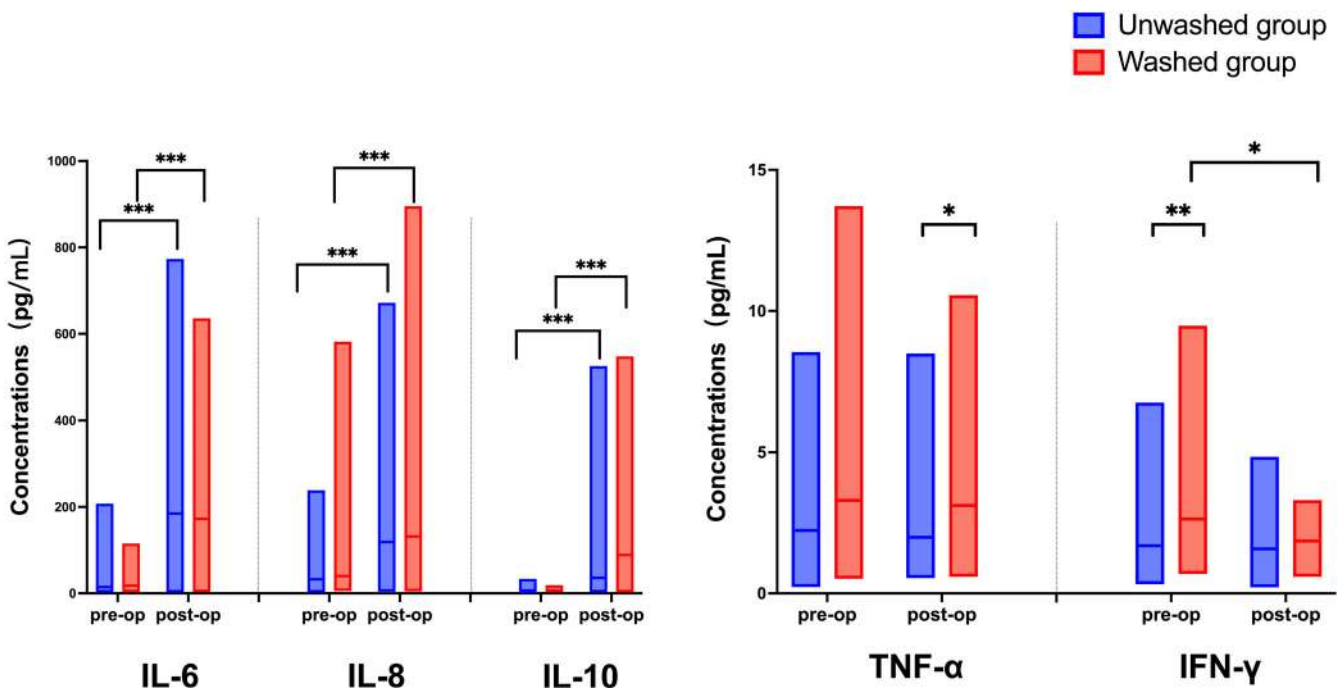

FIGURE 2 Inflammatory mediators' levels preoperative and postoperative in two groups. *Significant difference between groups, $p < 0.05$; **$p < 0.01$; ***$p < 0.001$. IL, inflammatory.

contractility [28]. VIS scores gauge physician intervention in cardiac output, with higher scores linked to adverse outcomes such as prolonged ventilation, hospital stay, sepsis and mortality [27, 29–32]. Elevated VIS scores (>20) independently predicted adverse outcomes after CPB [17]. Our study showed that using CPB priming fluid with washed RBCs significantly lowered VIS scores upon PICU arrival and 6 h later. Fewer patients in the washed group developed a high-risk VIS (>20) post-surgery (8.9% vs. 30.4%, $p = 0.003$). Multivariate analysis confirmed the independent contribution to reducing VIS within 6 h. Stapley et al. found that stored RBCs inhibit the Nitric Oxide (NO) pathway [29]; however, washing them reduced toxicity [30]. Sorin Xtra® likely reduces the toxic stimulation of harmful components in stored blood to the cardiovascular system of patients, improves the NO pathway, increases the function of vascular smooth muscle cells in patients, reduces vascular activity inhibition, increases the oxygen supply of RBCs to blood vessels, thereby improving hemodynamic and reducing the use of vasoactive drugs. Delayed chest closure was less common in the washed group, possibly because of improved circulatory stability. Bleeding volume and total CPB infusion remained similar; however, increased ultrafiltration in the washed group suggested improved fluid management, potentially contributing to postoperative stability and lower VIS scores. However, there was no difference in in-hospital mortality between the two groups, suggesting that priming RBCs washing may only affect early postoperative outcomes.

RBC storage leads to structural changes, microvesicle release and inflammatory reactions, with TNF, IL-1 and IL-6 increasing over time [31–33]. Cholette et al. [5, 34] reported that washing RBCs during cardiac surgery reduced inflammatory mediator levels, transfusions and mortality. We measured 12 inflammatory mediators before and after CPB and observed significant increases in IL-6, IL-8 and IL-10 levels in both groups post-surgery ($p < 0.001$), indicating substantial immune stress. Only TNF-α differed significantly between groups post-surgery, but multivariable analysis found no link between washed priming RBCs and reduced inflammatory stimulation during CPB. Priming RBCs alone may have a limited ability to remove inflammatory stimuli, and CPB may induce continuous inflammatory stimulation. Processing stored RBCs may damage their membranes, increasing the chances of inflammatory mediators entering the bloodstream. Different blood processing systems vary in their inflammatory mediator removal rates, with some systems potentially increasing their production [11]. Therefore, optimization of priming fluid handling to minimize CPB-induced inflammatory stress warrants further investigation.

In the present study, a standardized heparinization intervention was used during CPB. Therefore, the patient's postoperative coagulation index is primarily affected by systemic heparinization, and the impact of washing RBCs of the priming fluid on the patient's postoperative coagulation index is likely to be minimal.

Several limitations necessitate a cautious interpretation of our findings. First, this single-centre retrospective study used a specific CPB priming protocol, which limits its generalizability to institutions with different practices. Second, the focus on neonates, who require proportionally more prefilled RBCs than adults or adolescents, may hinder direct comparisons with other age groups. Finally, although our centre routinely analyses inflammatory and coagulation markers

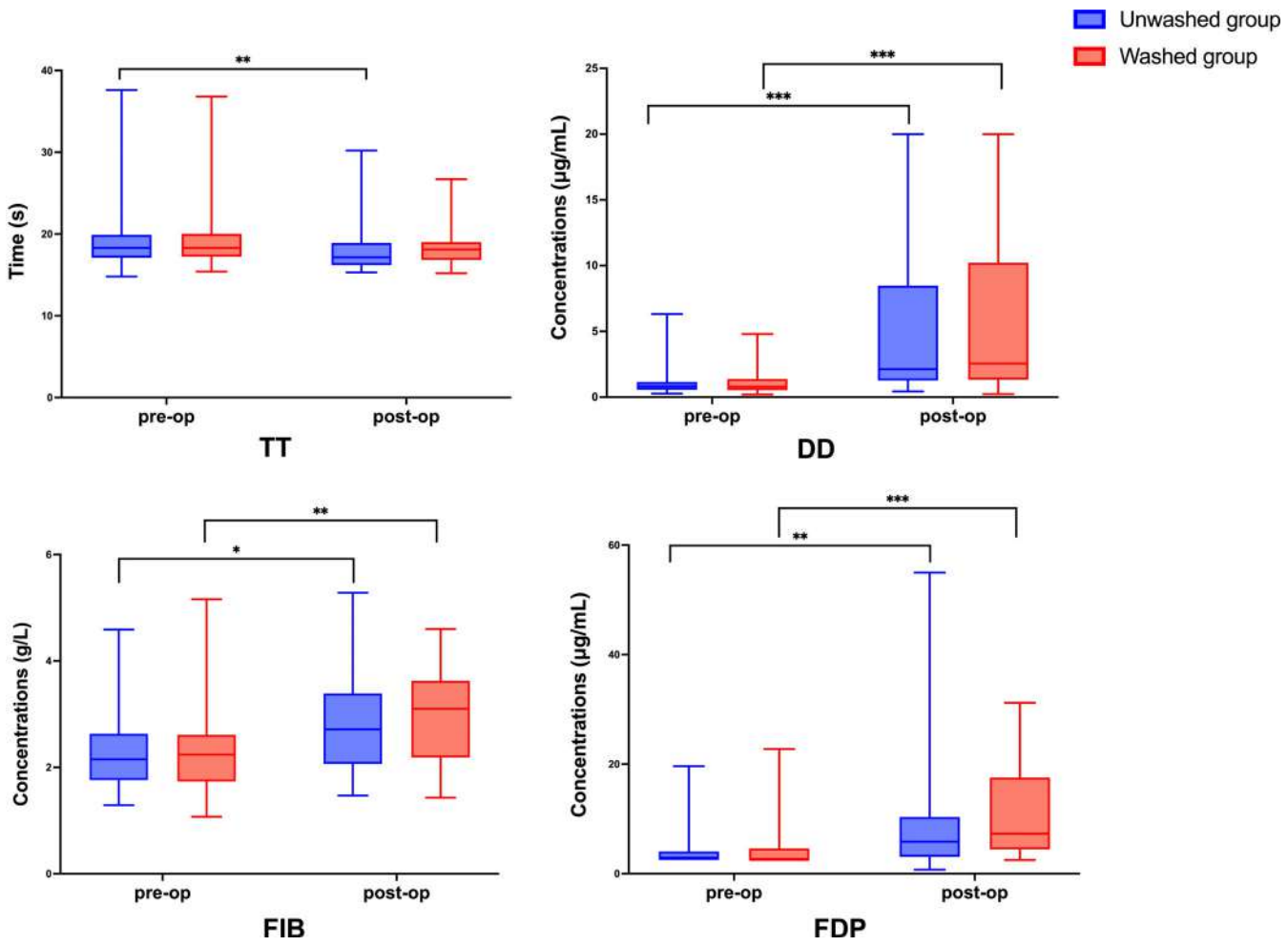

FIGURE 3 Coagulation parameters preoperative and postoperative in two groups. *Significant difference between groups, $p < 0.05$; **$p < 0.01$; ***$p < 0.001$. DD, D-dimer; FDP, fibrin degradation product; FIB, fibrinogen; TT, thrombin time.

before and after surgery, we lack data on these markers before and after blood processing, preventing definitive conclusions regarding the effect of the device on these indicators in processed stored RBCs. All blood products were uniformly collected and prepared by the city central blood bank. Consequently, this study did not include control groups for unwashed RBCs in saline, nor account for donor and manufacturing variability, potentially affecting stored RBC quality.

We conclude that the ATS can effectively increase Hb and Hct levels and remove RBC metabolites. The addition of washed RBCs to the priming fluid in neonatal CPB may be one of the reasons for reduced the early postoperative lactate increase and lower VIS. However, this intervention did not significantly improve intraoperative changes in the inflammatory and coagulation systems.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Dose-dependent inactivation of *Plasmodium falciparum* in red blood cell concentrates by treatment with short-wavelength ultraviolet light

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Abstract

Background and Objectives: *Plasmodium* species are naturally transmitted by *Anopheles* mosquitos. The parasite infects red blood cells (RBCs) and can be transfused with blood products. In non-endemic areas, the main risk of infection arises from travellers coming back and people immigrating from malaria-endemic regions. Endemic countries face a permanent risk of infection from transfusion-transmitted malaria (TTM). TTM may cause life-threatening complications in patients dependent on blood donations. This study aimed to investigate the efficacy of *Plasmodium falciparum* inactivation in RBC units by treatment with short-wavelength ultraviolet C (UVC) light in the absence of photochemical additives.

Materials and Methods: RBC units were spiked with *P. falciparum* to a parasite density of 0.1%–1% and irradiated with up to 4.5 J/cm^2 UVC. The parasite density of UVC-treated dilution series and untreated controls were compared over 3 weeks after irradiation.

Results: The lowest dose of 1.5 J/cm^2 UVC led to a 3.1 log reduction in parasite load compared with the untreated control. The inactivation capacity was dose-dependent. Strikingly, 4.5 J/cm^2 led to ≥5.3 log unit reduction, which was equivalent to a complete inactivation in two out of three experiments.

Conclusion: Pathogen reduction with UVC light was previously shown to be effective for different bacteria and viruses, but the inactivation of parasites in RBC concentrates was not addressed until now. The present study provides evidence for significant inactivation of *P. falciparum*-infected RBCs by UVC light.

Bernd Lepenies and Ute Gravemann are lead contact.

Keywords
blood safety, pathogen inactivation, pathogen reduction, *Plasmodium falciparum*, transfusion-transmitted malaria, UVC inactivation

Highlights

- Red blood cell concentrates were treated with ultraviolet C (UVC) light of 254 nm wavelength.
- UVC treatment dose-dependently inactivated *Plasmodium falciparum*.
- UVC offers a new strategy for preventing the transmission of malaria through transfusion.

INTRODUCTION

Apicomplexan parasites of the genus *Plasmodium* are the causative agents of the blood-borne infectious disease malaria, which are transmitted by female *Anopheles* mosquitos. Severe malaria cases are mostly caused by *Plasmodium falciparum* [1, 2]. The WHO reported 249 million cases for 2022 and a growing disease burden due to the COVID-19 pandemic, humanitarian crisis, climate change and resistance development of *Anopheles* mosquitos to insecticides [3]. Adults living in malaria-endemic regions often remain asymptomatic or develop mild symptoms, while infants, pregnant women and immunologically naïve people carry a high risk for severe disease [1, 2, 4]. Infected blood products can cause transfusion-transmitted malaria (TTM) [5, 6]. TTM facilitates severe disease progression as it directly leads to blood-stage malaria without the prior asymptomatic liver phase [6]. Infectious red blood cell (iRBC) concentrates and whole blood are the main source of TTM [7–10]. Nevertheless, all blood components carrying residual RBCs can be infectious because 10 parasites or less are sufficient for infection, as shown for *Plasmodium vivax* [11]. In the past 20 years, 20 cases of TTM, mostly caused by *P. falciparum*, were registered in Europe and the United States, both of which are non-endemic regions for malaria [6]. Temporal exclusions of donors from blood donations based on recent travel history limit TTM, but rising numbers of travellers and migrants from endemic areas also favour blood shortages [6, 12]. In a meta-analysis from 2010, it was demonstrated that 10% of blood donations were positive for *Plasmodium* in malaria-endemic countries in Sub-Saharan Africa [13]. Consistently, pathogen reduction technologies (PRTs) gained growing attention during the past years. Studies have shown significant inactivation of *P. falciparum* in whole blood using a combination of riboflavin and ultraviolet (UV) light [14, 15]. Both whole blood and RBC units treated with a combination of amustaline and glutathione exhibited robust inactivation as well, but added 24 h of processing time at room temperature [16, 17]. Inactivation using amotosalen with UVA light was efficient, but only tested in platelet concentrates and plasma, which contain residual numbers of erythrocytes only and have a much higher translucency compared with RBC concentrates [18]. Ultraviolet C (UVC) light was previously shown to be effective for *P. falciparum* inactivation in platelet concentrates [19]. The present study investigates the treatment of RBC units with UVC light at a wavelength of 254 nm. UVC light induces single- and double-strand DNA breaks, but also cyclobutane pyrimidine dimerization, the formation of reactive oxygen species and ultimately the loss of replication ability [20, 21]. It was previously shown that a dose of 4.5 J/cm^2 is sufficient to preserve a good in vitro quality of RBCs while facilitating significant pathogen inactivation [22]. In the present study, we describe a substantial and dose-dependent inactivation of *P. falciparum* in erythrocyte concentrates.

MATERIALS AND METHODS

Blood component preparation

Whole blood units of 500 in 70 mL citrate–phosphate-dextrose solution were collected from blood group 0+ donors screened in accordance with the German national guidelines (see Figure 1 for experimental design). Donations were stored at room temperature overnight (RT; 22 ± 2°C). One day after collection, RBC units were separated by high-speed centrifugation (Roto Silenta 630 RS/63 RS; Hettich, Tuttlingen, Germany) and automated blood component separation (Macopress Smarter; Macopharma, Tourcoing, France). Leucocyte depletion (via RCC flexible filter, Fresenius Kabi, Bad Homburg, Germany) was performed after the addition of 300 ± 10 mL additive solution PAGGS-C as previously described, which is used to improve the quality of UVC-treated RBCs [22]. The separation of RBCs was identical to the routine procedure except for the use of the newly developed additive solution PAGGS-C.

Parasite cultivation

Frozen aliquots of ring-stage parasites at 10% infected RBCs were thawed on ice for 10 min, centrifuged at 400 g for 5 min at RT and resuspended in 3.5% sodium chloride solution for washing. After centrifugation, the cell pellet was washed again with culture medium (500 mL RPMI 1640 [RPMI-XA, Capricorn, Ebsdorfergrund, Germany], supplemented with 2 mM L-glutamine [STA-B, Capricorn], 28 µg/mL hypoxanthine [6416.1, Carl Roth, Karlsruhe, Germany], 50 µg/mL gentamycine [G1397, Sigma Aldrich, Taufkirchen, Germany], 0.5% Albumax II [11550376, Thermo Fisher Scientific, Waltham, USA] and 5 mM HEPES [H4034, Sigma Aldrich]). Cultures were first kept in

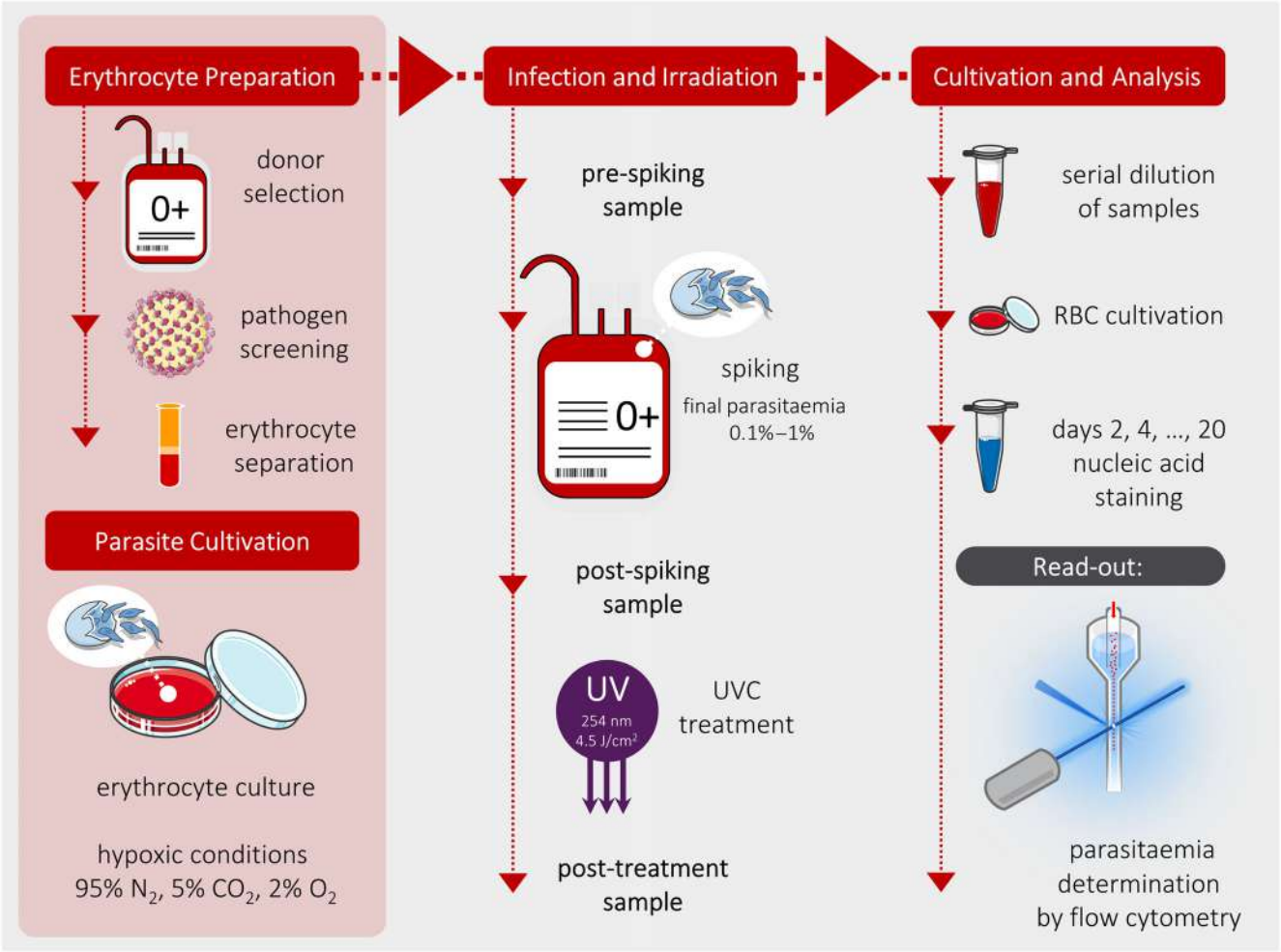

FIGURE 1 Experimental design of the study. Blood donors of blood group 0+ were randomly selected and routinely tested for different infectious diseases based on national guidelines. Red blood cell (RBC) concentrate was obtained by blood component separation and leukocyte depletion. *Plasmodium falciparum* parasites were expanded in an in vitro RBC culture with RPMI 1640 medium. Fresh RBCs were spiked to 0.1%–1% parasite density and sampled as indicated. Spiked RBCs were treated with ultraviolet C (UVC) light of 254 nm and 1.5 J/cm^2 per round with a maximum of 4.5 J/cm^2. Samples were serially diluted (1:10) and cultivated for a minimum of 20 days after inactivation. Every second day, a sample was drawn from each culture and stained for nucleic acids to determine the ratio of *P. falciparum*-infected RBCs among all RBCs.

