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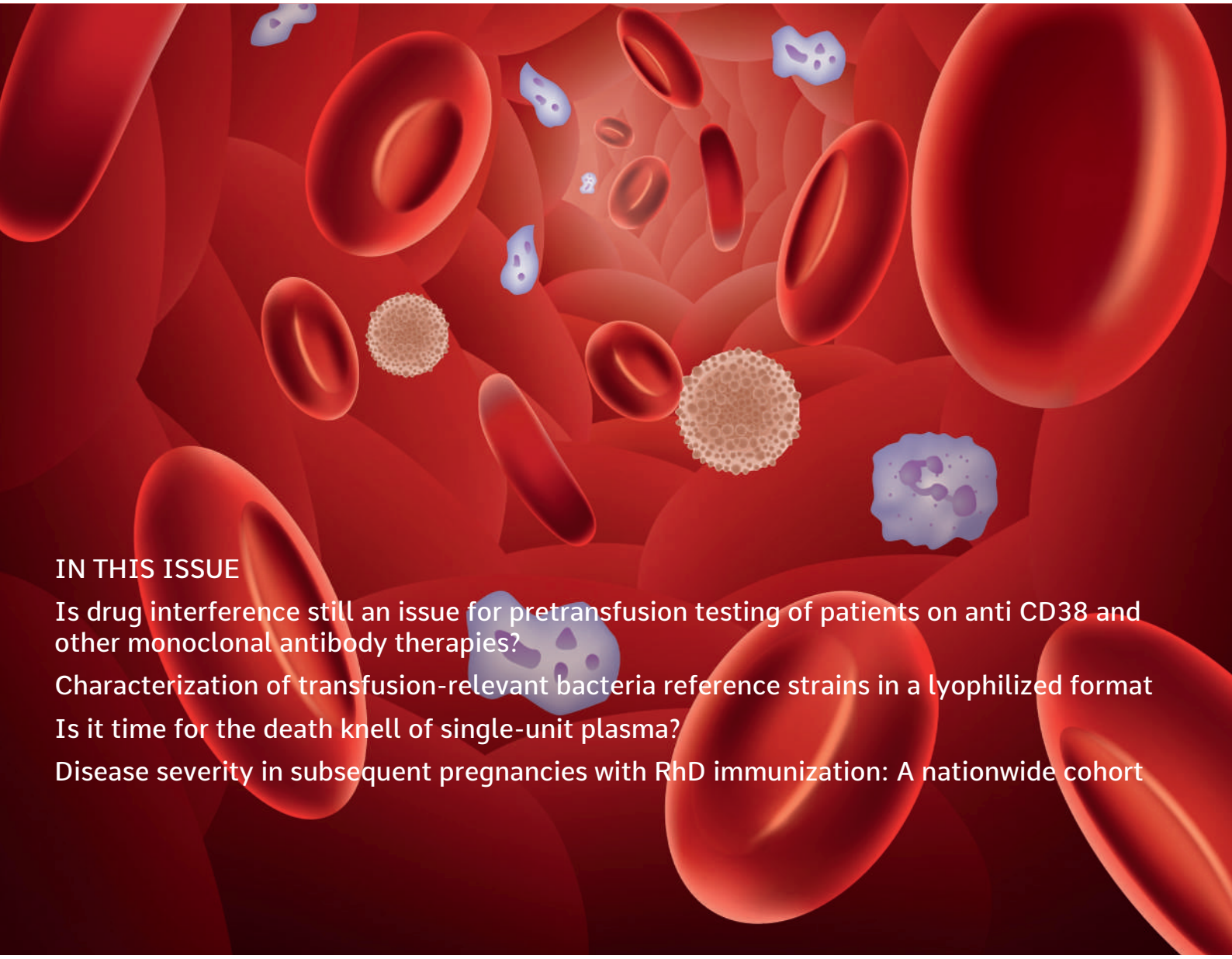

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Aims and Scope

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

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

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

Incentives for plasma donation

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Abstract

Background and Objectives: This work provides an overview of the incentives used for plasma donation in Europe and beyond. The overview can provide new ideas to blood establishments.

Materials and Methods: We conducted a systematic online search of incentives used and asked national experts to validate the data across all European Union countries as well as other European and non-European countries. We categorized the data into level of incentive (using the Nuffield Council on Bioethics' rungs [2011]) and country.