10-cm cell culture dishes (83.3902, Sarstedt, Nümbrecht, Germany) and for expansion in cell culture flasks (83.3912.002, Sarstedt) and RPMI 1640 medium with 2.8% haematocrit (blood group 0+ to which the parasites are adapted) under hypoxic conditions (93% N_2, 5% CO_2, 2% O_2). Asexual stages of strain 3D7 were used. Parasites became asynchronous during the 1.5-week expansion period and were kept in this state.

Parasite density determination

The parasite density was determined by flow cytometry (Attune NxT Flow Cytometer, Thermo Fisher Scientific), which is an accepted and precise method that allows for a high throughput of samples [23]. A volume of 150 µL of re-suspended culture was centrifuged at 400 g for 2 min at RT. The cell pellet was re-suspended in 200 µL of nucleic acid stain Syto61 (S11343, Thermo Fisher Scientific) at a final concentration of 0.5 µM. Samples were incubated for 10 min before analysis. The mean intensity threshold for Syto61-positive events was always set on the respective day based on a stained *P. falciparum*-negative sample. Analysis of flow cytometric data indicates that heavily UVC-treatment-affected parasites slowly denature within 1–2 weeks, but are not capable of replication anymore (data not shown). Thus, parasites are still positive for Syto61, but the signal decreases over time. Parasites could be unequivocally differentiated from background signals at ≥0.5% parasite density (Figure 2). For definite exclusion of false-positive samples, a threshold of 1% was chosen to define a culture as positive for *P. falciparum* replication upon treatment. The ratio of iRBCs per RBC_{total} multiplied by 100 was defined as the parasite density of a culture.

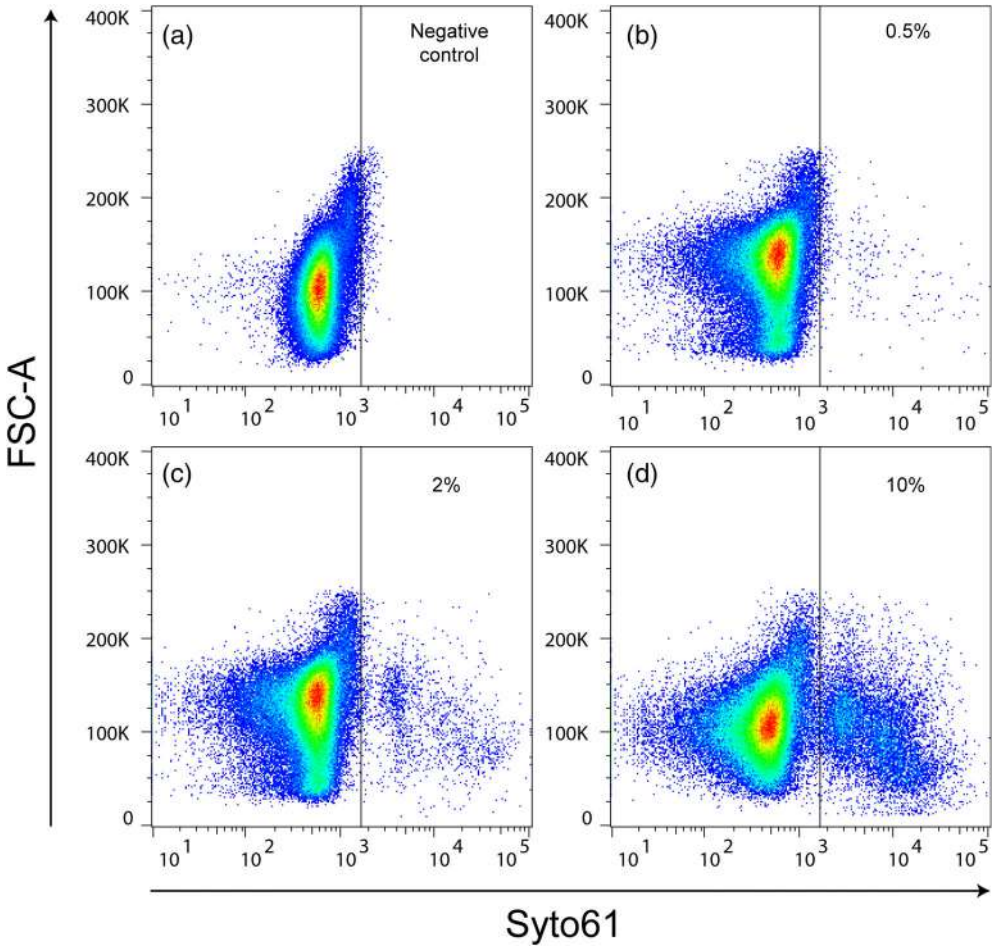

FIGURE 2 Differentiation of infected and uninfected red blood cells by Syto61 staining. Samples of all cultures were taken from D2 onwards every second day until no newly positive cultures were identified. (a)–(d) show example density plots of clearly negative (a) to positive samples with increasing parasite density in ascending order (b: 0.5%, c: 2%, d: 10% parasite density).

Parasite harvest and RBC concentrate spiking

On the day of inactivation (D0), parasite densities of all cultures were individually determined by flow cytometry. The highest in vitro achievable, yet still viable, parasite densities of 8%–14% were used for spiking. The spiking volume was determined based on the volume and haematocrit of the RBC concentrate and the parasite density of the pooled iRBC culture. Parasite cultures were centrifuged at 400 g for 30 min at RT. Cell pellets were re-suspended in 20 mL PAGGS-C and pooled to 10%–12% parasite density before spiking into the inactivation bag. The limit of spiked volume was set to 10% of the RBC unit volume. The final parasite densities of spiked RBC units were therefore 0.1%–1.0%.

Parasite inactivation and sampling

Samples of 4 mL were taken for cultivation at each step (see Figure 1 for experimental design). The negative control (NC) was taken from the inactivation bag after PAGGS-C dilution, but before spiking. The spiking control sample was taken after spiking when iRBCs and uninfected RBCs (uRBCs) had been carefully mixed. The pathogen inactivation treatment by UVC was performed using the MacoTronic illumination device (Macopharma) and the illumination bag from the THERAFLEX UV disposable kit (19 × 38 cm, Ref XUV 4005XU, Macopharma) [22]. Illumination was performed using UVC light at a wavelength of 254 nm, at a final dose of 4.5 J/cm^2. This dose was applied in one step ($n = 1$) or in three consecutive steps of 1.5 J/cm^2 ($n = 2$). During illumination, bags were agitated at 300 rpm to ensure homogeneous exposure of all cells to UVC light. A sample was collected after each illumination step. All samples were kept at RT to ensure comparable conditions during the inactivation procedure.

Sample preparation, cultivation and analysis

All samples were centrifuged at 400 g for 5 min at RT. For the NC sample, 400 µL were directly taken from the cell pellet and taken into culture in a 10 cm cell culture dish with 14-mL RPMI 1640 medium. Spike control and illuminated samples were diluted in a log-fold serial

dilution up to 1:10^8 in fresh uRBCs. A volume of 400 µL of the dilutions was taken into culture as described above. A summary of all samples and dilutions cultivated is given in Table S2. It was determined that a duration of 3 weeks was sufficient to expand a culture from a single parasite to 10% parasite density (data not shown). Therefore, cultures were analysed every 2 days for a minimum of 20 days. Flow cytometric analysis was performed as described above and cultures were considered positive at a parasite density of at least 1%. The titre of iRBC in the spike control was calculated from the parasite density of the spiking material and the spiking volume. For the spike control, the number of iRBCs per plated dilution was correlated with the time to reach 1% parasite density. Based on this correlation, the titre in UVC-treated samples was determined as follows: The number of iRBC in each dilution of UVC-treated samples was calculated from the number of days to reach a parasite density of 1% in the respective sample. The titre in each sample was calculated as a mean from all plated dilutions according to the following equation:

$$c(\text{Sample}) = \sum_{D=n}^{D=0} \frac{\text{iRBC}}{Vn} / n.$$

where c indicates titre of iRBC/mL, iRBC indicates number of infected RBC, D indicates serial dilution, n indicates number of plated dilutions and V indicates volume of plated dilutions (mL).

Samples that remained negative for 21 days were considered to be below the statistical detection limit of 0.007 iRBC/µL, based on a plated volume of 0.4 µL.

Data analysis and statistics

Statistical analysis was performed using commercially available software Microsoft Excel (version 2016 and Professional Plus 2019, Microsoft Office, Redmond, USA) and GraphPad Prism (version 8.4.3 and 9.0.0., Boston, USA) for Windows. Flow cytometric data were analysed with FlowJo (version 10.9.0, Ashland, USA). All data are expressed as mean ± standard deviation (SD). The reduction factors are calculated based on detected parasite density using the following equation:

$$\text{Log reduction} = \text{Log}(\text{pre} - \text{treatment titre of iRBC based on spiked parasite density}) - \text{Log}(\text{post} - \text{treatment titre of iRBC}).$$

Log reductions at or below the limit of detection are indicated accordingly. The statistical detection limit (95% probability), calculated for a plated volume of 400 µL, was determined according to Rabenau et al. [24].

RESULTS

To analyse the impact of UVC illumination on parasite inactivation in RBC concentrates, illumination was performed in 1.5 J/cm^2 steps and parasite growth was observed for approximately 3 weeks. NC samples were collected before spiking to ensure that RBCs were free of *P. falciparum* or other RBC-infecting parasites containing nucleic acid before spiking. The NC sample was used on all analysis days for flow cytometric gating and differentiating between Syto61-negative and -positive events (Figure 2).

The results of the inactivation study are shown in Table 1. Spiking of the RBC units with a parasite density of 0.1% yielded an average titre of 3 × 10^6 iRBC/mL. UVC treatment resulted in a dose-dependent parasite inactivation. An energy of 1.5 J/cm^2 reduced the infectivity of *P. falciparum* by 3.1 log steps, while 3.0 J/cm^2 reduced it by ≥4.8 log steps. In two out of three experiments, no infectivity was detected after UVC treatment with the standard dose of 4.5 J/cm^2, resulting in a log reduction factor of ≥5.3 ± 0.5 log steps. NC samples remained negative for Syto61 in all three replicates, indicating that the RBC units chosen for spiking were not infected at the time of blood donation (Figure 3, grey). The undiluted spike control reached the threshold of 1% parasite density on Day 2 in all replicates (Figure 3, green). The time to reach 1% parasite density was dependent on the number of iRBCs in the respective sample. Each log dilution took approximately 2 days longer than the previous log dilution to reach a parasite density of 1% (Figure 3, green). The spike control showed no parasite replication in all replicates, highlighting that the critical number of one remaining parasite for re-growth of the culture was exceeded no later than after a 10^8 log-fold dilution, respectively (Figure 3, green).

TABLE 1 Mean log titre of parasitized red blood cells calculated from a parasite density of 0.1%.

	Titre iRBC/mL				Log_{10} reduction factor			
UVC dose (J/cm^2)	**0**	**1.5**	**3**	**4.5**	**0**	**1.5**	**3**	**4.5**
Bag 1	3.3E+06	n.a.	n.a.	≤7.5	0.0	n.a.	n.a.	≥5.6
Bag 2	3.0E+06	5.3E+03	4.0E+02	70.6	0.0	2.8	3.9	4.6
Bag 3	4.0E+06	1.2E+03	≤7.5	≤7.5	0.0	3.5	≥5.7	≥5.7
Mean	**3.4E+06**	**2.2E+03**	**≤1.4E+02**	**28.5**	**0.0**	**3.1**	**≥4.8**	**≥5.3**
SD	**4.2E+05**	**2.3E+03**	**1.9E+02**	**29.8**		**0.4**	**0.9**	**0.5**

Note: Log reduction calculated from log titre pre-treatment–log titre post-treatment. Detection limit of 0.007 iRBC/µL is based on a plated volume of 0.4 mL. ≤Titre and ≥lLog reduction indicate samples with a parasite density below detection limit.
Abbreviations: iRBC, infectious red blood cells; UVC, ultraviolet C.

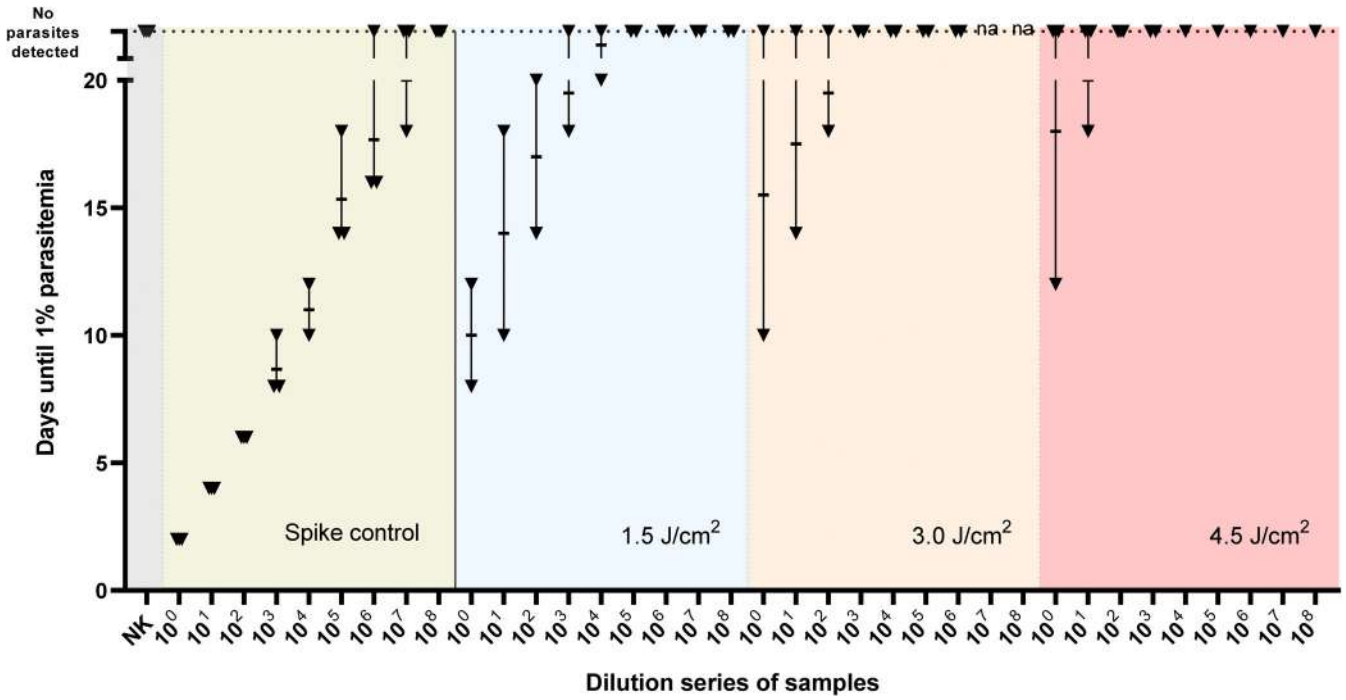

FIGURE 3 Ultraviolet C (UVC) light dose-dependently inactivates *Plasmodium falciparum* in red blood cell (RBC) concentrates. *P. falciparum*-parasitized red blood cell concentrates were illuminated with different doses of UVC light (green—untreated spike control; blue—1.5 J/cm^2; orange—3.0 J/cm^2; red—4.5 J/cm^2). Serial dilutions (1:10) of untreated (green) versus treated samples show dose-dependent inactivation of *P. falciparum* by UVC light. The *x*-axis represents the different dilutions and the *y*-axis days until 1% parasite density was reached by respective samples. Parasite density was determined based on a Syto61 nucleic acid staining. Referring to the undiluted sample, already 1.5 J/cm^2 led to a delay of 6–10 days until 1% parasite density was reached in comparison with the untreated sample that passed this mark on Day 2 already. For 3.0 and 4.5 J/cm^2, this threshold was reached even later or not at all, indicating stronger or complete inactivation of the parasite. Two biological replicates ($n = 2$) were performed for 1.5 and 3.0 J/cm^2, and three biological replicates ($n = 3$) were performed for the negative control, spike control and 4.5 J/cm^2, with each triangle representing one experiment. Error bars show the standard deviation of the two experiments.

DISCUSSION

TTM poses risk of infection, morbidity and mortality. The efficacy of different pathogen inactivation systems has been investigated in the past and may serve as an important means to limit malaria transmission [14–18]. The present study assessed the capacity of the newly developed PI system for RBC concentrates. The system uses short-wavelength UVC light without the addition of photochemicals to eliminate potential side effects, thereby simplifying and accelerating the inactivation procedure. All three replicates showed a highly similar replication rate of *P. falciparum* within the first 2 weeks in cultivation. IRBCs treated with UVC light at 4.5 J/cm^2 were markedly and, in two replicate experiments, even completely inactivated. Differences between replicates were seen, which is not unexpected because inter-recipient differences with respect to parasite replication rate, disease severity and varying host factors are seen in natural infections as well. The inactivation was clearly dose-dependent, with increasing log reduction factors from 1.5 to 4.5 J/cm^2.

The question remains to which extent the UVC-based pathogen reduction method is capable of reducing the risk of TTM and how it can be implemented. Notably, the mean log reduction factor of ≥5.3 ± 0.5 for 4.5 J/cm^2 is insufficient for blood products with high *Plasmodium* loads (e.g., RBC concentrates with 1% parasite density contain millions of parasites per microliter). Thus, the identification of donors with high parasitic loads based on the prevalence of clinical symptoms and by diagnostic methods such as light microscopy is still necessary to prevent transmission through high-density-infected RBC units [7, 25, 26]. However, laboratory screening tests for *Plasmodium* lack the sensitivity for low parasitaemia, which is a risk for TTM [27]. Therefore, UVC treatment could be an additional safety measure and significantly reduce or ideally eliminate the parasitic burden of RBC units derived from asymptomatic donors with low parasitaemia. Furthermore, refrigeration at 4°C, which is already the standard storage condition for RBC units, was described to decrease the number of infective parasites by 86% in 2 weeks [28]. Further studies are needed to estimate the combined inactivation potential of 4°C storage and UVC light treatment. Overall, we propose that a combination of UVC treatment, cold storage and already implemented diagnostics such as blood smears may have a significant impact on TTM eradication.

With 10% of blood products being *Plasmodium*-infected in malaria-endemic areas, UVC treatment could represent a significant advancement, especially in these areas [13]. In non-endemic areas, UVC treatment could be implemented not only to increase the safety of transfusions but also to revise blood donation regulations for

travellers and migrants from malaria-endemic countries to enable a sufficient supply of RBC units. As for all TTID mitigation strategies, there is a remaining risk of incomplete inactivation for high pathogen numbers exceeding the inactivation capacity of the pathogen reduction system [29]. Whether less stringent donor-deferral criteria in non-endemic countries can be applied when implementing UVC treatment will have to be carefully balanced against the paradoxical risk of increased TTM if more semi-immune donors are admitted to blood donation.

In general, the implementation of pathogen reduction processes into routine practice can be a challenge for the production side and may go along with additional costs and a moderate decrease in the quality of the respective blood component. However, it was previously shown that the in vitro quality of UVC-treated RBCs was in an acceptable range and did not affect RBC antigen expression [22].

Notably, the UVC-based pathogen reduction method used in this study was designed to treat RBC units and would require adaptation when applied to whole blood, which is often transfused in endemic countries [30]. However, before clinical implementation of this inactivation approach, additional studies will be necessary for the evaluation of the safety and efficacy of UVC-treated blood products.

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S.F., S.Z., T.J.S., A.S., W.H., B.L. and U.G. conceived and planned the experiments and analysed the data, S.F. and M.R. performed the experiments, B.L. and U.G. supervised the project and S.F. wrote the manuscript, with support from S.Z. All authors read and approved the final manuscript. Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST STATEMENT

S.F., S.Z., M.R., T.J.S., W.H. and B.L. have no conflict of interest to disclose. U.G. and A.S. received grants from the Research Foundation of the German Red Cross Blood Services (Deutsche Forschungsgemeinschaft der Blutspendedienste des Deutschen Roten Kreuzes) and Macopharma for the development of the UVC-based pathogen inactivation technology for platelets. U.G. and A.S. filed a joint patent application for the UVC-based technology for RBCs.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

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Re-evaluating treatment thresholds in patient blood management: Female patients experience more perioperative anaemia and higher transfusion rates in major elective surgery

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Abstract

Background and Objectives: By optimizing erythropoiesis, patient blood management (PBM) programmes can reduce transfusions, lower mortality and provide cost-effective care. While definitions of anaemia have historically varied by sex, for the purposes of PBM, anaemia is defined as a haemoglobin <130 g/L. Our objective was to describe whether perioperative anaemia and transfusion rates in the PBM setting vary by sex.

Materials and Methods: We conducted a retrospective study of the Ontario Nurse Transfusion Coordinators Program (ONTraC) database from 2018 to 2022. ONTraC collects data from 25 Ontario hospitals which together account for >70% of Ontario's provincial blood use (~400,000 units per year). We collected data on patients undergoing elective isolated coronary artery bypass graft surgery (CABG), open heart valve replacement, CABG plus valve replacement, single-knee arthroplasty and single-hip arthroplasty.

Results: From 2018 to 2022, 17,700 patients were included in the ONTraC program; 47% were females ($N = 8376$). Across almost all years and procedures, females were found to have a significantly lower pre-operative, nadir and discharge haemoglobin as compared with males, irrespective of PBM interventions. Transfusion rates were significantly higher for females; this was most pronounced in cardiac surgery.

Conclusion: Females experienced more perioperative anaemia and higher transfusion rates. Historic sex-specific definitions of anaemia may contribute to a greater

tolerance of anaemia in females. Prioritizing females for multimodal PBM and consistently achieving a pre-operative haemoglobin >130 g/L may reduce the amount of red blood cell (RBC) transfusions that female patients receive.

Keywords
anaemia, blood transfusion, cardiac surgical procedures, health equity, sex

Highlights

- In the setting of patient blood management (PBM), anaemia is defined as a haemoglobin concentration <130 g/L.
- Despite similar pre-operative targets, women in our study experienced more perioperative anaemia and higher transfusion rates than men.
- Multimodal PBM efforts, including patient and healthcare provider education, are required to achieve equitable patient care.

INTRODUCTION

Patient blood management (PBM) is a 'patient-centered, systematic, evidence-based approach to improve patient outcomes by managing and preserving a patient's own blood while promoting patient safety and empowerment [1].' Within the surgical setting, given associations between pre-operative anaemia and post-operative morbidity and mortality [2–4], the importance of PBM has been repeatedly underscored. In Ontario, the Ontario Nurse Transfusion Coordinators (ONTraC) program has been championing PBM at a network of 25 hospital sites for the past 22 years with success [5]. Under ONTraC's auspices, a significant reduction in red blood cell (RBC) transfusions has been observed at the provincial level, with transfusion rates decreasing from 25% to 0.4% for knee arthroplasties, and 60% to 27% for coronary artery bypass graft (CABG) surgery [5].

Central to the ethos of PBM, and reflected in ONTraC's algorithm, is the importance of addressing a patient's underlying cause for anaemia, whether by iron supplementation, B12 or erythropoiesis stimulating agents (ESAs) [6]. This reflects a targeted approach, particularly as etiologies of anaemia may vary. For instance, it is known that women are much more likely to experience iron deficiency (ID) and ID anaemia (IDA) compared with men [7–9]. In accordance with recent definitions of anaemia and an international consensus statement, ONTraC utilizes a pre-operative haemoglobin (Hb) target of 130 g/L, irrespective of patient sex [6, 10–12].

While there have been efforts to standardize treatment approaches and Hb targets within the PBM setting, how well this is being achieved remains unknown, particularly with regards to possible sex-based differences in treatment and transfusion. Furthermore, the literature continues to describe higher rates of ID and IDA amongst women, noting this to be a source of health inequity [8]. Given existing gaps in knowledge, our objective was to describe whether perioperative anaemia and transfusion rates in the PBM setting vary by sex.

METHODS

Setting and patient selection

The ONTraC program is well-described elsewhere [5]. Supported by the Ministry of Health, ONTraC supports registered nurses of Ontario hospitals who are dedicated to PBM. Collectively, the hospitals encompass over 70% of provincial blood use in the province, accounting for approximately 400,000 units per year. Each nurse coordinator works with perioperative health care teams to implement PBM principles. The ONTraC program focuses largely on elective total hip arthroplasty (THA), total knee arthroplasty (TKA), coronary artery bypass grafts (CABG) surgery, valve replacement and, more recently, benign gynaecological procedures. Data are systematically collected for 60 consecutive patients per year per hospital site; this data collection strategy was implemented for feasibility, and to provide a random sample across hospitals. The program was initiated in 2002, with data until 2022 available. Of note, all hospitals operate under the same general guidance that transfusion should be administered for an Hb <70 g/L, with a threshold of <80 g/L for patients with pre-existing cardiovascular disease or evidence of impaired tissue oxygenation, and the guidance that one unit should be transfused at a time [13]. Patients were treated as per the ONTraC algorithm [6]. Generally, in the absence of any contraindications, patients receiving EPO should have received iron (oral or IV), as well as tranexamic acid (TXA) intraoperatively.

Analysis

We retrospectively analysed data across all major elective surgeries between 2018 and 2022. Subgroup analyses were conducted amongst patients undergoing cardiac surgery given higher transfusion rates amongst this patient population. Pre-operative, nadir and discharge Hb, rates of transfusion, ESA use and intravenous (IV) iron use

are presented descriptively. A two-sided *t*-test was used to compare Hb values between sex, and a chi-square test was used to compare transfusion rates and rates of IV iron and ESA. A *p* value of <0.05 was deemed statistically significant. All analyses were done utilizing SAS (SAS Institute Inc., Cary, NC, USA).

RESULTS

From 2018 to 2022, 17,700 patients were included in the ONTraC database; 47% were females ($N = 8376$). Mean age was comparable between sexes, at 66 for males and 67 for females. Knee and hip arthroplasties represented the majority of procedures (68%, $N = 11{,}996$). Over 50% of knee and hip surgeries were seen at least 21 days before surgery, with a small percentage (approximately 11%–13%) being seen less than 7 days before surgery. Over 35% of cardiovascular surgeries were seen at least 21 days before surgery; however, a much higher proportion (27%–30%) of referred patients undergoing cardiovascular surgery had a lead time of less than 7 days, with 25% being seen between 7 and 14 days.

Across almost all years and procedures, females were found to have a significantly lower pre-operative, nadir and discharge Hb as compared with males, irrespective of PBM interventions (Table 1). Transfusion rates were significantly higher for females; this was most pronounced in cardiac surgery (Table 1). Examining CABG (2018–2022) as a case example, the pre-operative, nadir and discharge Hb for men and women can be visualized in Figure 1. Transfusion trends were consistent across years, acknowledging the intervening coronavirus disease 2019 (COVID-19) pandemic. Per transfused patient, hip and knee surgeries typically were administered 1–2 units of blood, whereas cardiovascular surgeries were typically transfused 2–3 units of blood.

In an analysis of all patients undergoing cardiac surgery, female patients were approximately twice more likely to be transfused perioperatively even with a normal pre-operative Hb level (>130 g/L). Amongst patients undergoing CABG, transfusion rates at this

TABLE 1 Red blood cell transfusion rates and haemoglobin (Hb) values per type of operation and patient sex.

		N		Transfusion rate (%)		Difference	Pre-op Hb [mean ± SD]		Nadir Hb [mean ± SD]		Discharge Hb [mean ± SD]	
Procedure	Year	M	F	M	F	F–M	M	F	M	F	M	F
CABG	2018	499	100	20.4	44.0	23.6*	138 ± 17	125 ± 16*	99 ± 15	82 ± 12*	99 ± 14	92 ± 12*
	2019	487	111	19.3	39.6	20.3*	139 ± 17	127 ± 15*	91 ± 15	82 ± 12*	100 ± 14	94 ± 11**
	2020	394	70	23.4	45.7	22.3*	138 ± 16	131 ± 13**	90 ± 15	84 ± 13*	97 ± 14	95 ± 12
	2021	475	95	21.9	49.5	27.6*	141 ± 17	131 ± 13*	92 ± 16	83 ± 11*	100 ± 15	94 ± 11*
	2022	469	95	21.1	49.5	28.4*	139 ± 17	127 ± 15*	91 ± 15	82 ± 12*	100 ± 14	94 ± 11*
CABG + valve	2018	235	73	48.1	64.4	16.3**	134 ± 19	129 ± 11**	83 ± 16	76 ± 10*	95 ± 14	90 ± 11**
	2019	225	57	39.6	66.7	27.1*	133 ± 15	128 ± 12	84 ± 13	77 ± 11*	95 ± 12	91 ± 10**
	2020	85	32	41.2	75.0	33.8*	138 ± 16	125 ± 16**	84 ± 14	80 ± 11	94 ± 11	90 ± 10
	2021	166	38	42.2	71.1	28.9*	138 ± 17	125 ± 16*	86 ± 15	77 ± 10*	97 ± 13	91 ± 10**
	2022	154	38	40.9	71.1	30.2*	138 ± 17	128 ± 11**	83 ± 14	78 ± 10*	95 ± 13	89 ± 9**
Valves	2018	288	167	25.3	41.9	16.6*	136 ± 18	128 ± 14*	91 ± 16	82 ± 11*	100 ± 15	93 ± 12*
	2019	267	171	26.2	42.1	15.9*	137 ± 16	127 ± 13*	91 ± 16	81 ± 12*	99 ± 14	92 ± 11*
	2020	164	91	26.8	56.0	29.2*	141 ± 15	129 ± 11*	91 ± 15	79 ± 10*	100 ± 13	92 ± 10*
	2021	218	145	23.9	44.1	20.2*	142 ± 16	130 ± 12*	92 ± 16	81 ± 11*	101 ± 15	94 ± 11*
	2022	208	88	20.2	43.2	23.0*	140 ± 14	127 ± 20*	90 ± 15	80 ± 10**	100 ± 14	93 ± 11*
Knee	2018	468	759	0.2	0.8	0.6	144 ± 13	133 ± 12*	116 ± 13	106 ± 12*	116 ± 13	107 ± 12*
	2019	486	811	1.2	0.4	−0.8	145 ± 15	133 ± 12*	116 ± 15	107 ± 12*	119 ± 15	109 ± 12*
	2020	486	814	0.4	0.4	0	143 ± 14	133 ± 11*	119 ± 15	109 ± 12*	120 ± 14	110 ± 12*
	2021	455	693	0.2	0.3	0.1	144 ± 15	133 ± 11*	121 ± 19	110 ± 13*	122 ± 14	110 ± 12*
	2022	420	738	0	1.1	1.1**	144 ± 16	131 ± 12*	121 ± 15	109 ± 13*	121 ± 15	109 ± 13*
Hip	2018	596	637	1.2	1.7	0.5	144 ± 13	132 ± 13*	114 ± 15	102 ± 13*	115 ± 14	103 ± 13*
	2019	580	688	0.7	1.0	0.3	146 ± 12	133 ± 12*	114 ± 14	102 ± 13*	115 ± 14	104 ± 12*
	2020	438	553	0.2	2.0	1.8**	144 ± 14	133 ± 12**	118 ± 14	105 ± 13*	119 ± 14	106 ± 12*
	2021	548	631	0.5	2.2	1.7**	145 ± 14	133 ± 12*	119 ± 15	105 ± 14*	119 ± 14	106 ± 13*

Note: Hb presented in g/L.
Abbreviations: CABG, coronary artery bypass graft; F, female; M, male; Pre-op, pre-operative.
*$p < 0.001$; **$p < 0.05$.

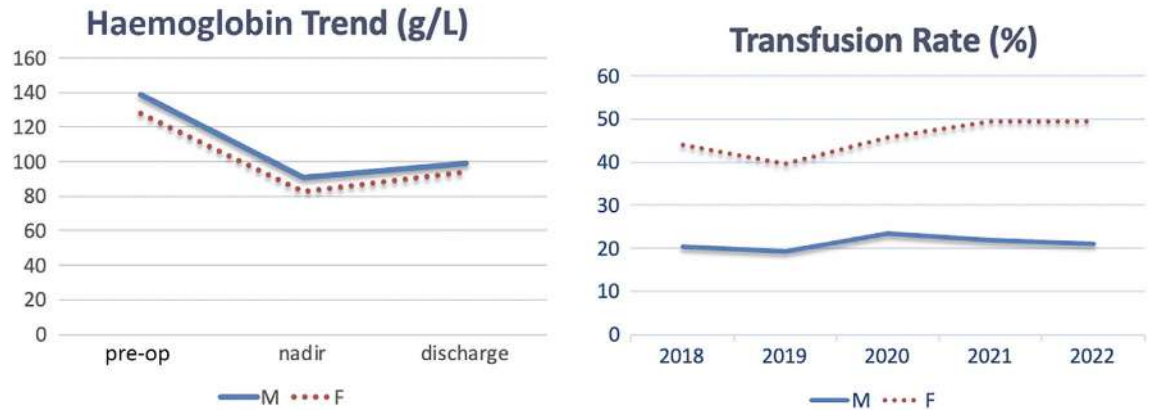

FIGURE 1 Haemoglobin (Hb) trend and transfusion rates by sex in coronary artery bypass graft (CABG) surgeries.

pre-operative Hb were 33.3% and 14.3% in female and male patients respectively ($p < 0.001$). Amongst patients undergoing isolated valve procedures, transfusion rates were 30.1% and 17.6% ($p < 0.001$). Amongst patients undergoing both CABG and valve procedures, transfusion rates were 55.6% and 34.3% ($p < 0.001$).

Female patients generally received more IV iron and ESA's pre-operatively (Table 2). Trends in supplementation varied across years, as did IV iron practices. While significantly more females received ESAs across almost all years and procedures, and more frequently received IV iron before orthopaedic surgery, they did not consistently receive more IV iron before non-CABG cardiac surgery; this was particularly true for valve surgery (Table 2).

TABLE 2 IV iron and ESA use per type of procedure.

Procedure	Year	IV iron (%)		ESA (%)	
		M	F	M	F
CABG	2018	4.2	8.0	3.6	16.0*
	2019	1.9	9.0*	3.1	17.1*
	2020	4.6	11.4**	5.3	8.6
	2021	4.2	14.7*	3.2	13.7*
	2022	4.9	11.6**	4.7	9.5**
CABG + valve	2018	3.4	8.2	4.7	24.7*
	2019	2.2	8.8**	7.6	10.5
	2020	1.2	21.9*	5.9	31.3*
	2021	5.4	15.8**	8.4	5.3
	2022	6.5	15.8	5.2	15.8**
Valves	2018	1.7	4.2	4.5	15.0*
	2019	2.6	4.1	3.8	9.4**
	2020	3.1	11.0**	3.7	8.8
	2021	4.1	8.3	4.6	14.5*
	2022	2.9	4.6	4.3	11.4**
Knee	2018	1.1	4.5*	2.8	6.9**
	2019	1.9	5.4**	2.5	6.8*
	2020	2.7	3.4	2.1	4.2**
	2021	1.1	5.3*	0.7	5.9*
	2022	1.4	5.0**	1.0	2.4
Hip	2018	1.3	4.9*	1.7	6.8*
	2019	1.9	4.1**	1.7	7.0*
	2020	1.8	4.3**	1.4	5.4*
	2021	1.8	3.7	1.6	5.9*
	2022	2.3	5.9**	1.2	2.9**

Abbreviations: CABG, coronary artery bypass graft; ESA, erythropoietin stimulating agent; IV, intravenous.
*$p < 0.001$; **$p < 0.05$.

DISCUSSION

Despite the same Hb treatment target amongst male and female patients within our program, female patients experienced more peri-operative anaemia and higher transfusion rates across major elective surgeries. In a subgroup analysis of cardiac surgery, female patients were more likely to be transfused. This was despite (1) most patients reaching a pre-operative Hb level of 130 g/L (or close to it, at 125–130 g/L in most cardiac surgeries) and (2) female patients being more likely to receive pre-operative PBM interventions such as ESA and IV iron, with IV iron rates generally increasing in the last few years.

Whether the observed difference in IV iron receipt between sexes is reflective of actual patient need deserves questioning. Population estimates of anaemia amongst males and females have varied; within Canada, most recent statistics show that at least 7% of Canadians are iron deficient, ignoring inflammation status, with males largely unaffected [14]. Given these statistics, as well as the likelihood of higher rates of ID within the PBM patient population, it is possible that women (and men) should be receiving even more IV iron than what was observed here.

Irrespective, this does not explain why female patients were twice as more likely to be transfused across major surgeries when their pre-operative Hb thresholds were close to being met. The topic of optimal Hb thresholds to proceed with elective surgery has recently been debated. In their recent study in the *British Journal of Anaesthesia*, Cavalli et al. found that, amongst over 6000 patients undergoing

cardiac surgery, utilizing the World Health Organization (WHO) anaemia thresholds of <120 g/L for females and <130 g/L in males disproportionately disadvantaged females [15]. Females were more likely to be transfused, with a pre-operative Hb concentration of 133 g/L and 127 g/L being associated with a 15% probability of intraoperative transfusion in females and males respectively [15].

One posited mechanism by which females may have greater transfusion needs is by haemodilution [15–17]. As the priming volume of the cardiopulmonary bypass (CPB) circuit is not adjusted for sex, female patients may become more haemodiluted and meet RBC transfusion triggers faster [15–17]. Furthermore, female patients may have a lower blood volume and lower RBC mass [15]. Some authors have suggested that modifying the extracorporeal circuit and CPB and utilizing strategies such as restrictive fluid administration may reduce the risk of transfusion [17]. In this context, it has been noted that achieving an Hb >130 g/L may be even more important in women than it is amongst men [16].

Other strategies may involve examining the root cause of overtransfusion. In their study of gender disparities in RBC transfusion in major elective surgery, Gombotz et al. found that in all surgical groups (CABG, total hip arthroplasty, total knee arthroplasty), the transfusion rate was significantly higher in women than in men [18]. This was felt to be secondary to liberal transfusion strategies, a lower baseline RBC volume in women, as well as clinicians applying the same transfusion thresholds irrespective of patient gender. While sex or gender-specific transfusion thresholds are not guideline-based, a more nuanced approach considering risks and benefits of transfusion could be undertaken, particularly in younger patients of child-bearing potential, those with known antibodies and, most importantly from a PBM perspective, those with uncorrected IDA.

Finally, adjuncts to minimize bleeding, such as TXA, should be considered. In their study of haemostasis in patients after CABG, Wang et al. showed that females had less blood loss than males with TXA treatment [19]. While they still had higher transfusion rates attributed to already described mechanisms (haemodilution on the CPB circuit), less plasma was transfused in the setting of TXA treatment [19]. Given that women have been described to be less likely to receive TXA in other settings [20], utilizing TXA uniformly in the intraoperative setting may help further mitigate any sex-related disparities.

Our study has multiple limitations. First and foremost, due to limitations with our data collection, we are able to report on sex, but not gender, nor other sociodemographic variables. Second, due to the limitations of our database, we do not have important covariates, including ferritin levels or underlying diagnoses (e.g., malignancy, renal disease), to help distinguish between etiologies of anaemia. Third, given that many patients were likely transfused in the operating room, we do not have bleeding data or markers of end-organ damage (vitals, real-time labs) that may have influenced the need for transfusion, nor do we have information on any differences in total blood volume (TBV), which has been found to be influential in previous studies. However, previous work, including that by Cavalli et al., has found TBV to be generally lower in females [15]. Finally, we do not have provider specific data to capture variations in transfusion practices, nor the timing of transfusion. Ideally, even if patients did not have sufficient time to be optimized pre-operatively (e.g., due to timing of referral, or other logistical factors), IV iron could still be administered in the post-operative period; ESAs may be avoided due to concerns around thromboembolic risk. We advocate that future registries systematically collect this data, particularly as there remain many unknowns.

Given that ONTraC collects data on transfusion rates for each hospital, future audits will more closely examine those hospitals which are transfusing more frequently for equivalent surgical procedures; this may serve as a prototype for other institutions undertaking quality improvement measures. Future work should also explicitly examine the impact of combination therapy versus a single treatment alone, acknowledging the impact of TXA, particularly in light of recent publications such as the HiFIT trial [21]. Finally, timing of referral should be captured in additional studies; while the majority of our orthopaedic surgeries were being referred at least 3 weeks before surgery, approximately a third of our cardiovascular patients were seen less than a week before surgery, despite females receiving more treatment, a longer lead time would allow for greater optimization amongst both sexes.

In conclusion, our study of patients enrolled in the ONTraC database shows that, in major elective surgery, female patients have lower pre-operative, nadir and post-operative Hb and are more likely to be transfused. Furthermore, amongst patients undergoing cardiac surgery, female patients are more likely to be transfused even if their pre-operative Hb is normal (i.e., Hb >130 g/L). Utilizing restrictive transfusion thresholds, providing important adjuncts such as TXA and potentially modifying the CPB circuit may help reduce the amount of RBC transfusions that female patients receive. Ongoing registries to monitor transfusion by sex and gender are needed to systematically evaluate the success of PBM interventions; they should also encompass important covariates such as iron status, sociodemographic variables, referral patterns/available time for optimization and patient and provider characteristics. Finally, the importance of uniformly utilizing an Hb >130 g/L (at minimum) should be emphasized; this should particularly be the case for cardiac surgery, and future studies should explore whether aiming for a higher Hb is of value. Although there has been a movement towards utilizing the Hb >130 g/L threshold, including by the National Advocacy Committee on Blood and Blood Products in Canada [11], the most recent cardiac surgery guidelines continue to utilize the WHO definition of anaemia (<120 g/L in women, <130 g/L in men) [22]. Haematologists, transfusion medicine specialists, anaesthesiologists, surgeons and other healthcare providers must work collaboratively to reduce transfusion burden, ensuring a common language and understanding of anaemia thresholds is an important first step.