Results: We analysed more than 490 organizations across 26 countries. Our findings reveal different incentives used in these countries. Snacks and pre-donation health checks are commonly provided. In addition, loyalty programmes, small gifts, vouchers, lotteries, travel compensations and time off from work extend the strategic incentive portfolio. Only seven countries offer financial compensation ranging from the equivalent of 10–35€ for European countries. In countries with a decentralized model, where more than one organization collects plasma, we observe that more diversified incentive strategies are generally used, including monetary and non-monetary incentives. In countries with a centralized model, where only one organization is allowed to collect plasma, financial compensation is usually not offered. Centralized plasma

collection without financial compensation relies on a wider range of non-monetary incentives than with financial compensation.

Conclusion: The country group analysis offers valuable insights into the relationship between incentive strategies and the prevailing centralized versus decentralized plasma collection model. This overview provides a broader understanding of incentives used by blood establishments and offers avenues for future practice.

Keywords

donor recruitment, Europe, incentives, plasma collection

Highlights

- All 26 investigated countries that are currently collecting plasma offer post-donation snacks and pre-donation health checks that are later communicated to the donors; these are either mandatory or serve as a basic incentive strategy.
- In most cases, countries with a centralized plasma collection model do not offer financial compensation, relying on a wider range of non-monetary incentives.
- In countries with a decentralized plasma collection model, more diverse incentivization strategies are generally observed regardless of financial compensation.

INTRODUCTION

Plasma donations are essential for the production of plasma-derived medicinal products or for the treatment of critical illnesses [1]. In the European Union (EU), there is an estimated annual shortfall of over 5 million litres of plasma to meet the demand [2]. As an economically important raw material, plasma is at high risk of supply disruption [1, 3].

Currently, the EU largely depends on plasma collected outside of Europe, mostly supplied by paid donors in the United States, which accounts for about 40% of the total demand [4, 5]. Due to potential global health crises such as coronavirus disease 2019 (COVID-19), or other geo-political turmoil, it is important for the EU to achieve a level of self-sufficiency and strategic independence in terms of plasma by increasing donations. The SUPPLY (Strengthening Voluntary Non-Remunerated Plasma Collection Capacity in Europe) project, which is co-funded by the EU and started in 2022, aims to increase plasma collection and strengthen the resilience of voluntary non-renumerated plasma collection programmes by blood establishments throughout the EU. This study is part of the SUPPLY project and focuses on the role of *monetary* and *non-monetary* incentives used to collect plasma in the EU.

Blood establishments strive to increase both the number of donors and the frequency of donations per donor. One individual can donate plasma up to 60 times per year in Germany and as many as 104 times per year in the United States [4], but only a fraction of these potential yearly donations is actually collected. For example, in Australia, plasma donations can be made as frequently as every 2 weeks, yet from 2017 to 2018, the average number of donations by Australian plasma donors was approximately four, with half of the donors making only one, two or three donations a year [6]. Addressing this gap between potential and actual donations is critical.

However, little is known on how to improve donors' willingness to donate plasma. Blood establishments aim to increase donations through the use of incentive strategies. By definition, incentives aim to motivate individuals to behave in a certain way [7]. For instance, they can increase the motivation to donate and hereby help to overcome the costs associated with the donation [8]. It is important to note that depending on how incentives are communicated to the donor, they can be perceived as incentives, encouragements or rewards (aimed at increasing [pre-]donation motivation) or more as compensation (aimed at overcoming potential barriers, i.e., costs associated with the donation). From a donor's perspective, we use the term *incentive* when referring to either strategy as they both ultimately aim to increase donations. In the case of plasma donation, incentives are offered to increase individuals' willingness to donate plasma and can take the form of *monetary* or *non-monetary* incentives. *Monetary* incentives can be given in the form of direct cash payment, vouchers or travel compensations, whereas *non-monetary* incentives can include gifts (e.g., snacks, mugs or bags), on-site refreshments or health checks [9, 10].

Existing literature summarized incentives in 17 countries concluding that non-monetary incentives, such as small gifts or health checks, were the primary approach adopted by most countries to encourage blood donation. In contrast, a smaller group of nations opted for monetary incentives, with cash incentives being the prevailing choice [10]. Similarly, a recent study examines incentive policies for whole blood donors in 63 countries and 50 US states. This extensive data set shows that half of the sampled countries utilize financial incentives, encompassing cash and tax benefits. In addition, time off from work is a commonly extended benefit to blood donors [11]. Limited research dealt with specific incentive types for plasma donation exploring the potential of an in-centre discount voucher reward, which might increase retention and donation frequency among new and repeat

plasma donors. The Australian pilot trial indicated a notable acceptance rate (~70%) for the vouchers. Although this successfully reduced the time taken to return for donation, it did however not significantly enhance the likelihood of donors returning to donate [12].