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CONFLICT OF INTEREST STATEMENT

Yulia Lin has research support from Canadian Blood Services and Octapharma and is a consultant with Choosing Wisely Canada.

DATA AVAILABILITY STATEMENT

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

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ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Detecting serologically difficult ABO blood groups using single-molecule real-time sequencing technology

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Abstract

Background and Objectives: Recently, third-generation long-read sequencing technology has been increasingly applied to the detection of various blood group systems. Because of its long read length and use of single-molecule sequencing, it is capable of obtaining the sequences of blood group genes in their entirety as well as of distinguishing haplotypes. Therefore, here, we collected ABO blood group samples that were difficult to classify serologically and analysed the sequences of the coding regions of the ABO genes as well as the sequences upstream and downstream of the coding regions.

Materials and Methods: Samples with ABO antigen typing and reverse serum typing discrepancies were screened in a total of 21 patients. All samples were subjected to serological testing and preliminary ABO genotyping (polymerase chain reaction with sequence-specific primers [PCR-SSP]), followed by single-molecule real-time (SMRT) sequencing to obtain complete ABO gene sequences. PCR sequence-based typing (PCR-SBT) was performed to validate the results.

Results: Of the 21 samples, 15 had common ABO types, and 6 had rare ABO subtypes. One new allele, *ABO*B.NEW (c.861C>T)*, and one allelic base recombination event was identified. Forty-two haplotype sequences were obtained via SMRT sequencing with intronic single-nucleotide variants (SNVs) specific to the ABO allele, and all of the exon region sequences were consistent with the PCR-SBT results.

Conclusion: SMRT sequencing is capable of accurately obtaining complete ABO gene sequences, distinguishing haplotypes and identifying allelic recombination.

Keywords

ABO subtypes, haplotypes, single-molecule real-time sequencing, single-nucleotide variants

Highlights

- Third-generation long-read sequencing can obtain complete ABO gene sequences and distinguish haplotypes. It can be a good aid in serologically difficult ABO blood grouping.
- A novel allele *ABO*B.NEW (c.861C>T)* was discovered and has been uploaded to GenBank (OR565861).
- A total of 114 specific intronic region single-nucleotide variants for different ABO alleles were found using single-molecule real-time sequencing.

INTRODUCTION

Accurate blood group identification is a prerequisite for ensuring the safety of clinical blood transfusion, and the ABO blood group system, the first blood group system discovered in humans [1], is an important part of clinical blood grouping. With the development of science and technology, the means of blood group testing has expanded from traditional serological methods to molecular biology methods, and the study of blood group genes using polymerase chain reaction with sequence-specific primers (PCR-SSP), Sanger sequencing and next-generation sequencing (NGS) has become more widespread [2–13].

The ABO gene consists of seven exons and six introns and is located on human chromosome 9, 9q34.1-9q34.2, with a total length of approximately 25 kilobases (kb). Exons 6 and 7 encode most of the catalytically active glycosyltransferase region. This is the major part of the ABO gene product that determines the glycosyltransferase function and thus the production of ABO antigens [14]. Only exons 1–7, particularly exons 6 and 7, are tested in most ABO blood group gene sequencing. The complete sequence of the ABO coding region as well as the regulatory regions upstream and downstream of the coding region have been less well studied.

Single-molecule real-time (SMRT) sequencing, a third-generation sequencing technology, yields an average read length of up to 100 kb and highly accurate sequencing results, overcoming challenges in distinguishing haplotypes and multiple recombination events because of its sequencing of single molecules [15–17]. Fichou et al. were the first to apply third-generation sequencing technology to the detection of blood group genes, using this technology to obtain the full-length haplotype reference sequence of the ACKR1 gene (Duffy blood group system) [18]. Zhang et al. used the long-read length of third-generation single-molecule sequencing to assemble the full-length sequence of RHD-RHCE for the first time and study it in detail [19]. Subsequently, Tounsi et al. developed a convenient and rapid technique for full-length sequence detection of RHD genes using third-generation single-molecule sequencing [20]. Ji et al. also elucidated the transcriptional mechanism of the RHD DEL phenotype using third-generation sequencing [21]. Moreover, Gueuning et al. obtained the first full-length standard reference sequences of the six major isoforms of the ABO gene using third-generation sequencing and performed an evolutionary analysis of the gene [22].

The above-mentioned studies demonstrate the successful full-length sequencing of several common subtypes of blood group genes using third-generation sequencing technology, but the acquisition and analysis of complete sequences for rare ABO subtypes remains scarce. Therefore, in this study, ABO blood samples that were difficult to identify via serology were screened for rarer ABO subtypes. The target ABO gene was sequenced using SMRT technology, which not only yielded the complete sequence of the ABO gene from the start codon to the stop codon, including the sequences upstream and downstream of the coding region, but also distinguished the maternal and paternal haplotypes of the ABO gene.

MATERIALS AND METHODS

Sample collection

A total of 21 blood samples with incompatible ABO antigen typing and reverse serum typing were obtained from unpaid blood donors at the Blood Centre of Hefei City, Anhui Province, China, and from patients requiring blood transfusion for clinical purposes at the First Affiliated Hospital of Anhui Medical University, Anhui Province, China. Blood was collected intravenously in 3-mL EDTA-k^2 anticoagulation tubes and stored at 4°C. Blood serology was performed after sampling, and the samples were stored at −80°C for DNA extraction within 72 h. This study was approved by the Scientific Ethical Committee of The First Affiliated Hospital of Anhui Medical University. Informed consent was obtained from all participants.

Serological analysis

ABO antigen typing and reverse serum typing was performed on an ORTHO VISION MAX automated blood typing analyser (Ortho-Clinical Diagnostics) using the Ortho BioVue® System ABO-Rh/Reverse Grouping Cassette (Ortho-Clinical Diagnostics). If weak antigens or antibodies are detected, they must be further confirmed by the test tube method and the absorption/elution test. The relevant procedures were performed according to the National Clinical Laboratory Procedures Standards [23].

Genomic DNA extraction

The target genomic DNA was extracted from whole blood using a DNA extraction kit (Tianjin Super Biotechnology Developing Co., Ltd.) in strict accordance with the kit instructions. The concentration of the extracted DNA was determined using a Qubit 4 fluorometer (ThermoFisher Scientific), and the DNA concentration suitable for sequencing analysis was 30–50 ng/μL, with A260/A280 values of 1.6–2.0.

ABO genotyping using the PCR-SSP method

Target genomic DNA amplification and ABO blood group genotyping were performed using the Human Erythrocyte ABO Blood Group Genotyping Kit (PCR-SSP) (Tianjin Super Biotechnology Developing Co., Ltd.). The handling was carried out strictly in accordance with the kit instructions.

Sequencing the exon region of the ABO gene with the polymerase chain reaction sequence-based typing method

The PCR sequence-based typing (PCR-SBT) method belongs to the Sanger sequencing technology. Exons 1–7 of the ABO gene were

amplified and sequenced in both directions using the BigDye Terminator Cycle V3.1 sequencing kit (Application Biosystems, Foster City, CA, USA). The primer sequences are referenced to the Reference [24]. Sequence data were analysed using the SEQscape2.1 software (Application Biosystems, Foster City, CA, USA). The ABO alleles were determined from the nucleotide sequences of the polymorphic loci according to the International Society of Blood Transfusion (ISBT) standards for erythrocyte immunogenetics and blood group terminology [25] (Reference sequence:GenBank ID: NG_006669.2).

SMRT sequencing and bioinformatics analysis of the ABO gene

Three primer pairs were designed based on the ABO gene sequence (GenBank ID: NG_006669.2) [26]. The sequence from the start codon to the stop codon was covered by the overlapping amplicons of the primer pairs (9, 9.5 and 11.5 kb) (Figure 1, Table 1). Amplification was performed using the KOD FX enzyme (TOYOBO). The PCR amplification system and parameters are shown in Tables 2 and 3.

The construction of SMRT libraries was performed via a one-step method. DNA damage repair, end repair and adapter ligation were combined in one step to generate pre-sequencing libraries containing unique barcode adapters. The reaction mixtures were prepared before use and were 10 μL in total, containing 4 μL of PCR product, 5 μmol/L barcoded adaptor (Integrated DNA Technologies), 1× T4 DNA ligase buffer (Enzymatics), 1 mmol/L ATP (New England Biolabs), 200 μmol/L dNTP (New England Biolabs), 2.5 units of T4 polynucleotide kinase (Enzymatics), 0.75 units of T4 DNA polymerase (Enzymatics) and 180 units of T4 DNA ligase (HC) (Enzymatics).

A total of 120–250 ng of purified PCR product was mixed with the enzyme mixture. The reaction mixture was then incubated at 37°C for 20 min, 25°C for 15 min and 65°C for 10 min. Afterwards, exonuclease I (Enzymatics) and exonuclease III (Enzymatics) were added to remove the failed ligation products, and the final prelibrary was purified with 0.6× AMPure PB beads (Enzymatics). For multiple sample sequencing, prelibraries were pooled together according to equal masses. After pooling, the prelibraries were purified two times with 0.45× AMPure PB beads.

The final library was subsequently subjected to sequencing with the Sequel Binding Kit 2.2 (Pacific Biosciences) and the Internal Control Kit 1.0 (Pacific Biosciences). A total of 150 pM DNA–polymerase complexes were finally loaded and sequenced with the Sequel II platform (Pacific Biosciences) in a 20-h movie.

Primary analysis of the output data was carried out with the SMRTLink v10.1.0 software (Pacific Biosciences). The raw reads were first demultiplexed, and barcode sequence analysis was carried out automatically at the end of the runs, followed by subread analysis to generate circular consensus sequencing (CCS) reads using the CCS software application (filtration criterion: quality value>19). Filtered CCS reads were aligned to the human reference genome (GRCh38) with pbmm2 to specifically batch the desired blood-related gene CCS fragments. The target CCS reads were realigned to the reference genome (ISBT) using pbmm2. For the identification of single-nucleotide variants (SNVs) and small indels, variant calling was carried out with DeepVariant v1.2.0 (https://github.com/google/deepvariant/blob/r1.2/docs/deepvariant-quick-start.md). All alleles were named according to the standards of the ISBT working group,

TABLE 2 Polymerase chain reaction (PCR) amplification system.

	Single sample (μL)
KOD neo FX buffer	10.5
dNTPs	4
Total primer	9
gDNA	1
KOD Neo FX	0.5

TABLE 3 Polymerase chain reaction (PCR) parameters.

Temperature (°C)	Time	Cycle number (×)
94	2 min	1
98	10 s	25
68	12 min	
68	10 min	1
8	∞	

FIGURE 1 Three primer pairs were designed covering the 5′-UTR to 3′-UTR of the ABO gene, and three overlapping amplicons of 9, 9.5 and 11.5 kb were amplified.

TABLE 1 Primer design for ABO gene single-molecule real-time (SMRT) sequencing.

Sequence primers	Forward primer	Reverse primer
Primer 1	5′catccctttcaccttggcattt3′	5′agctacattgaccagagagaga3′
Primer 2	5′gcccaccaaaactccctggaa3′	5′ccagttcctgccagggagagga3′
Primer 3	5′gtgtgaaactcatcaaaacc3′	5′cgcagggattgcagtgagg3′

which develops and maintains guidelines for blood group antigens and allele nomenclature [25].

RESULTS

ABO serology, PCR-SSP, PCR-SBT and SMRT sequencing results from 21 samples

Fifteen common ABO phenotypes were identified (nine group A, three group B and three group AB). Six rare ABO subtypes were identified with the following genotypes: *A1.02/B.NEW(c.28G>A)*, *cisAB.05/B.NEW(c.861C>T)*, *A1.02/B3.01*, *BEL.03/O.01.02*, *A1.02/BW.07* and *BA.04/O.01.01*. The specific test results for each method for these samples are shown in Table 4. Given that the PCR-SSP kit was designed with primers specific for only common ABO subtypes, the typing could not be interpreted in three samples (1, 4, 5), and the typing did not match the final genotype in three samples (2, 3, 6). Four samples (1, 2, 3, 5) for which the PCR-SBT results disagreed with the SMRT sequencing results are described in detail in the following sections.

TABLE 4 Serology, polymerase chain reaction with sequence-specific primers (PCR-SSP), PCR sequence-based typing (PCR-SBT) and single-molecule real-time (SMRT) sequencing results from 21 samples.

		ABO serology						
		Forward		Reverse				
Sample	Phenotype	Anti-A	Anti-B	A cell	B cell	PCR-SSP	PCR-SBT	SMRT
1	AB_{weak}	4+	mf	–	–	\	A1.02/B.01	A1.02/B.NEW
2	$A_{weak}B$	2+	4+	1+	–	AB	B(A)new/B.01	cisAB.05/B.NEW
3	AB3	4+	–	4+	4+	AB	A1.02/B.01	A1.02/B3.01
4	Bel	–	–	4+	–	\	BEL.03/O.01.02	BEL.03/O.01.02
5	AB_{weak}	4+	–	–	–	\	A1.02/B.01	A1.02/BW.07
6	B(A)	2+	4+	1+	–	BO_1	BA.04/O.01.01	BA.04/O.01.01
7	A	4+	–	2+	2+	AA	A1.02/A1.02	A1.02/A1.02
8	AB	4+	4+	3+	2+	AB	A1.02/B.01	A1.02/B.01
9	B	–	4+	+	+	BO_1	B.01/O.01.01	B.01/O.01.01
10	A	–	–	–	4+	AO_2	A1.02/O.01.02	A1.02/O.01.02
11	A	–	–	–	4+	AO_1	A1.02/O.01.01	A1.02/O.01.01
12	AB	4+	4+	–	1+	AB	A1.01/B.01	A1.01/B.01
13	B	–	4+	3+	1+	BO_2	B.01/O.01.02	B.01/O.01.02
14	A	4+	–	+	–	AO_1	A1.02/O.01.01	A1.02/O.01.01
15	AB	4+	4+	+	+	AB	A1.02/B.01	A1.02/B.01
16	B	–	4+	+	+	BO_2	B.01/O.01.02	B.01/O.01.02
17	A	4+	–	3+	3+	AO_1	A1.02/O.01.01	A1.02/O.01.01
18	A	4+	–	3+	3+	AO_1	A1.02/O.01.01	A1.02/O.01.01
19	A	4+	–	2+	3+	AO_2	A1.02/O.01.02	A1.02/O.01.02
20	A	4+	–	3+	3+	AO_2	A1.02/O.01.02	A1.02/O.01.02
21	A	4+	–	2+	3+	AO_1	A1.01/O.01.01	A1.01/O.01.01

TABLE 5 The results of the six ABO subtypes with variations in the exon region by single-molecule real-time (SMRT) sequencing.

Sample	Phenotype	Allele name	Location	Nucleotide change	Amino acid change	GenBank ID/rsID
1	Bweak	ABO*B.NEW(c.28G>A)	Exon 1	c.28G>A	p.Gly10Arg	JN652594
2	B	ABO*B.NEW(c.861C>T)	Exon 7	c.861C>T	p.Ala287Ala	OR565861
3	B_3	ABO*B3.01	Exon 7	c.1054C>T	p.Arg352Trp	rs56390333
4	Bel	ABO*BEL.03	Exon 7	c.502C>T	p.Arg168Trp	rs573234689
5	Bweak	ABO*BW.07	Exon 7	c.1055G>A	p.Arg352Gln	rs1019994127
6	B(A)	ABO*BA.04	Exon 7	c.640A>G	p.Met214Val	rs964984014

Haplotype sequence analysis of ABO alleles

SMRT sequencing of the ABO gene in 21 samples yielded 42 haplotype sequences, including 2 *ABO*A1.01*, 14 *ABO*A1.02*, 6 *ABO*B.01*, 7 *ABO*O.01.01*, 6 *ABO*O.01.02*, 1 *ABO*B.NEW (c.28G>A)*, 1 *ABO*B.NEW (c.861C>T)*, 1 *ABO*cisAB.05*, 1 *ABO*B3.01*, 1 *ABO*BEL.03*, 1 *ABO*BW.07* and 1 *ABO*BA.04*. The exonic SNVs of each haplotype sequence are shown in Table S1, with key variant sites in bold. Two alleles were found that are not yet listed in the official ABO (ISBT001) blood group 624 allele list (v1.1 17102) [25], and the corresponding new variant sites are highlighted in red.

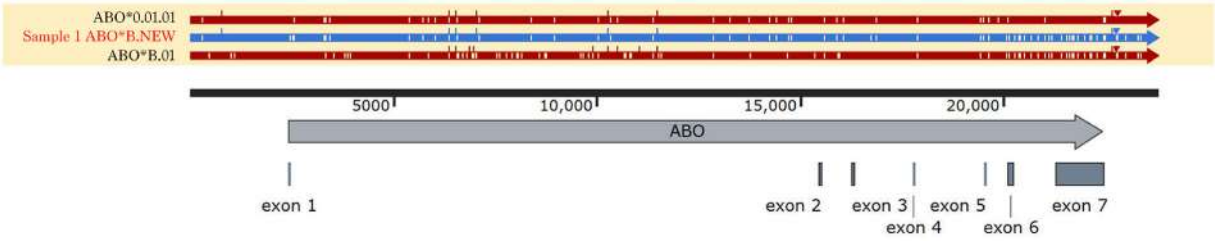

FIGURE 2 Schematic diagram of allelic recombination in sample 1. The sequences from intron 1 to intron 5 has the characteristics of the ABO*O.01.01 but the sequences from exon 6 to exon 7 have characteristics for ABO*B.01. The recombination event maybe happens from c.240-219G>A to c.240-25A>G.

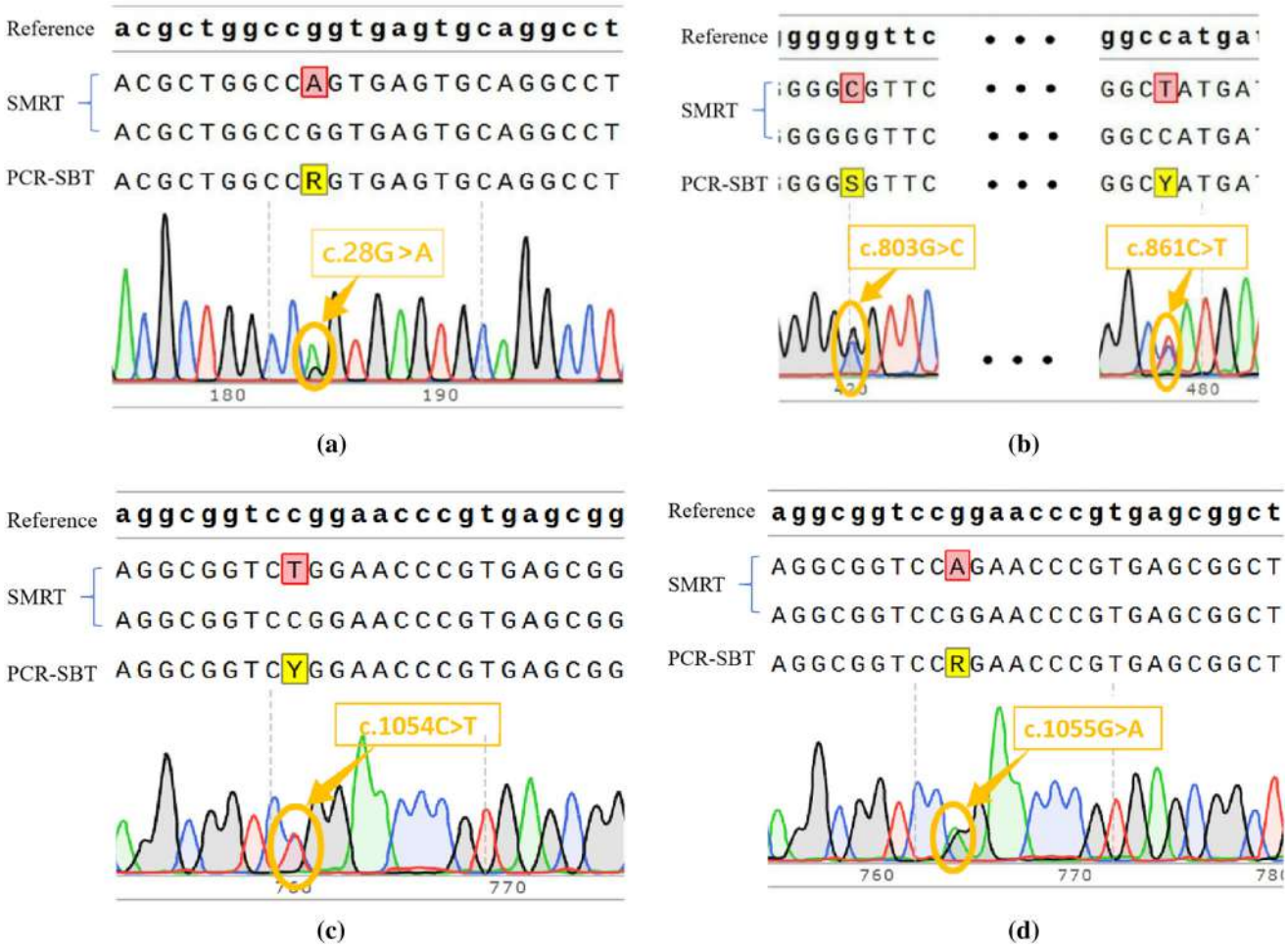

FIGURE 3 (a) In sample 1, the polymerase chain reaction sequence-based typing (PCR-SBT) method was unable to determine the haplotype because of c.28G+A heterozygosity. (b) In sample 2, the PCR-SBT method was unable to determine the haplotype because of c.803G+C and c.861C+T heterozygosity. (c) In sample 3, the PCR-SBT method was unable to determine the haplotype because of c.1054C+T heterozygosity. (d) In sample 5, the PCR-SBT method was unable to determine the haplotype because of c.1055G+A heterozygosity. SMRT, single-molecule real-time.

TABLE 6 The specific intronic region single-nucleotide variants (SNVs) for different ABO alleles (Reference sequence:GenBank ID: NG_006669.2).

Genotype	Location	Nucleotide change	RS ID	Genotype	Location	Nucleotide change	RS ID
ABO*A1.02	Promoter region	c.1-1201A>G		ABO*O1.02	Promoter region	c.1-840G>T	
	Promoter region	c.1-1326_1-1321del			Promoter region	c.1-593C>T	
	Promoter region	c.1-1011_1-975del			Intron1	c.28+175C>T	rs537895
	Intron1	c.28+748C>T	rs532436		Intron1	c.28+3660C>A	rs663054
	Intron1	c.28+1180C>T	rs507666		Intron1	c.28+3840G>A	rs551100
	Intron1	c.28+3981G>A	rs550057		Intron1	c.28+5888C>A	rs596141
	Intron1	c.28+5152G>T	rs9411378		Intron1	c.28+5984C>A	rs66697526
	Intron1	c.29-4303G>A	rs2519093		Intron1	c.28+6043T>C	rs488775
ABO*B.01	Promoter region	c.1-1948G>A			Intron1	c.28+6467T>C	rs574311
	Intron1	c.28+1429G>A	rs8176644		Intron4	c.203+359T>G	rs626792
	Intron1	c.28+4268A>G	rs79343853		Intron4	c.203+751T>G	rs638756
	Intron1	c.28+5163G>T	rs587611953		Intron4	c.204-512C>T	rs517414
	Intron1	c.28+6123T>C	rs8176662		Intron4	c.204-221G>A	rs514708
	Intron1	c.29-4746T>G	rs8176671		Intron4	c.204-192T>C	rs641943
	Intron1	c.29-4618G>A	rs8176672		Intron4	c.204-177T>G	rs641959
	Intron1	c.29-3938C>T	rs13299342		Intron5	c.240-249C>T	rs8176714
	Intron1	c.29-86G>A	rs8176693		Intron5	c.240-105C>A	rs8176717
	Intron5	c.240-25A>G	rs75179845		Intron5	c.240-28G>A	rs8176718
	Intron6	c.374+42G>T	rs8176722		Intron6	c.374+89T>A	rs2073825
	Intron6	c.374+271A>G	rs8176730		Intron6	c.374+188G>A	rs8176726
	Intron6	c.374+280C>T	rs2073823		Intron6	c.374+226C>T	rs8176727
	Intron6	c.375-425A>G	rs8176733		Intron6	c.374+235C>G	rs8176728
	Intron6	c.375-152G>A	rs7855255		Intron6	c.374+493T>C	rs8176732
	3′-UTR	c.1065+31G>A	rs8176751		Intron6	c.375-336G>A	rs8176734
	3′-UTR	c.1065+194_1064+197del			Intron6	c.375-42A>G	rs8176736
	3′-UTR	c.1065+312G>A	rs187099314		Intron6	c.375-40G>A	rs8176737
	3′-UTR downstream region	c.1065+824G>C			3′-UTR	c.1065+328_1065+350del	
	3′-UTR downstream region	c.1065+1338G>A			3′-UTR	c.1065+352_1065+386del	
	3′-UTR downstream region	c.1065+1406C>T			3′-UTR	c.1065+216A>C	rs373302536
ABO*O1.01	Promoter region	c.1-1471C>G			3′UTR	c.1065+228C>G	rs370952072
	Intron1	c.28+3285C>T	rs8176649		3′-UTR	c.1065+229A>C	rs199555421
	Intron1	c.28+3568G>A	rs7046674		3′-UTR	c.1065+345G>A	rs62636488
	Intron1	c.28+5951C>T	rs7036642		3′-UTR	c.1065+351G>A	rs62636487
	Intron1	c.29-6491T>A	rs8176668		3′-UTR	c.1065+359G>C	rs112981202
	Intron1	c.29-2184A>G	rs8176681		3′-UTR	c.1065+364T>C	rs62641788
	Intron1	c.29-1727G>A	rs8176682		3′-UTR	c.1065+372C>T	rs111926917
	Intron1	c.29-1206A>G	rs8176686		3′-UTR	c.1065+376A > G	rs62641786

(Continues)

TABLE 6 (Continued)

Genotype	Location	Nucleotide change	RS ID	Genotype	Location	Nucleotide change	RS ID
	Intron1	c.29-1054_ c.29-1038del			3′-UTR	c.1065+384A>G	rs62641785
	Intron1	c.29-746T>C	rs8176690		3′-UTR	c.1065+414C>T	rs113820458
	Intron1	c.29-658G>A	rs8176691		3′-UTR	c.1065+431G>T	rs62641782
	Intron2	c.99-363C>T	rs2073828		3′-UTR	c.1065+443C>T	rs7466265
	Intron2	c.99-356C>G	rs2073827		3′-UTR	c.1065+449A>G	rs113403969
	Intron2	c.99-186C>A	rs2073826		3′-UTR downstream region	c.1065+1337C>T	
	Intron3	c.155+575C>T	rs8176702		3′-UTR downstream region	c.1065+1411A>C	
	Intron4	c.203+115C>A	rs8176707		3′-quantitative reverse transcription PCR UTR downstream region	c.1065+1442C>T	
	Intron4	c.204-9T>C	rs4962040				
	Intron5	c.240-219G>A	rs8176715				
	Intron6	c.375-269G>A	rs7873635				

A novel B allele and an allelic recombination were identified

We obtained six haplotypes of the ABO subtypes using SMRT sequencing. The locations, nucleotide changes and amino acid changes in key variant sites are shown in Table 5. The novel allele *ABO*B.NEW (c.861C>T)*, which is a synonymous variant located in the exon 7 region, was identified in sample 2, but it is not yet clear whether this variant affects protein expression. The full sequence has been uploaded to GenBank (OR565861) by the authors. In addition, the *ABO*B.NEW(c.28G>A)* haplotype of sample 1 showed the c.28G>A variant, which was not indexed by ISBT but was submitted to BGMUT upon its discovery by Cai's team at Shanghai Blood Station [27, 28]. The characteristic sequence of *ABO*O1.01* appeared in the intron 1 to intron 5 segment of *ABO*B.NEW(c.28G>A)* haplotype, and allelic recombination may have occurred (Figure 2).

Reasons for discrepancies between the PCR-SBT and SMRT sequencing results in four samples

Four samples showed discrepancies between the PCR-SBT and SMRT sequencing results and were therefore analysed further (Figure 3). The PCR-SBT method is not able to accurately identify which haplotype the variant site is located on, but rather relies on the probability calculations of the biosignature software to infer the final genotype. Therefore, the difference in the final interpretation is not a result of inaccurate SMRT sequencing. On the contrary, SMRT sequencing can be used to sequence both haplotypes of a chromosome pair separately and accurately distinguish the haplotype in which the variant site is located, which is an advantage over traditional sequencing methods.

The specific intronic region SNVs for different ABO alleles

Intronic regions and regions upstream and downstream of coding regions were analysed for 42 ABO haplotype sequences. The SNVs in these regions that were specific for different ABO alleles were counted. Among them, *ABO*A1.02*, *ABO*B.01*, *ABO*O1.01* and *ABO*O1.02* had 8, 21, 19 and 66 specific intronic region SNVs, respectively, as shown in Table 6.

Comparative mapping of ABO allele haplotypes

Variants in 42 ABO haplotype sequences were summarized and plotted for comparison (Figure 4). For various ABO subtype genes, specific variant sites located in exonic regions as well as in intronic regions can be visualized. Notably, related sequences upstream and downstream of the coding region were also obtained via SMRT sequencing, and these sequences also exhibited specific sequence patterns.

DISCUSSION

With the rapid development of molecular biology techniques, an increasing number of studies on ABO genes have been reported.

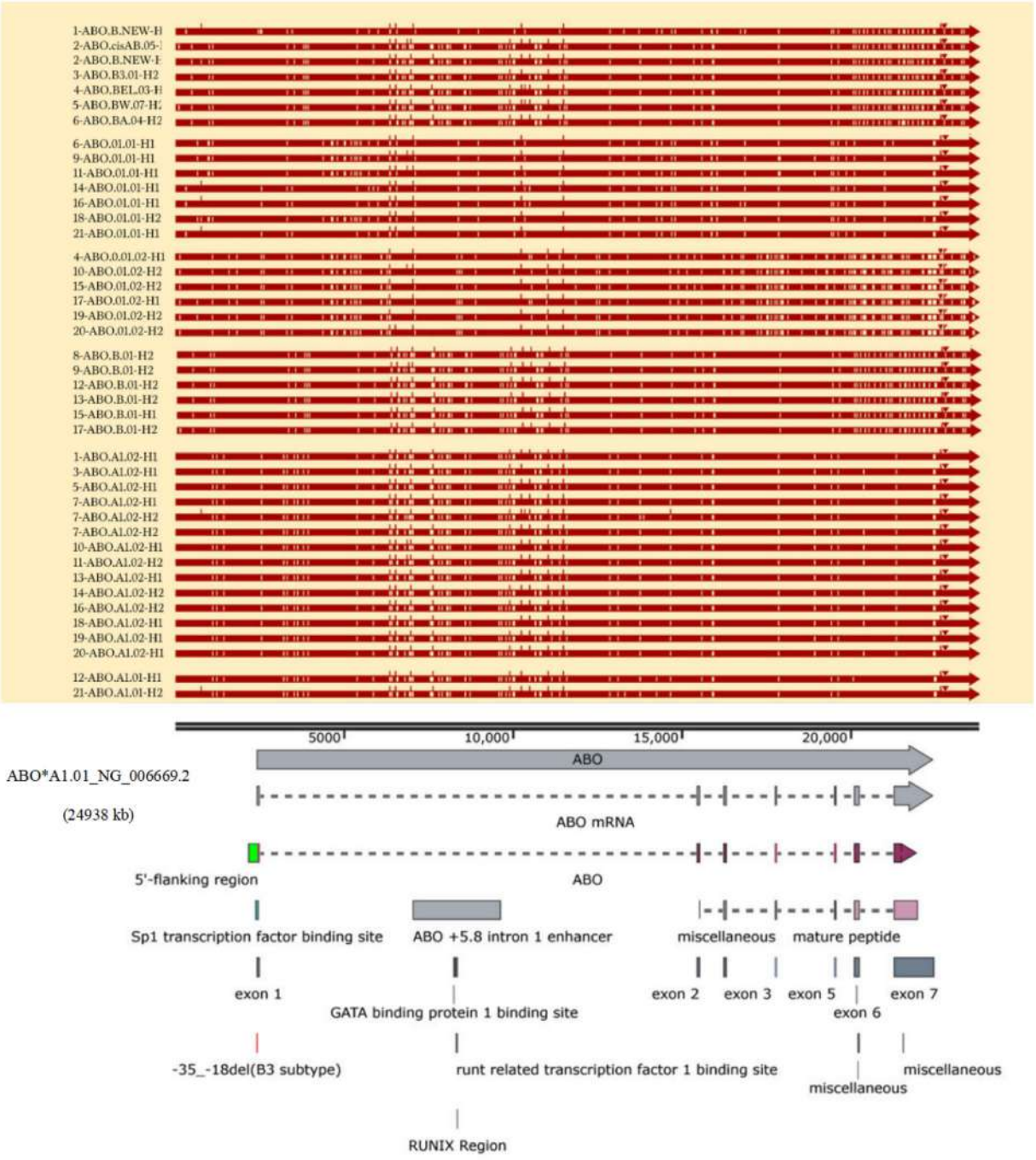

FIGURE 4 Comparative mapping of ABO allele haplotypes.

However, most of these studies are still limited to exon sequences and do not include the analysis of introns or sequences upstream and downstream of the coding region; moreover, reports on the full-length genes of rare ABO subtypes are even scarcer. The total length of the ABO blood group gene is approximately 25 kb, of which intron 1 is the longest, at up to 13 kb, making it difficult to obtain the complete gene sequence using conventional sequencing techniques. Although studies have successfully used NGS technology to obtain the full-length sequence of the ABO gene, haplotype analysis for the ABO gene must still be limited to samples of individuals with a homozygous genotype, as NGS technology cannot distinguish between haplotypes [13]. Compared to previous generations of sequencing technology, the average read length of third-generation sequencing technology is as high as 100 kb, and therefore this sequencing technology can successfully distinguish between haplotypes [15–17], which is a unique advantage when used to study ABO gene sequences. In addition, Oxford nanopore technology (ONT) is equally capable of long-read sequencing and distinguishing haplotypes. However, due to the high error rate (the raw error rate is around 5%, and R10.3 chip delivers up to 96% sequencing accuracy), ONT is not

suitable for accurate sequencing of ABO genes but is more suitable for rapid sequencing in emergency situations. Although SMRT sequencing has base read errors, these errors are random and can be self-corrected by Circular Consensus Sequencing (sequencing accuracy >99.9%). Therefore, in the present study, the complete sequence of the coding region of the ABO gene, as well as sequence upstream and downstream of the coding region, of the 21 samples was obtained using SMRT sequencing and differentiated into 42 haplotypes. Comparison with PCR-SBT showed consistent detection results, demonstrating the high accuracy of SMRT sequencing.

Full-length sequences were obtained from six samples with rare ABO subtypes using SMRT sequencing. These complete ABO subtype sequences have been poorly accessed and studied to date. A new ABO allele (*ABO*B.NEW, c.861C>T*) with a key synonymous variant was identified. In a recent study by Clark, it was found that synonymous variants could alter a protein folding mechanism in vivo, leading to changes in cellular fitness [29]. However, there are no studies mentioning whether synonymous variants in ABO alleles affect antigen expression. So whether the c.861C>T variant affects protein expression needs to be further investigated. In sample 1, an ABO allele not included in the ISBT (*ABO*B.NEW, c.28G>A*) had a variant profile and phenotype consistent with those of the study by Cai's team [27], who evaluated the expression of the cDNA containing the variant by quantitative reverse transcription PCR and concluded that the c.28G>A variant affects splicing [28]. We also performed an elution test on this sample and confirmed the weak expression of B antigens. However, the present study revealed that the characteristic sequence of *ABO*O1.01* appeared in this haplotype, located in the segment from intron 1 to intron 5, which was considered a possible allelic recombination and was also consistent with the findings of He's team [13]; therefore, the molecular mechanism of this isoform needs to be further investigated.

Different ABO genes have specific SNVs, whether in the exonic region, the intronic region or upstream and downstream of the coding region. However, studies on specific SNVs in intronic regions or upstream and downstream of coding regions are rare. Forty-two full-length ABO haplotype sequences were distinguished by SMRT sequencing, and a total of 114 specific SNVs in the intronic region and upstream or downstream of the coding region were analysed using these ABO haplotype collections. These specific SNVs are useful for evaluating ABO allele splicing and genotyping during sequencing, as well as for the detection of ABO gene recombination and other structural variants, and for the design of specific primers for ABO gene analysis. For example, a recent study by Matzhold et al. established an allele-specific long-range SBT method based on four SNP positions in introns 1, 2 and 4 of the ABO gene that were found to be suitable for distinguishing the different ABO haplotypes [30]. For some rare ABO subtypes, there may also be some specific intronic SNVs and sequence patterns [31–38]. However, due to the insufficient sample size, statistical analysis could not be performed, and additional samples of the same genotype need to be collected for full sequence acquisition and analysis in subsequent studies. Moreover, the samples collected to date remain limited; to establish a full-length standardized reference sequence set of common ABO gene subtypes in the Chinese population, a larger number of samples covering a wider range of genetic diversity should be collected in subsequent studies.

In summary, SMRT sequencing allows access to accurate and complete ABO gene sequences, differentiation of haplotypes and identification of allelic recombination. The 7 rare and 35 common ABO haplotype full-length sequences collected in this study and made publicly available should provide a valuable resource for molecular diagnosis and genetic evolution studies of ABO genes in Chinese populations.

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Z.W. performed the research and wrote the first draft of the manuscript, Z.W. and Y.C. designed the research study and collected the sample, Z.W., Y.C. and Y.X. acquired and analysed the data and M.B. 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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SUPPORTING INFORMATION

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

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SHORT REPORT

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Antibodies against glutamic acid decarboxylase in intravenous immunoglobulin preparations can affect the diagnosis of type 1 diabetes mellitus

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Abstract

Background and Objectives: Intravenous immunoglobulins (IVIgs) contain various autoantibodies, including those against glutamic acid decarboxylase (GADAb), a valuable biomarker of type 1 diabetes mellitus. Passive transfer of GADAb from IVIgs to patients poses a risk of misdiagnosis, and information on the specific titres of GADAb and their impact on diagnostic accuracy remains limited. This study aimed to provide further insights into the origin of GADAb detected in patient serum following IVIg infusion.

Materials and Methods: GADAb titres in IVIg products from Japan and the United States were measured using enzyme-linked immunosorbent assay-based assays. For reliable quantification, GADAb titres in pooled plasma were quantified and compared with those in the IVIg products. The determined titres were used to estimate the likelihood of passively detecting acquired GADAb in individuals receiving IVIgs.

Results: GADAbs were prevalent in IVIg products; however, the titres varied significantly among different lots and products. Importantly, IVIg-derived GADAb was estimated to remain detectable in patient serum for up to 100 days following a dosage of 2000 mg/kg.

Conclusion: Clinicians should consider that IVIg preparations may contain GADAb, which can lead to false-positive results in serological assays. Careful interpretation of the assay results is key to the definitive diagnosis of type 1 diabetes mellitus.

Keywords

anti-GAD antibody, intravenous immunoglobulin, type 1 diabetes mellitus

Highlights

- Intravenous immunoglobulins (IVIgs) contain various autoantibodies, including those against glutamic acid decarboxylase (GADAb), a valuable biomarker of type 1 diabetes mellitus.
- Titres of autoantibodies targeting GADAb vary among different IVIgs and among batches of the same product.
- GADAb originating from IVIgs can persist for a couple of months in IVIg recipients.

Tatsuki Miyamoto and Yuki Fukunaga contributed equally to this work.

INTRODUCTION

Intravenous immunoglobulins (IVIgs) are concentrated as polyclonal immunoglobulin G (IgG) preparations. They help restore and modulate immune system functions and are prescribed for the treatment of various diseases ranging from infections to autoimmune disorders [1]. IVIg contains millions of IgG antibodies, including autoantibodies used to diagnose autoimmune disorders [2, 3]. The presence of autoantibodies in IVIg preparations presents a diagnostic challenge, as these antibodies can be passively transferred to recipients, potentially leading to the misdiagnosis of autoimmune conditions [2–4]. Therefore, characterizing the types of autoantibodies in IVIgs is important for enhancing diagnostic precision in patients treated with IVIgs.

Autoantibodies targeting glutamic acid decarboxylase (GADAbs) are particularly relevant because of their valuable role as a biomarker of type 1 diabetes mellitus. Several studies have confirmed the presence of GADAbs in IVIg products [3, 5, 6]. Clinical observations, including positive GADAb conversions in X-linked agammaglobulinemia recipients of IVIg, further support these findings [7]. These studies suggest that IVIg administration can lead to iatrogenic GADAb positivity; however, limitations remain, such as the reliability of the methods used to quantify GADAb and the lack of comprehensive data on variations in GADAb titres across different IVIg lots and brands. These issues continue to impede the accurate evaluation of the potential impact of IVIg therapy on the diagnostic accuracy of type 1 diabetes mellitus.

This study aimed to gain further insight into the titres of GADAbs in IVIg preparations and assess the potential for detecting passively acquired GADAbs in the serum of IVIg recipients. Our findings will help reduce the misinterpretation of serological test results, thereby minimizing the risk of misdiagnosis and inappropriate treatment of type 1 diabetes mellitus.