Although some research has started to investigate the influence of incentive strategies on (blood) donations (e.g., [8], [13] and [14]), especially in the context of plasma donation, more research is needed, and a criterion-based distinction between incentive strategies is required. Hence, following a recent call to further study different types of incentives for plasma donors [11], we focus on providing a consolidated review of the respective use of different *monetary* and *non-monetary* incentives in the EU, and beyond, to gain a broader understanding of similarities and differences, as well as to identify synergies and learning effects. Moreover, we analyse the plasma collection market across countries. This article (1) provides an overview of the *monetary* and *non-monetary* incentives implemented in 26 countries that are currently collecting plasma, (2) relates the incentives to the different rungs of the intervention ladder according to the Nuffield Council on Bioethics (2011) [15] and (3) discusses different market models (centralized vs. decentralized) for plasma collection.

We study more than 1000 blood establishments belonging to more than 490 different organizations across 26 countries (we provide insights on 44 countries, with 26 countries actively collecting plasma, that generate the main results of this article) including the EU, the rest of Europe and global countries of interest outside of Europe. After conducting a systematic online search of the incentives used in each country, we let national experts officially validate the data. The identified incentives have been grouped and evaluated along the six rungs of the Nuffield Council on Bioethics' (2011) intervention ladder for promoting donations [15] covering altruistic (rung 1–4) and non-altruistic (rung 5 and 6) interventions. We apply this framework to plasma donation, and account for different plasma collection models (centralized vs. decentralized) across countries. We discuss recommendations for blood establishments based on the identified *monetary* and *non-monetary* incentive strategies.

MATERIALS AND METHODS

To provide an overview of incentive strategies throughout all countries of interest (EU, European non-EU and global), we followed a three-step approach. We were unable to sample all non-EU countries due to lack of contacts and/or non-response. Outside Europe, we included the United States and Canada because of their plasma collection models (decentralized) and the highly competitive environment, and Australia because of its relevance in the existing literature on donor studies. The first step was a thorough desk review of official websites to find as much information as possible. Then the dataset was enriched with input from country experts, mainly provided by the European Blood Alliance and obtained via e-mail or personal communication. Finally, we identified suitable contacts in each country to validate the data we had systematically collected. Each contact received the corresponding country's data for validation. We then incorporated the comments and suggested changes to enhance the dataset's completeness.

As a result, Table 1 provides a consolidated summary of the incentive strategies implemented in each target country. The first columns of the table indicate whether (validated) data are available for the respective country categorized as EU countries, other European countries and global countries. This is followed by information on whether plasma donations are collected by private, government-owned or non-profit organizations and whether the country has a centralized or decentralized plasma collection model. In centralized models, only one blood organization is responsible for plasma collection, whereas in decentralized models, multiple organizations operate. Our key findings include the specific incentives used in each country categorized along the six rungs of the Nuffield Council's on Bioethics (2011) intervention ladder for promoting donations [15]. The concept was developed focusing on different incentives to encourage individuals to donate bodily material. We assigned the identified incentives to the respective rungs based on the associated level of altruism for each incentive [16].

The full dataset (available here: http://doi.org/10.25592/uhhfdm.13407) is comprised of over 1000 blood establishments from 490 distinct organizations and offers comprehensive details including general contact information, organization type, plasma collection model and implemented incentives for each centre.

RESULTS

We identify various incentives in the countries of interest and classify them as *monetary* and *non-monetary* incentives following Chell et al. [17] (see Table S1).

Although cash payments by definition serve as *monetary* incentives to increase donation behaviour, we also observe more indirect forms of *monetary* compensation. Some aim at maintaining a financial neutrality for the donor (travel cost reimbursement, paid time off work during donation), others at creating a synergy between two altruistic donations (redirection of compensation towards a charitable cause) or aim at donors' financial gain (tax deductions, additional paid time off). In some countries, there are also less common *monetary* incentives, such as free medication delivery or the possibility of receiving a state-sponsored pension after years of donating. Vouchers, lotteries and loyalty programmes can be interpreted as either *monetary* or *non-monetary* depending on their design [17]. If the incentives can be redeemed at specific shops and provide actual discounts, they are considered *monetary*. However, if they can be redeemed for specific events, additional health check results or gifts (e.g., keychains, magnets, coffee mugs, picnic blankets), then they are considered *non-monetary*. Pre-donation (mandatory) health checks, snacks, small gifts and entertainment (e.g., streaming movies, music, free available wi-fi) that donors receive while donating are *non-monetary* incentives. Recognition, such as certificates or badges, and thank you messages are also considered non-monetary.

TABLE 1 Synthesized overview of the incentives used in all countries of interest with currently active plasma donation program.