MATERIALS AND METHODS

Preparations of IVIg products and pooled plasma

Five domestic donor-derived IVIg product lots (Product A; Japan Blood Products Organization, Tokyo, Japan) and one US donor-derived IVIg product lot (Product B) were used in this study. Because both were supplied as pre-dissolved liquids with identical IgG concentrations of 100 mg/mL, dissolution procedures were not required. Five distinct sets of pooled plasma batches were prepared, each generating a single lot of Product A. Each set consisted of a combined pool of plasma composed of eight or nine distinct pooled plasma units used to manufacture a single lot of IVIg.

Determination of GADAb titres in IVIg preparations and associated pooled plasma

Serial dilution of Products A and B was preliminarily performed using phosphate-buffered saline containing 1% bovine serum albumin in twofold increments. After confirming linearity, the GADAb titres of eightfold diluted samples were quantified in triplicate. For comparison, GADAb titres in five sets of pre-combined pooled plasma samples corresponding to the ingredients of five lots of Product A were also measured. Measurements were performed in triplicate without dilution.

All assays, except for the linearity confirmation of Product A, were conducted in an external laboratory to ensure objectivity. Assays were performed using the GADAb enzyme-linked immunosorbent assay (ELISA) Cosmic kit (Cosmic Corporation, Tokyo, Japan), according to the manufacturer's protocol, which is approved for in vitro diagnostics and has a quantification range of 5.0–2000 U/mL. A cut-off value of 5.0 U/mL represents GADAb titres in non-diabetic individuals.

Estimation of serum titres in IVIg recipients and detection period

The post-infusion pharmacokinetics were estimated as described previously [2]. In summary, the serum titres at three time points, namely immediately after infusion (Day 0), after equilibration (Day 5) and after the IgG half-life period (Day 22), were simulated using the following three equations, which consider the GADAb titres and dosage of each IVIg product:

$$\text{Serum titre immediately after infusion(Day 0)} = \frac{\text{GADAb titres in each IVIg lot} \times \text{IVIg dosage}}{100 \times 40}.$$

$$\text{Serum titre after equilibration (Day 5)} = \text{Serum titre just after infusion} \times 0.4.$$

$$\text{Serum titre after IgG half-life(Day 22)} = \text{Serum titre after equilibration} \times 0.5.$$

The following assumptions were made for the equations used in this study: (i) IVIg was administered at a dose of 200, 400, 800, 1000, 1200, 1600 or 2000 mg/kg body weight; (ii) IVIg contained 100 mg/mL of IgG; (iii) each recipient's plasma volume was estimated at 40 mL/kg body weight; (iv) equilibration of IgG between intra- and extravascular components occurs 5 days after administration; (v) 40% of IgG molecules remained within blood vessels, while 60% resided in extravascular spaces and (vi) the half-life of IgG molecules was 22 days.

The detection period, defined as the duration for which passively acquired GADAb from IVIg remained measurable in the recipient's serum, was calculated using the following equation:

$$\text{Cut-off value} = \text{Serum titre after equilibration} \times \left(\frac{1}{2}\right)^{\frac{\text{Detection period}}{22}}.$$

Ethics statement

This study was approved by the ethics committee of the Japan Blood Products Organization.

RESULTS

High-dose hook effect confirmed with IVIg preparations

Two main techniques, radioimmunoassay (RIA) and ELISA, are commonly used to quantify GADAb titres in human serum. We opted for ELISA in this study due to the December 2015 shift from RIA- to ELISA-based assays in Japan [8, 9]. To determine whether this ELISA method is applicable to IVIg preparations containing 10% IgG, we preliminarily assessed the linearity of our serological assay system by measuring serially diluted domestic donor-derived IVIg products (Product A). We observed a high-dose hook effect, in which undiluted and two-fold diluted samples yielded falsely lowered GADAb levels (Figure 1a, blue line). This reproducible phenomenon is not brand specific, as we also observed with a US donor-derived IVIg product (Product B; Figure 1a, orange line). This finding highlights the importance of preliminary examinations for reliable GADAb measurement in IVIg products.

GADAb titres in IVIg preparations and associated pooled plasma

Following confirmation of assay linearity, we determined an optimal dilution factor of 1:8 for subsequent analyses and measured GADAb titres. Quantification of the IVIg products revealed substantial yet variable GADAb levels across the different lots and preparations (Table 1).

To further enhance the reliability of our results, we prepared five sets of pooled plasma samples, each generating a single lot of Product A. Considering that IVIg preparations contain 10-fold more IgG (100 mg/mL) than healthy donor serum (ca. 10 mg/mL), GADAb titres were expected to be roughly 10-fold higher in IVIg. This predicted

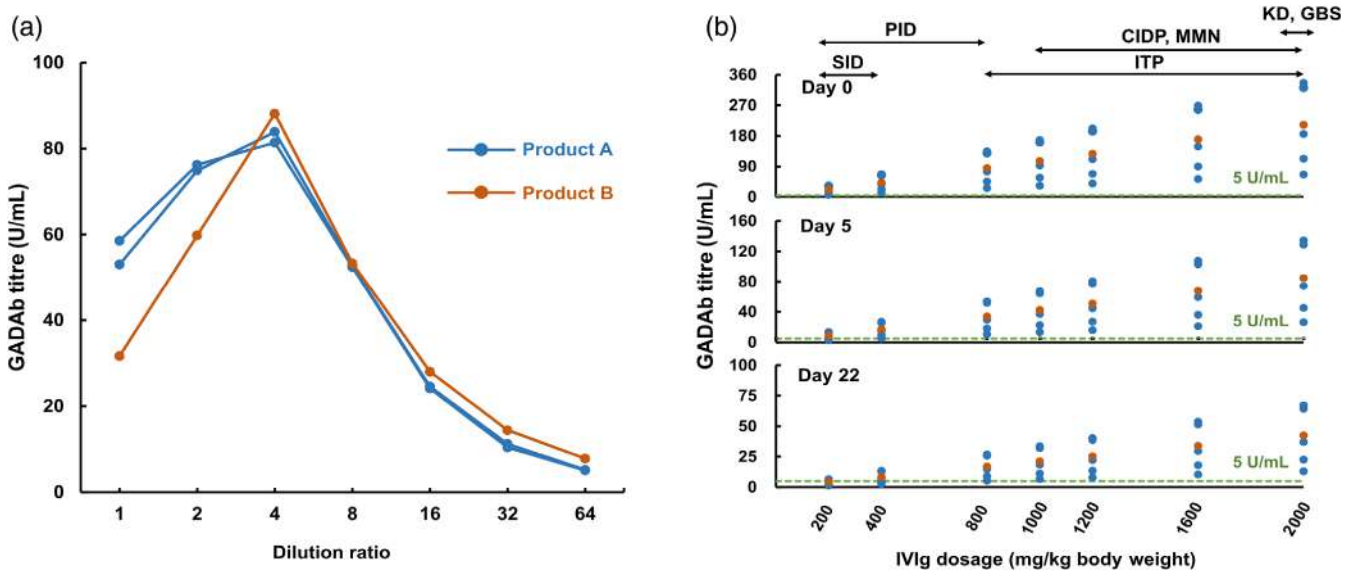

FIGURE 1 Autoantibodies targeting glutamic acid decarboxylase (GADAb) titres in intravenous immunoglobulin (IVIg) preparations and modelled titres in IVIg recipients. (a) The GADAb titres of two IVIg brands were measured after serial dilution in preliminary experiments. The quantification results of Product A are indicated by blue lines ($n = 2$) while the quantification results of Product B are represented by orange line ($n = 1$). (b) Estimated GADAb titres in IVIg recipients at Days 0, 5 and 22. The blue circles represent five lots of Product A, while the orange circles represent one lot of Product B. The assay cut-off is shown with green broken lines. Representative IVIg indications are shown with abbreviations: CIDP, chronic inflammatory demyelinating polyradiculoneuropathy; GBS, Guillain–Barré syndrome; ITP, primary immune thrombocytopenia; KD, Kawasaki disease; MMN, multifocal motor neuropathy; PID, primary immunodeficiency syndromes; SID, secondary immunodeficiencies.

TABLE 1 GADAb titres in IVIg products and associated pooled plasma.

Test items		Product A					Product B
		A-i	A-ii	A-iii	A-iv	A-v	B-i
Titre (U/mL)	IVIg	371.5 ± 10.7	672.5 ± 17.1	644.5 ± 15.1	225.9 ± 5.2	132.0 ± 2.1	424.5 ± 3.3
	Pooled plasma	38.5 ± 1.2	51.7 ± 1.6	57.5 ± 3.1	25.0 ± 0.5	11.0 ± 0.6	-[a]

Abbreviations: GADAbs, autoantibodies targeting glutamic acid decarboxylase; IVIg, intravenous immunoglobulin.
[a]Pooled plasma comprising a Product B lot was not specified and not measured.

TABLE 2 Estimated detection period of GADAb in IVIg recipients (days).

	Product A					Product B
Dosage (mg/kg body weight)	A-i	A-ii	A-iii	A-iv	A-v	B-i
200	<22	31.4	30.1	<5	<5	<22
400	34.6	53.4	52.1	<22	<22	38.8
800	56.6	75.4	74.1	40.8	23.7	60.8
1000	63.7	82.5	81.1	47.9	30.8	67.9
1200	69.4	88.3	86.9	53.6	36.6	73.7
1600	78.6	97.4	96.1	62.8	45.7	82.8
2000	85.7	104.5	103.1	69.9	52.8	89.9

Abbreviations: GADAbs, autoantibodies targeting glutamic acid decarboxylase; IVIg, intravenous immunoglobulin.

increase was observed across the lots (Table 1), confirming the accuracy of our measurement approach.

These observations suggest that IVIg administration is likely to influence diagnoses to varying degrees.

Possible detection durations of passively acquired GADAb

To assess the diagnostic impact of passively acquired GADAb, we simulated the decline in serum GADAb titres in IVIg recipients at three key time points. We found that passively acquired GADAb was detectable after IVIg administration (Figure 1b) and remained measurable in patient serum for more than 100 days following the most commonly used dosage of 2000 mg/kg (Table 2).

DISCUSSION

Type 1 diabetes mellitus is a chronic autoimmune disorder characterized by the destruction of insulin-producing cells in the pancreas, leading to the production of autoantibodies against the pancreatic components, including GADAb, insulinoma-associated antigen-2 and zinc transporter 8 [9, 10]. GADAbs are the most extensively employed in Japan, due to their uncomplicated measurement procedures and coverage under healthcare insurance. Its utilization is particularly useful for distinguishing a subtype of type 1 diabetes mellitus, known as slowly progressive insulin-dependent diabetes mellitus, from type 2 diabetes mellitus [8, 9]. This allows for an accurate diagnosis by physicians and appropriate treatment of patients.

This study revealed the presence of GADAb in IVIg, with significant variations in titres across different production lots and brands. Importantly, passively acquired GADAbs were estimated to persist for several months. These findings highlight the importance of considering recent IVIg administration, maintaining patient follow-up and quantifying GADAb levels in IVIg products for accurate diagnosis when interpreting positive GADAb test results.

The bivalent ELISA is an established method for measuring GADAb titres in human serum; however, we found that this methodology was not directly applicable to IVIg products, as we observed an unexpected high-dose hook effect (Figure 1a). As the principles and procedures of bivalent ELISA are common across kits, our observations identified a possible risk of underestimating GADAb levels in IVIg, potentially leading to misidentification of the antibody source as either patient-produced or IVIg-originated.

IVIgs are purified from large, pooled plasma collected from healthy individuals, often thousands of blood donors. As donors complete questionnaires designed to exclude individuals with diabetes mellitus or receiving relevant treatments, it is difficult to identify the exact reason for GADAb detection in all sets of pooled plasma and IVIg products. A possible explanation might be that GADAbs originate from a small subset of non-diabetic individuals, as reported previously [11], which could then become concentrated during the fractionation and purification steps of IVIg manufacturing.

The neonatal Fc receptor (FcRn) plays a crucial role in extending IgG half-life by binding to and recycling IgG antibodies. It is recognized as a significant factor contributing to the variability in IgG clearance rate observed among different therapeutic monoclonal antibodies [12]. Consequently, genetic differences in FcRn expression might impact the variability in GADAb clearance. Previous studies have highlighted the presence of five distinct alleles in the 5′ flanking region of the *FCGRT* gene, which encodes the FcRn protein, characterized by variable number of tandem repeats (*VNTR1–5*). Among these alleles, *VNTR2* and *VNTR3* are the most common in Japanese populations, with frequencies of 3.2% and 96.8%, respectively. The *VNTR3* variant demonstrates higher transcriptional activity compared with *VNTR2* [13, 14]. This finding is consistent with clinical studies, which showed prolonged degradation of administered IgG in patients with *VNTR3/3* genotypes as opposed to those with non-*VNTR3/3* genotypes [14]. Consequently, passively acquired GADAbs might clear more rapidly in patients with *VNTR2/3* alleles, and it is essential to note that the simulation results presented in Table 2 are subject to change.

While GADAbs occur in neurological disorders, including stiff person syndrome and cerebellar ataxia, IVIg administration poses a minimal risk of triggering them because of significantly lower simulated GADAb levels in recipients than in patients with these conditions [5, 15]. This is further supported by the absence of such case reports within our

pharmacovigilance department, despite extensive IVIg use in Japan. Further study and surveillance are needed to assess the potential for these disorders associated with passively acquired GADAb.

In summary, our study revealed the presence of GADAbs in IVIgs, demonstrating that IVIg administration can produce false-positive results in GADAb tests. IVIg-derived GADAbs are estimated to remain detectable in patient serum for up to 100 days, following the most commonly used dosage of 2000 mg/kg. Clinicians should be aware of this risk to avoid the misdiagnosis of diabetes mellitus and should carefully establish a diagnosis by considering other clinical factors.

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T.M., A.M. and K.M. conceived the research, T.M. and Y.F. designed the experiments, Y.F. performed the experiments, T.M. and Y.F. analysed the data, T.M. wrote the manuscript, A.M. and K.M. supervised the research and reviewed 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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SHORT REPORT

Vox Sanguinis ISBT International Society of Blood Transfusion

Routine maternal ABO/Rhesus D blood typing can alert of massive foetomaternal haemorrhage

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Abstract

Background and Objectives: Spontaneous massive foetomaternal haemorrhage (SM-FMH) is a rare yet critical condition that poses substantial risk to foetal health and survival. Existing data indicate that many cases may be undiagnosed. The current study aimed to investigate and validate the utility of identifying mixed field red blood cell (RBC) agglutination during maternal blood typing as a diagnostic aid for SM-FMH.

Materials and Methods: Retrospective analysis of medical records from neonates born at our tertiary, university-affiliated medical centre between 2016 and 2023 was performed. Diagnosis of SM-FMH was based on neonates born with severe anaemia (haematocrit [HCT] <15%) within the first 24 h post-delivery with positive maternal Kleihauer–Betke (KB) test. Maternal ABO/Rhesus D (RhD) blood typing results were scrutinized with the primary objective of assessing the ability to identify dual RBC populations in cases clinically diagnosed with SM-FMH.

Results: Among 29,192 neonates studied, a mere 0.02% (5 cases) exhibited severe SM-FMH. Notably, a mixed field RBC agglutination was discerned in 80% (4/5) of these cases.

Conclusion: This study underscores the significance of detecting mixed field RBC agglutination during antepartum maternal ABO/RhD blood typing as a potential indicator for SM-FMH. Increased awareness among blood bank technology specialists and obstetricians regarding these laboratory findings could prove instrumental in saving foetal lives.

Keywords

blood typing, foetomaternal haemorrhage, mixed field agglutination

Highlights

- The diagnosis of spontaneous massive foetomaternal haemorrhage (SM-FMH) may be complex and require high index of suspicion.
- A finding of mixed field red blood cell (RBC) agglutination on routine maternal ABO/Rhesus D (RhD) blood typing should alert the physician to suspect SM-FMH.
- A finding of mixed field RBC agglutination on maternal ABO/RhD blood typing may supplement foetal ultrasound Doppler and foetal monitoring in the diagnosis of SM-FMH.

INTRODUCTION

Spontaneous massive foetomaternal haemorrhage (SM-FMH) is defined as transfer of foetal blood into maternal circulation [1]. This phenomenon, virtually occurring in all gravidas, is of clinical significance only in 0.3% of pregnancies [2]. Up to 14% of foetal demise is estimated to result from SM-FMH [3].

Based on the blood loss volume, FMH is classified as non-significant or massive. A standardized definition of SM-FMH has not been established yet. While the foetal blood transfer exceeding 30 mL is considered clinically significant, it does not necessarily correlate with severe foetal morbidity and mortality [4]. One factor that has an impact on the clinical significance is the timeframe during which haemorrhage occurs. Foetuses experiencing sudden, acute bleeding may not have the chance to activate compensatory mechanisms, as opposed to those with slower, more chronic blood loss [1]. Christensen et al. [5] defined SM-FMH as day-of-birth neonatal haematocrit (HCT) levels <30% combined with a positive maternal Kleihauer–Betke (KB) test [6] or by measuring above 2% of foetal blood in maternal circulation, using flow cytometry [7].

The SM-FMH diagnosis is complex, requiring rapid and accurate assessments, as this may be detrimental to the foetus. Yet gravidas may present with non-specific symptoms during the evaluation [2]. Current clinical diagnostic methods include foetal cardiotocography and measurement of middle cerebral artery peak-systolic velocity (MCA-PSV) [8]. Despite the available diagnostic tools, numerous severe FMH cases may be missed [9].

The standard ABO/Rhesus D (RhD) blood typing test, conducted at the blood bank, is aimed to detect the presence of A, B and D antigens on individual's red blood cells (RBCs) [10]. While test results are generally straightforward, in some clinical settings, an additional RBC population (either antigen-positive or -negative) can be revealed (mixed field RBC agglutination). Such finding may be attributed to several clinical scenarios, including blood transfusion of a matched but not identical ABO/RhD unit of RBCs, or haematopoietic stem cell transplantation with major, minor or bidirectional ABO/RhD incompatibility. Since the finding of a mixed field RBC agglutination (presence of two RBC populations) in routine ABO/RhD blood typing is usually unexpected, such cases are thoroughly investigated by blood bank technology specialists.

Cases

The index event describes a 27-year-old woman at 34 + 2 weeks of her second pregnancy who arrived at our obstetric department with complaints of reduced foetal movements. Following non-reassuring foetal monitoring and ultrasound, she was hospitalized for evaluation of foetal well-being. As part of the work-up, a routine ABO/RhD blood typing was performed. While foetal monitoring was non-reassuring, the ABO/RhD blood typing revealed the presence of two RBC population (mixed field agglutination). The significance of this finding was transferred from the blood bank specialist to the attending obstetrician. As the patient had not received any recent blood transfusion and considering the clinical presentation, suspicion of possible SM-FMH was raised, and the patient underwent emergency caesarean section (ECS). The neonate was born with severe anaemia, and the KB test demonstrated 7% of foetal cells in maternal circulation.

Based on this case, we aimed to evaluate the possible contribution of mixed field RBC agglutination finding during maternal ABO/RhD blood typing, assisting in the diagnosis of SM-FMH. After approval from the local ethical committee, we analysed electronic medical records (EMRs) from our tertiary, university-affiliated medical centre, for the period between 2016 and 2023. We searched for neonates presenting with severe anaemia (HCT <15%) within the first 24 h post-delivery. The SM-FMH diagnosis was considered only in case of a positive maternal KB test [6, 7]. Notably, according to our institutional regulations, the KB test is performed to all mothers delivering neonates with HCT <30%. KB assay was conducted by introducing an acidic buffer of pH 3.3 to dried and fixated maternal peripheral blood and analysing the remaining stained foetal RBCs on a background of 'ghosted' maternal RBCs under light microscope. Normal reference values range between 0% and 0.1%. In addition, flow cytometry quantitation of foetal cells was applied to validate FMH and estimate the volume of foetal bleeding.

Detailed results of antepartum ABO/RhD blood typing, conducted using ID-cards (Figure 1) and the IH-1000 automated analyser (Bio-Rad Laboratories, Hercules, CA, USA), were available at the Blood Bank archive. Hence, in this study, ABO/RhD blood typing data, including images, were re-evaluated for all cases of confirmed SM-FMH.

The EMR review identified among 29,192 neonates born during the study period, 5 babies (0.02%) with HCT levels <15%. In all these cases, the KB test was positive, with >1% of foetal cells found in the maternal blood. Due to existing ABO/RhD incompatibility between the mother and the neonate in all five cases, the evaluation for the presence of a mixed field RBC agglutination population was feasible. A mixed field RBC agglutination was detected in 4/5 (80%) mother-neonate pairs, in whom the KB test result was ≥3.3% (Table 1). In the first case, as described above, a male infant was born on week 34 + 2 by ECS, with bradycardia (60–100 beats/min), no respiratory efforts and extreme paleness. He was immediately started on mechanical ventilation (MV) and moved to the Neonatal Intensive Care Unit (NICU). His HCT at birth was 11%, and he received RBC transfusion. One day post-transfusion, MV was discontinued and he was discharged in a good general condition 32 days post-delivery. In this case, a mixed field RBC agglutination was found in A-antigen typing, as the foetus' RBCs were type O and mother's were type A (Figure 1a).

In the second case, a term female infant was born on week 37 by ECS due to abnormal monitoring results. All measured neonatal parameters were normal apart from extreme pallor. Because of the postnatal HCT level of 11.3%, she was transfused with RBCs and discharged in a good general condition 5 days after birth. In this case, a mixed field RBC agglutination was related to the RhD difference between the mother and the foetus (Table 1; Figure 1b).

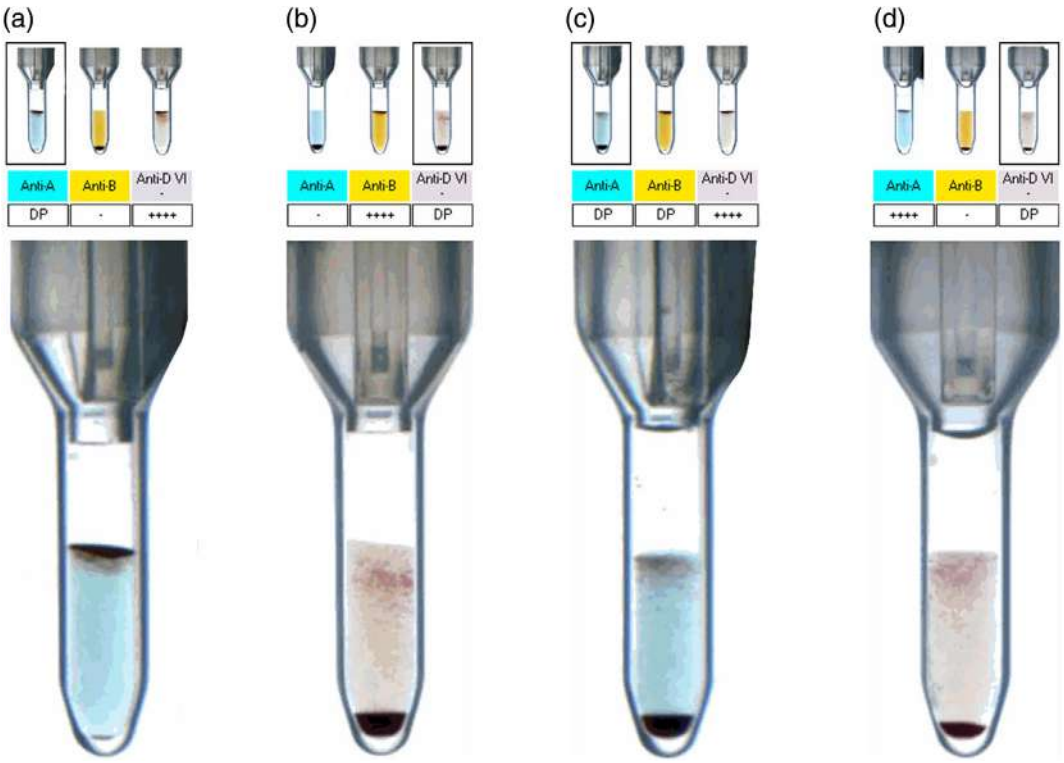

FIGURE 1 Maternal ABO/Rhesus D (RhD) blood typing results showing presence of mixed field red blood cells (RBCs) agglutination in ID-cards. The mixed field agglutination may be seen as a line in the upper part of the gel column, presenting agglutinated (antigen-positive) RBCs and as a pellet in the bottom part of the gel column, presenting non-agglutinated (antigen-negative) RBCs. (a) Mother's RBCs are type A, and a population of type O cells, originating from the foetus, is seen in the bottom part of the A-antigen typing column. (b) Both mother's and the foetus's RBCs are type B, but the mother is RhD-negative (a pellet in the bottom part of the RhD-antigen typing column) and RhD-positive cells of the foetus are seen in the upper part of the gel column. (c) Mother's RBCs are type B, and a population of type A cells, originating from the foetus, is seen in the upper part of the A-antigen typing column. (d) Both mother's and foetus's RBCs are type A, but the mother is RhD-negative (a pellet in the bottom part of the RhD-antigen typing column) and RhD-positive cells of the foetus are seen in the upper part of the RhD-antigen typing column.

TABLE 1 Cases of spontaneous massive foetomaternal haemorrhage with mixed field agglutination in ABO/RhD blood typing.

Case	G/A week	Birth weight (g)	APGAR score	Neonate haematocrit (%)	Neonate blood type	Maternal blood type	Mixed field	KB result (%)	PRBC transfusion (mL)
1	34 + 2	2199	1/5	11.0	O POS	A POS	YES	7	150
2	37	3095	8/9	11.3	B POS	B NEG	YES	8.90	50
3	36 + 1	2115	4/5	12.0	A POS	B POS	YES	3.30	100
4	38 + 5	3365	1/5	12.0	A POS	A NEG	YES	5.80	100
5	33 + 4	2055	1/4	10.1	AB NEG	B NEG	NO	1.2	150

Note: APGAR denotes APGAR score at 1 and 5 min after birth.
Abbreviations: APGAR, appearance, pulse, grimace, activity and respiration; G/A, gestational age; KB, Kleihauer–Betke; NEG, negative; POS, positive; PRBC, packed red blood cells; RhD, rhesus D.

In the third case, a term female infant was born by ECS due to reduced foetal movements. She was extremely pale, with decreased muscle tone, no respiratory efforts and required short cardio-resuscitation. The neonate was immediately moved to the NICU and transfused with RBCs due to HCT of 12%. She was discharged in a good general condition 8 days post-delivery. In this case, the mother was type B, but a population of type A cells, originating from the foetus, was detected in her blood during routine ABO/RhD blood typing (Table 1; Figure 1c).

In the fourth case, a term male infant was born by ECS due to reduced foetal movements. He was extremely pale, with decreased muscle tone, bradycardia (60 beats/min) and no respiratory efforts. He required MV, was immediately moved to the NICU and received RBC transfusion due to HCT of 12%. He was discharged in a good general condition 8 days post-delivery. ABO/RhD blood typing results demonstrated foetal RhD-positive cells in the blood sample of the A RhD-negative mother (Table 1; Figure 1d).

The fifth case presented a patient at 33 weeks of gestation, admitted to our hospital for decreased foetal movements. Sonographic biophysical profile was adequate; however, foetal monitoring was non-reassuring. The patient was hospitalized for continuous foetal monitoring and antenatal corticosteroids. Following several hours, repeated decelerations were noted in the foetal heart rate, and the patient underwent ECS. The neonate was immediately transferred to the NICU and received RBC transfusion due to HCT of 10.1%. The KB test was positive. ABO/RhD blood typing results revealed B RhD-negative in the mother and AB RhD-negative in the neonate. KB was 1.2%, and no mixed field agglutination was detected (Table 1).

DISCUSSION

The current report presents a novel application of routine ABO/RhD blood typing results for the clinical diagnosis of SM-FMH, which is considered one of the most life-threatening events in obstetrics. The SM-FMH incidence identified in this study (1:5000 births), is consistent with previously reported values [9]. The finding of a mixed field RBC agglutination population in maternal blood along with the antepartum clinical context may contribute to early diagnosis of this obstetric complication, requiring immediate intervention. The described phenomenon was observed in the majority of SM-FMH cases identified in this study.

The prenatal diagnosis of SM-FMH is challenging and may be missed in approximately 40% of cases [9]. The most common complaint reported in the literature is decreased perception of foetal movements. Likewise, in our study, all confirmed SM-FMH cases have been identified due to such complaint [10]. Objective findings indicative of SM-FMH include alterations in foetal monitoring data, such as cardiotocographic parameters, specifically, foetal heart rate variability and the sinusoidal pattern, suggesting foetal anaemia [4]. Yet these changes in the foetal heart rate are recorded in <10% of foetal anaemia cases [2]. In late 1990s, MCA-PSV measurement was introduced to clinical practice as a non-invasive tool for foetal anaemia detection [8], demonstrating very high sensitivity [11]. While this modality is considered most efficient in foetal anaemia diagnosis, it requires special personnel training. KB is the gold standard laboratory test used for FMH confirmation. Yet similar to MCA-PSV, it requires specialists with technical expertise, who are not readily available 24/7.

At the same time, maternal ABO/RhD blood typing is an integral part of routine lab assessments, performed at our centre for all women during antepartum hospitalization. Our personal experience, after examining over 29,000 blood samples, enabled us to detect a minimum of 3.3% of foreign RBCs in an evaluated blood sample during ABO/RhD typing. However, the identification of this phenomenon requires training and vigilance on the part of the blood bank staff.

To the best of our knowledge, this is the first report linking the finding of a mixed field RBC agglutination in ABO/RhD blood typing to the SM-FMH diagnosis.

The proposed approach, allowing to detect 4/5 SM-FMH cases in our cohort, is limited by the requirement of ABO/RhD incompatibility between the mother and the foetus. As previously reported, rates of maternal and foetal ABO incompatibility varies between ethnicities. In Caucasians, ABO incompatibility is present in approximately 12% of pregnancies, and 15%–20% of pregnancies present ABO discrepancy between a type O mother and type A or B foetus. Finally, the prevalence of RhD incompatibility may range between 10% and 15% [12].

The small number of identified SM-FMH cases could be another limitation; however, it reflects the rarity of this severe clinical condition, found in 0.02% of 29,192 live neonates included in this analysis.

The current report demonstrates that the detection of a mixed field RBC agglutination in the maternal blood sample during routine ABO/RhD blood typing could point to the occurrence of a life-threatening SM-FMH. Such finding should prompt an immediate alert among blood bank specialists performing this test in pregnant women, particularly those in the third trimester. Obstetricians should be aware of the significance of such lab results, as this may be crucial for the management of women harbouring a mixed field RBC agglutination and life-saving for the foetus.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts to declare.

DATA AVAILABILITY STATEMENT

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

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SHORT REPORT

Vox Sanguinis ISBT International Society of Blood Transfusion

Higher rates of laboratory-confirmed cases of syphilis in Western Canadian blood donors compared with Eastern Canadian blood donors following a period of societal re-opening

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Abstract

Background and Objectives: There is a growing infectious syphilis outbreak in Western Canada. Although blood donors are screened for syphilis risks, some blood donors will still be confirmed test-positive for syphilis. This study compares the characteristics of confirmed test-positive syphilis donations in both Western Canada and Eastern Canada, November 2022–August 2023.

Materials and Methods: Donors were defined as Western or Eastern Canadian. Blood donations were tested for syphilis using the PK-TP assay (Beckman Coulter PK7300 Automated Microplate System). Confirmatory *Treponema pallidum* particle agglutination (TPPA) and rapid plasma reagin (RPR) assays were performed by one of two reference laboratories. An RPR titre ≥1:8 was used as a proxy for possible infectious syphilis.

Results: Rates of laboratory-confirmed syphilis were higher in Western ($n = 43$, 13.4/100,000 donations) versus Eastern donors ($n = 19$, 4.7/100,000 donations; Fisher's exact test, two-sided, $p \leq 0.0001$). Most syphilis confirmations were in first-time donors (Western Canada $n = 31/43$, 72.1%, Eastern Canada 12/19, 63.2%).

Conclusion: Although rates of laboratory-confirmed syphilis were higher in Western versus Eastern donors, Western donors did not have higher rates of infectious syphilis. Further studies might assess whether donors with laboratory-confirmed syphilis understood pre-donation screening questions or were completely unaware of a past infection.

Keywords

blood donors, Canada, confirmation, syphilis

Highlights

- Rates of laboratory-confirmed syphilis were higher in Western versus Eastern donors.
- Western donors did not have higher rates of infectious syphilis.
- Further studies should be undertaken to assess whether donors understood pre-donation screening questions or whether these donors were completely unaware of a past infection.

INTRODUCTION

Currently, there is a growing infectious syphilis outbreak in Canada. In 2022, there were 36.1/100,000 cases of infectious syphilis in the general population. This represented a 109% increase from 2018 (Figure 1) [1, 2]. Between 2011 and 2023, the rates of confirmed syphilis in Canadian Blood Services (CBS) donors also rose in both first-time and repeat donors (Figure 1) [3]. However, these rates of confirmed syphilis positivity in blood donors were not stratified into infectious versus resolved or past cases. Between 2018 and 2022, increases in rates of infectious syphilis in the general population were more profound in Western Canada (British Columbia [82%], Alberta [105%], Saskatchewan [1444%], Manitoba [69%]) than in Eastern Canada (Ontario [69%], Quebec [24%], New Brunswick [78%], Nova Scotia [32%]). One Eastern province (Newfoundland and Labrador) noted a 63% decrease in infectious syphilis for this period. From 2020 to 2022, increases in infectious syphilis in the general Canadian population occurred during the coronavirus disease 2019 (COVID-19) pandemic and were impacted by reduced availability of sexually transmitted and blood-borne infection (STBBI) services [1].

CBS tests all donations using a syphilis serologic assay [3]. At every donation, donors are also asked the following question, 'In the last 12 months have you had or been treated for syphilis or gonorrhea?' [4]. In Canada, societal re-opening occurred over spring–summer 2022 and completed by October 2022. As Canadian society re-opened, it is possible that Canadian blood donors (whole blood and plasma) may have been unaware of their syphilis infection status before donating. It is also not clear whether a Western preponderance of infectious syphilis would also be found in Canadian blood donors. Therefore, this study compares the characteristics of confirmed test-positive syphilis donations in both Western Canada (Manitoba West) and Eastern Canada (East of Manitoba) for November 2022–August 2023. This period covers a time when Canada opened in the COVID-19 era. Another key point is that this study analyses confirmed syphilis-positive in a period following the implementation of sexual behaviour-based screening (SBBS), including a more gender neutral donor questionnaire implemented on September 11, 2022 [5].

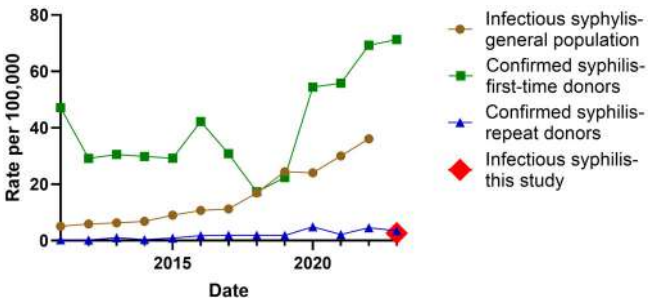

FIGURE 1 Rates of syphilis in Canadian blood donors and the general population: 2011–2023. Brown circles and lines represent reported rates of infectious syphilis in the general Canadian population per 100,000 people. Green squares and lines represent rates of confirmed antibody-positive syphilis in first-time Canadian Blood donors calculated per 100,000 donations. Blue triangles and lines confirmed antibody-positive syphilis in repeat Canadian Blood donors calculated per 100,000 donations. The enlarged orange diamond represents the rate of infectious syphilis in the study period per 100,000 donations. No general population data for 2023 were available. Rates of infectious syphilis were not previously published by Canadian Blood Services.

MATERIALS AND METHODS

Blood donor screening and testing for syphilis

Before donation, blood donors were screened for sexual risks and diagnosis/treatment in the last 12 months. CBS tests all donations for syphilis; up until 2022, all donors with repeat reactive (RR) results, with or without confirmation, were deferred indefinitely as of January 1, 2023, donors with RR results that were determined to be negative could be re-instated after 6 months. The number of donations (whole blood and source plasma) tested were derived from the National Epidemiology Donor Database (NEDD).

For the duration of the study period, the Beckman Coulter PK7300 System, a microhaemagglutination assay, was used for the qualitative laboratory testing of blood donors for the detection of *Treponema pallidum* Immunoglobulin G (IgG) and Immunoglobulin M (IgM) antibodies in Ethylenediaminetetraacetic acid (EDTA), plasma and serum (PK-TP assay, Beckman Coulter, Brea, CA, USA). Confirmatory testing at one of two reference laboratories (ProvLab Alberta, Edmonton, AB, Canada & Public Health Ontario's laboratory, Toronto, ON, Canada) used the *T. pallidum* particle agglutination (TPPA) and the rapid plasma reagin (RPR) assays [6]. An RPR titre of ≥1:8 was used as a proxy for possible infectious syphilis (primary, secondary, early latent). For continuity purposes, RPR was utilized for all laboratory results and was based on Government of Canada syphilis screening guidance [7].

Due to site specific laboratory information system design, specimens submitted to ProvLab Alberta were also tested by the Abbott™ ARCHITECT™ Syphilis Treponemal Test (Abbott Park, IL, USA) a chemiluminescent microparticle immunoassay (CMIA) capable of detecting both IgG and IgM against *T. pallidum*. Specimens at ProvLab were then tested by TPPA and RPR regardless of the CMIA result.

Laboratory result review

Two clinical microbiologists and an epidemiologist reviewed laboratory results; final scoring of syphilis confirmatory testing was based on consensus. Confirmatory scoring occurred at least 1–2 months after primary screening results collation (e.g., August 2023 data were scored in September/October 2023). A secondary analysis of RPR-positive only specimens was also undertaken.

Definition of Western versus Eastern donors

Numbers of donations and donors were generated from the CBS NEDD. Donors were defined as Western or Eastern based on the

location of their donation using Statistics Canada based on Canada subarea classifications [8]. Gender was categorized as male or female according to the donor record. CBS data were collected from all provinces except for Quebec and the Northern territories (Yukon, Northwest Territories and Nunavut) where CBS does not collect donations.

Historic syphilis data

Historic syphilis data from first-time and repeat donors were collected from the CBS Epidemiology Donor Database with the most recent data available publicly on the CBS Surveillance Report. This database did not include data on infectious syphilis [3]. Rates of infectious syphilis in the general Canadian population were collated from Government of Canada data [1, 2].

Data analysis

Data were collated using Excel (Microsoft, Redmond WA, USA). General descriptive statistics, Fisher's exact tests and Mann–Whitney *U* were performed via GraphPad Prism 10 (GraphPad Software, Boston, MA, USA).

RESULTS

Specific data points for Western and Eastern Canadian syphilis-positive blood (whole blood and source plasma) donors at CBS are identified in Table 1. One confirmed Western Canadian syphilis-positive stem cell donor with no reactive RPR value was removed from the analysis. In total, 62 confirmed syphilis-positive blood donors were included in the study. Slightly more specimens were collected from Eastern blood donors ($n = 406{,}662$) than Western donors ($n = 319{,}814$). Rates of laboratory-confirmed syphilis were higher in Western ($n = 43$, 13.4/100,000 donations) versus Eastern donors ($n = 19$, 4.7/100,000 donations, Fisher's exact test, two-sided, $p < 0.0001$). Most syphilis confirmations were reported in first-time donors (Western Canada $n = 31/43$, 72.1%, Eastern Canada 12/19, 63.2%). Of the 43 confirmed syphilis-positive Western Canadian donor specimens, 41 (95.3.0%) were also syphilis CMIA-positive.

Using an RPR of ≥1:8 as a marker of infectious syphilis, the overall rate of infectious syphilis in the study period was 2.6/100,000 donations (Figure 1). There was no difference in rates of possible infectious syphilis in Western ($n = 8$, 2.5/100,000 donations) versus Eastern Canada ($n = 11$, 2.7/100,000 donations; Fisher's exact test, two-sided, $p = 0.82$).

As seen in Figure 2, when any RPR value was scored, a lower proportion of syphilis-confirmed Western Canadian donors (17/44, 38.6%) versus Eastern donors (19/19, 100%) had any RPR-positive result. Values of measurable positive RPR titres were then compared between Eastern and Western Canadian donors. When compared with Eastern donors, measurable RPR values were not biased towards lower values in Western donors. There was no difference in the median distribution of RPR-positive titres of syphilis RPR results between Eastern and Western Canadian donors (Mann Whitney test, $p = 0.92$, Mann–Whitney $U = 158$).

TABLE 1 TPPA-positive syphilis serology-confirmed specimens.

Year	Month	Confirmed syphilis	Confirmed EC	Confirmed WC	CMIA-positive WC	RPR-positive EC	RPR titres EC	RPR ≥8 EC	RPR-positive WC	RPR titres WC	RPR ≥8 WC
2022	November	7	3	4	4	3	1:4, 1:4, 1:4	0	2	1:128, 1:128	2
	December	3	0	3	3	0	NA	0	2	1:1, 1:128	1
2023	January	8	2	6	6	2	1:32, 1:64	2	1	1:64	1
	February	3	2	1	1	2	1:1, 1:2	0	0	NA	0
	March	2	1	1	1	1	1:16	1	1	1:4	0
	April	4[a]	1	3	3	1	1:8	1	2	1:4, 1:128	1
	May	6	2	4	4	2	1:8, 1:32	2	2	1:64, 1:1	1
	June	9	4	5	4	4	1:1, 1:4, 1:16, 1:32	2	1	1:16	1
	July	11	3	8	8	3	1:32, 1:64, 1:128	3	5	1:1, 1:2, 1:2, 1:4, 1:16	1
	August	9	1	8	7	1	1:1	0	1	1:2	0
Total		62	19	43	41[b]	19		11	17		8

Abbreviations: CMIA, chemiluminescent microparticle immunoassay; EC, Eastern Canada; NA, not applicable; RPR, rapid plasma reagin; TPPA, *Treponema pallidum* particle agglutination; WC, Western Canada.

[a]Does not include one stem cell donor with an RPR-negative specimen.

[b]Of the 43 specimens, 2 were CMIA-negative with signal-to-cut-off values of 0.32 and 0.09.

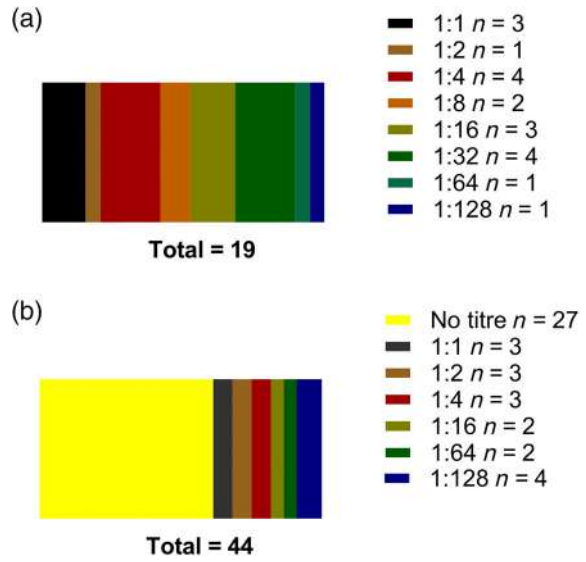

FIGURE 2 Distribution of rapid plasma reagin (RPR) titrable and RPR non-detectable blood donor specimens. (a) Eastern Canadian donors. All 19 syphilis-positive donor specimens had a measurable RPR titre. (b) Western Canadian donors. Of the 44 syphilis-positive donor specimens, a lower proportion of syphilis-confirmed Western Canadian donors (17/44, 38.6%) had any RPR-positive result (Fisher's exact test, two-sided, $p < 0.0001$). When RPR-negative specimens were excluded from analysis, there was no difference in the median distribution of RPR-positive titres of syphilis RPR results between Eastern and Western Canadian donors (Mann–Whitney test, $p = 0.92$, Mann–Whitney $U = 158$).

When any RPR titre was assessed, rates of 4.7/100,000 (19/406,662 donations) in Eastern donations were not significantly different than rates in Western donors (5.3/100,000 donations; 17/319,814 donations; Fisher's exact test, two-sided, $p = 0.74$).

Of the 62 syphilis-confirmed blood donors, there was no difference in the distribution of infectious (RPR ≥1:8) syphilis serology (first-time donor 12/19 [63.2%] vs. repeat donor 7/19 [36.8%]) and non-infectious (RPR <1:8) syphilis serology (first-time donor 31/43 [72.1%] vs. repeat donor 12/43 [27.9%]) (Fisher's exact test, two-sided, $p = 0.56$).

Of the 62 syphilis-confirmed blood donors, the majority identified as male (male $n = 50$, 86% vs. female $n = 12$, 19.4%). There was no gender-related difference in the proportion of donors within the infectious (male $n = 13$ [13/19, 68.4%] vs. female $n = 6$ [6/19, 31.6%]) and non-infectious (male $n = 37$ [37/43, 86.0%] vs. female $n = 6$ [6/43, 14.0%]) groups (Fisher's exact test, two-sided, $p = 0.16$).

Of the 62 syphilis-confirmed donors, the median age of the infectious syphilis group was younger (median age: 28.0 years, 25th–75th percentiles: 25.0–35.0 years) than from the non-infectious group (median age: 39.0 years, 75th- 25th percentiles: 29.0–54.0 years) ($p = 0.002$, Mann–Whitney $U = 209$).

DISCUSSION

Rates of infectious syphilis in Canadian blood donors were low when compared with rates of infectious in the general population (Figure 1). Although rates of laboratory-confirmed syphilis were higher in Western Canadian versus Eastern Canadian donors, Western donors did not have higher rates of infectious syphilis. These trends in blood donors differ from the trend to increasing rates of infectious syphilis in Western Canada [1]. There was also no difference in the median RPR values for Western Canadian versus Eastern Canadian donors. It is also unclear why a larger proportion of Eastern Canadian donors had any RPR-positive value than Western Canadian donors. These higher rates of confirmed, but non-infectious syphilis, in Western Canada represent distant or previously treated infections. One possibility for this difference is that the blood donor population in Canada is not impacted by the same sexual networks, either spatially or temporally, as the general Canadian population [9].

In this study, donors with infectious syphilis were more likely to be younger than donors in the non-infectious group. In the general population, infectious syphilis is most common in Canadians aged 25–29 years [2]. Although not significant, infectious syphilis was more common in males (68.4%) versus females (31.6%), and this is similar to rates seen in the general Canadian population from 2022 (males [65%] vs. females [35%]) [1].

The outbreak of infectious syphilis in Canada, and in Western Canada in particular, is multifactorial and risks factors for syphilis in blood donor populations may not mirror risk factors in the general population. A Public Health Agency of Canada report on the outbreak of infectious syphilis noted that there were a greater proportion of cases in males and females identifying as heterosexual and an increase in cases of congenital syphilis. Cases appeared to occur where barriers to accessing care and delays in management allowed for sustained transmission. Impacted groups included people experiencing inequalities in social determinants of health (e.g., discrimination, unstable housing, poverty) and multiple and/or anonymous partners.

Although there may also be an association with human immunodeficiency virus infection [10], in Canada, rates of new human immunodeficiency virus (HIV) infections in donors and the general population have not increased [11]. The increase in infectious syphilis runs parallel to increases in congenital syphilis from 2018 onward particularly in the western provinces of Alberta (increase 420%) and Manitoba (increase 199%) and the eastern province of Ontario (increase 2345%). Before the COVID-19 pandemic, there was a notable increase in the proportion of syphilis-related stillbirths (SRSB) in the western city of Winnipeg, Manitoba [12]. Those SRSB events were associate with risk factors including a lack of prenatal medical care, unstable housing/shelter and other wellness issues (mental illness, substance use and sexually transmitted co-infections) [12]. Those risk factors differ from previously described risk factors in Canadian blood donors where risk factor associated with confirmed syphilis in first-time donors included histories of intravenous drug use, gay and bisexual male-to-male (gbMSM) sex and birth in a high-prevalence country. In repeat donors, confirmed syphilis positivity was associated with gbMSM sex. We note though that less than 10% of Canadian blood donors are first-time donors [3, 11].

An RPR value of <1:8 is considered a low titre and in the presence of concurrently positive treponemal assays (e.g., TPPA, PK-TP, CMIA) likely represents a true low titre. Although some donor studies have

used any RPR titre as a proxy for infectious syphilis [13], in some scenarios, this may lead to misclassification of syphilis disease staging. For example, if a donor is successfully treated for syphilis (and therefore non-infectious), they may lose their RPR titres completely or may still have reactive low titres of RPR in a serofast state (not fully seroreverted) [14]. In the CBS setting, it is possible that Canadian blood donors with low RPR titres were in a serofast state [4]. Furthermore, in Canada, the 'Syphilis guide: Screening and diagnostic testing' guidelines note that an RPR titre ≥8 is 'more likely to be infectious syphilis' [7].

A lower proportion of syphilis-confirmed Western Canadian donors had any RPR-positive result (Table 1 and Figure 1). Since both laboratories were part of standard North American proficiency networks, it is unlikely that differences in RPR titres would be due to laboratory-based factors [15]. In support of this, there was no difference in the median distribution of measurable titres (e.g., no direction bias in laboratory results) of syphilis RPR results (RPR-negative specimens not included in analysis) between Eastern and Western Canadian donors. One other factor may play a role in why roughly 60% of syphilis-positive Western donors do not have measurable RPR titres. At CBS, the wording of the donor screening question in, 'In the last 12 months have you had or been treated for syphilis or gonorrhea?' does allow for donors with a history of syphilis infection or treatment to donate cellular blood products as long as it was more than 12 months ago [4]. Prior effective treatment of syphilis would have made donors non-infectious and would have led to significant decreases in (≥4-fold titre reduction from pre-treatment to post-treatment follow-up) RPR titres [7, 16, 17]. The profile of a positive treponemal-specific screening assay, a positive confirmatory test (e.g., TPPA) and an RPR-negative might also represent late latent/tertiary syphilis [7]. In asymptomatic healthy donors who thoughtfully and correctly respond 'No' to multiple health/wellness and sexual-based risk criteria questions, this test profile is less likely to be early primary syphilis [7]. Thus, these RPR-negative western Canadian blood donors likely reflect past infections linked to the large western Canadian syphilis outbreak [2].

Two of the 43 confirmed syphilis-positive Western Canadian donor specimens were CMIA-negative (Table 1). Both specimens belonged to repeat donors (prior donation 16 and 7 years earlier). Test profiles were PK-TP-positive (treponemal assay), TPPA-positive (treponemal assay) and RPR non-reactive (non-treponemal assay). In blood donation settings, *T. pallidum* microhaemagglutination assays (TPHA) and enzyme immunoassays/chemiluminescent assays are generally considered to have equivalent sensitivities [18]. In some rare cases, discordant results between TPHA and chemiluminescent immunoassays have been sporadically described [19].

This study has several limitations including a relatively short period of analysis from societal opening following the peak of the COVID-19 pandemic and for a period when all syphilis serology testing was done on one consistent platform (PK-TP assay on the Beckman Coulter PK7300 System). Although a small number of syphilis-confirmed specimens were analysed, they represent all confirmed syphilis-positive specimens for this time at CBS. Further follow-up of donors was not undertaken, and clinical histories for syphilis exposure, risk or treatment were not obtained. This study was also not able to differentiate between serology results due to sexually transmitted versus endemic syphilis [20]. The study also did not include data from donors in Quebec or the Northern territories. Canada is a diverse country with different social and economic differences between regions. This study does not assess the impact of these complex factors on the current Canadian syphilis outbreak [21–23].

Even in the setting of a large Canadian syphilis outbreak, the rates of infectious syphilis in blood donors are low when compared with the general population (Figure 1). The study did not intend to compare patterns of infectious syphilis before and after the implementation of an SBBS approach at CBS in September 2022 [5]. It is therefore unclear if rates of infectious syphilis have changed over time within the Canadian blood donor population. In this study, we speculate that pre-donation screening for syphilis infection risks may reduce the risks of collecting blood from a donor with infectious syphilis. The authors propose that further studies should be undertaken to assess whether donors with a history of laboratory-confirmed syphilis or treatment of syphilis based on public health contact tracing understood pre-donation screening questions or whether these donors were completely unaware of a past infection. Given the lack of recent evidence for syphilis transfusion transmission in North America, and the differing patterns of confirmed syphilis in blood donors versus the general population, blood operators should continue to reassess the relevance of testing every donation for syphilis [24].

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S.J.D. classified cases, analysed data, conceptualized and wrote the first draft of the manuscript; C.C. classified cases and edited the manuscript; V.T. and H.Y.Z. oversaw confirmatory syphilis testing and edited the manuscript; G.H. and I.R. oversaw laboratory testing, collated data and edited the manuscript; S.O. classified cases and edited the manuscript.

CONFLICT OF INTEREST STATEMENT

Steven J. Drews has consulted to Roche on malaria and emerging arboviruses and has received research support from Abbott to support a serology project focused on COVID-19. He is a paid speaker to Danaher, focusing on the impact of genetic drift on viral diagnostics. Steven J. Drews and Carmen Charlton have both engaged Roche as investigators.

DATA AVAILABILITY STATEMENT

This study makes use of aggregate data. These aggregate data are available from the corresponding author on request.

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REPORT

Vox Sanguinis ISBT International Society of Blood Transfusion

The International Society of Blood Transfusion (ISBT) Public Health Research Toolkit: A report from the Surveillance, Risk Assessment and Policy Sub-group of the ISBT Transfusion Transmitted Infectious Diseases Working Party

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Abstract

Background and Objectives: Data provided from blood donors have contributed to the understanding of public health epidemiology and policy decisions. A recent example was during the severe acute respiratory syndrome-related coronavirus (SARS-CoV-2) pandemic when blood services monitored the seroprevalence in blood donors. Based on this experience, blood services have the opportunity to expand their role and participate in public health surveillance and research. The aim of this report is to share available resources to assist blood services in this area.

Materials and Methods: The Surveillance, Risk Assessment and Policy (SRAP) Subgroup of the International Society of Blood Transfusion (ISBT) Transfusion Transmitted Infectious Diseases (TTID) Working Party developed a Public Health Research

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Toolkit to assist blood services and researchers interested in expanding their role in public health research.

Results: The ISBT Public Health Research Toolkit provides resources for what blood services can offer to public health, examples of donor research studies, the utility of donor data and website links to public health agencies. The toolkit includes a customizable template for those interested in establishing and managing a biobank.

Conclusion: The ISBT Public Health Research Toolkit includes resources to increase the recognition of the role blood donors can play in public health and to help blood services gain commitment and funding from various agencies for new research and surveillance.

Keywords

biobanks, donors, public health, research, toolkit

Highlights

- Blood services have an opportunity to increase the utilization of blood donors and to leverage their data to contribute to public health surveillance and research.
- The International Society of Blood Transfusion Public Health Research Toolkit provides resources for blood services and researchers for getting started.
- The toolkit provides information on what blood services can offer to public health, and how they can enhance the ability to support emerging outbreaks once discovered.

INTRODUCTION

Historically, the surveillance of blood donors has contributed to the understanding of public health epidemiology in the general population, in many cases providing data that contributed to policy decisions. Donor screening data for hepatitis B virus (HBV) [1], hepatitis B virus (HCV), human immunodeficiency virus (HIV), West Nile virus (WNV), *Babesia* [2] and hepatitis E virus (HEV) [3] are examples of such contributions. Analysis of donor demographics has also provided a better understanding of the effect of donations on donor health and transfusion recipients, as has been done with the Scandinavian Donation and Transfusion database [4]. Furthermore, health-focused investigations, including those conducted within the Danish Blood Donor Study, examine the impact of various risk factors such as obesity, smoking and contraception on infection rates [5].

Blood donors are an ideal sentinel or convenience population for surveillance for blood-borne disease markers and biomarkers in a healthy population. As it may not always be possible to gather data from an entire population, utilizing data from this population can prove sufficient, aiding in the formulation of public health decisions. In addition, blood donors, being altruistic, are often willing to not only provide their life saving donation but also contribute data to research. It is important to note, however, as blood donors must pass strict criteria to donate blood, researchers need to be aware of the 'healthy-donor-effect' that may act as a bias when designing a study [6] However, donors can also reduce many confounding factors that can affect the study outcome compared with recruiting patients as a study population.

Blood services are well suited to collaborate in this type of research as they have an infrastructure already in place for recruiting donors; collecting, transporting and testing large numbers of samples on a daily basis; many frequent repeat donors, which permits longitudinal studies; information systems for capturing data and a range of expert personnel who support these operations. In addition to being trained in their area of specialty, all staff are trained following standard operating procedures, good manufacturing practices and biosafety procedures. In some facilities, procedures may already be in place for collecting blood samples and blood products for research purposes, including obtaining research consent at the time of donation.

With some exceptions, blood services are largely under-utilized for public health surveillance. Reasons for this include lack of awareness amongst public health agencies as to the potential of blood donors, lack of knowledge of the data available and siloed interaction between public health and blood services that focuses on transfusion issues. Importantly, public health authorities do not always understand the screening requirements for blood donation, which vary between countries. During the severe acute respiratory syndrome-related coronavirus (SARS-CoV-2) pandemic, there was a heightened awareness as blood services around the world stepped up to conduct seroprevalence studies. These studies supported their local health authorities in monitoring the population exposure to the virus, the durability of immunity in asymptomatic donors and vaccine-induced immunity through cross-sectional and longitudinal seroprevalence studies [7]. This collaboration with public health agencies brought to the forefront that blood services have an opportunity to increase the utilization of blood donors and to leverage their data to contribute to public health surveillance and research, as well as emergency preparedness against emerging transfusion-transmitted pathogens. It is also an opportunity

for blood services to expand their services to support their blood operations.

In response, the Surveillance, Risk Assessment and Policy (SRAP) Sub-group of the International Society of Blood Transfusion (ISBT) Transfusion Transmitted Infectious Diseases (TTID) Working Party, developed a public health research toolkit designed to provide resources and links aimed at assisting blood services interested in initiating public health collaboration.

The ISBT Public Health Research Toolkit

The toolkit provides a framework to enable researchers and/or blood services to increase the recognition of the role that blood donors can play in public health and to efficiently gain commitment and funding from various agencies for new research and surveillance. The toolkit consists of two main sections—information on how and what blood services can offer to public health, and how they can enhance the ability to support emerging outbreaks once discovered.

What blood services can offer to public health

This first section includes information for creating a fact-based document that can be shared with public health agencies and is titled, 'The value of using blood donors and blood establishments for potential research studies'. This document includes much of the information addressed in this article; however, a blood service can customize it to meet their individual needs. The information may also be useful to gain commitment and funding from various agencies for new research and surveillance. Other information includes two publications: the first shares information from a recent global public health research survey to understand how blood services are partnering with public health agencies outside the scope of coronavirus disease 2019 (COVID-19) [8]. The second publication provides a stepwise approach to assess the characteristics of donors relative to the general population and to define appropriate blood donor research studies [9].

This section also includes literature references with examples of the use of donors for public health research and ideas for research studies. In addition, there is a list of resources for emerging infectious diseases from the Association for the Advancement of Blood and Biotherapies (AABB), the European Centre for Disease Prevention and Control (ECDC), Centers for Disease Control and Prevention (CDC) and the National Institutes of Health (NIH).

Other references include some global agencies with links to their website and a list of blood service laboratories from various countries that are actively involved in research and have research capabilities. For quite a few of these countries, the research function is attached to the national blood operator, and in others, such as the United States and Spain, there are multiple blood establishments and providers of research. Some other countries have academic research in universities that is focused on blood transfusion (i.e., Germany, Canada, United States).

How to enhance the ability to support emerging outbreaks once discovered

This second section provides information for developing a biobank where samples from donors are stored and are available for internal and external research studies. Several blood services and institutions retain donor samples indefinitely, many of which are repeat donors. Some bioregistries include existing national research funded by governments, such as the Recipient Epidemiology and Donor Evaluation Study repository. These samples may be used for longitudinal studies to monitor the progression of a new or emerging pathogen in the general population, like the studies with SARS-CoV-2.

The biobank is a stand-alone service, separate from the blood services' routine operations. The decision to establish a biobank as a service requires careful consideration and its success depends on how well a biobank fits as a service within the blood facility's infrastructure and culture. An organization may decide to provide samples only, samples and data or just data. In this section, a reference to considerations for the development of a biobank is available with links to some publications, one addressing ethical concerns and data protection issues [10].

In addition, for those blood services just starting out, there is a customizable framework for establishing and managing a biobank. The template is a modification of the Héma-Québec 'Management and Governance Framework for the Plasma Donor Biobank—Héma-Québec' [11]. The template includes a description of a biobank and its objectives; governance and management of a biobank; collection, retention and use of samples and data and responsibilities of biobank users.

Summary

The ISBT Public Health Research Toolkit provides multiple resources to help blood services interested in expanding its scope to include surveillance and public health research. The intent is to update the toolkit as more information becomes available. ISBT members involved in research are encouraged to contribute information that may be useful for others.

Access to the toolkit is free and is located on the ISBT website. There are two ways to access the toolkit. The first is from the ISBT website main page (isbtweb.org). On the ISBT main page, (1) select Working Parties in the top ribbon, (2) select TTID Working Party on the next page, (3) select the link to the Public Health Research Toolkit in the right-hand menu on the TTID page. The second way is direct access via a link at https://www.isbtweb.org/isbt-working-parties/transfusion-transmitted-infectious-diseases/public-health-research-toolkit.html.

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the manuscript; C.E., S.A.G., J.A.H., A.L., S.F.O., C.O., G.P., W.A.R., B.R.S. and C.H. critically reviewed the manuscript.

CONFLICT OF INTEREST STATEMENT

Jean Stanley, Susan A. Galel and Connie Higgs are employees and shareholders of Roche Diagnostics. Christian Erikstrup has received unrestricted research grants from Abbott Diagnostics and Novo Nordisk, administered by my institution, no personal fees. Michael P. Busch is an employee of Vitalant and has received research funding from NIH, CDC, FDA, Grifols and Hologic.

DATA AVAILABILITY STATEMENT

Access to the ISBT Public Health Research Toolkit is available to all members of ISBT and may be accessed at https://www.isbtweb.org/isbt-working-parties/transfusion-transmitted-infectious-diseases/public-health-research-toolkit.html.

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EVENTS

See also: https://www.isbtweb.org/events.html

23–26 October 2024	Brazilian Congress of Haematology, Hemotherapy and Cell Therapy (Hemo 2024). https://www.hemo.org.br/2024/index.ingles.php
14–15 November 2024	BlooDHIT 2024. https://bloodhit.com/registration/
20–21 November 2024.	RCPath & SHOT Advances in Transfusion Symposium. https://www.rcpath.org/event/advances-in-transfusion-medicine-2024-joint-rcpath-shot-symposium.html
21–23 November 2024	TRANSCON 2024. https://www.transcon2024.in/
14–15 January 2025	EDQM Blood Conference: Innovation in Blood Establishment Processes. https://www.edqm.eu/en/edqm-blood-conference