	Market attributes				Incentives[a]										
		Organization type			Rung 6	Rung 5	Rung 4	Rung 4	Rung 2	Rung 4	Rung 3	Rung 3	Rung 2	Rung 2	Rung 2
Country	Centralized plasma collection model	Profit	Government	Non-profit	Cash payment [amount in euros][b]	Vouchers [amount in euros][b]	Lottery [amount in euros][b]	Gifts [amount in euros][b]	Health check	Loyalty program	Travel compensation	Time off work	Snacks	Entertainment	Recognition
EU countries															
Austria	No	×		×	30–35				×[c]	×			×	×	
Belgium	Yes			×	-	2.50		1	×[d]	×	×		×		
Czechia	No	×		×	30	×	×	×	×[d]	×			×	×	
Denmark	Yes		×	×	-		×	4–160		×			×		
Estonia	Yes		×		-	5–10	×	2	×[c]	×			×		
France	Yes		×		-			×	×[d]		×		×	×	×
Germany	No	×	×	×	Up to 45	10	Up to 100	5–20	×[c]	×	×		×		
Hungary[e]	No	×			13–26	×	×		×	×			×	×	
Italy	No		×	×	-	0–20		0–5	×[c]	×		×[f]	×		×
Latvia	Yes		×		17				×[d]			×[g]	×	×	
Lithuania[e]	Yes		×		12				×	×					×
Luxembourg[e]	Yes			×	-				×				×		
The Netherlands	Yes			×	-			×	×[d]	×	×		×		
Poland	Yes		×						×	×	×	×[g]	×		×
Portugal	No		×	×									×		
Slovakia	No	×	×		-			×					×		
Slovenia	Yes		×		-								×		
Spain	No		×		-			1	×[c]	×			×		×
Sweden	No			×	10–15	Max. 10	×	Max. 10			×		×		
Non-EU countries in Europe															
North Macedonia[h]	Yes		×		-				×	×		×[f]	×		
Norway	No		×					×					×		
Switzerland	Yes			×	-			×					×		
United Kingdom: England	Yes		×						×	×			×	×	×

TABLE 1 (Continued)

	Market attributes				Incentives[a]										
		Organization type			Rung 6	Rung 5	Rung 4	Rung 4	Rung 2	Rung 4	Rung 3	Rung 3	Rung 2	Rung 2	Rung 2
Country	Centralized plasma collection model	Profit	Government	Non-profit	Cash payment [amount in euros][b]	Vouchers [amount in euros][b]	Lottery [amount in euros][b]	Gifts [amount in euros][b]	Health check	Loyalty program	Travel compensation	Time off work	Snacks	Entertainment	Recognition
Worldwide countries															
Australia	Yes		×						×[d]	×		×	×		
Canada	No	×		×	48–305				×[d]	×					
United States[e]	No	×			Up to 95				×	×					

Note: We did not include referral programmes in this table. The following countries provide referral programmes that vary in the incentives offered: Belgium, Czechia, Denmark, Germany, Hungary, the Netherlands, Slovakia and Canada. However, in the Netherlands, donors do not receive any reward for recruitment. They are provided with brochures, etc. to recruit other donors.

[a]An incentive is marked in black only if it is used in every establishment in the country; if not, the incentive is marked in grey.

[b]Local currencies converted to Euro.

[c]Mandatory health exam plus option of receiving additional health check information.

[d]Mandatory pre-donation health exam.

[e]Data are not validated by national experts.

[f]Donors receive extra time off work for their donation; for example, in Latvia, donors receive up to five paid days off per year.

[g]The time spent for donation does not need to be reworked.

[h]In planning.

Nuffield Council on Bioethics (2011) intervention ladder

Next, we propose a categorization of the incentives along the six rungs of the Nuffield Council on Bioethics (2011) intervention ladder for promoting donations [15]. The incentives ranging from rung 1 to rung 4 are defined as more altruistic interventions, including interventions targeting individuals who already donate and/or are more altruistically motivated. In contrast, incentives of rungs 5 and 6 are considered non-altruistic strategies as individuals' motivation to donate is connected to an incentive's appeal [16]. Thus, the evaluation of rungs 1–4 and rungs 5 and 6 also focuses on assessing whether, or to what extent, the donor's material, financial and personal well-being is neutralized or improved after their donation when given a specific incentive. Although rung 6 refers to direct financial compensation, the definition of all other rungs may include (indirect) monetary or non-monetary incentives. In addition, depending on their design, some incentives may be included in more than one rung.

As rung 1 aims to spread information about the need for donations, this step rather aligns with communication or marketing activities. Rung 2 focuses on recognizing altruistic donations through various incentives like snacks, mandatory pre-donation health checks, entertainment programmes, redirection of compensation towards a charitable cause or thank you messages. Rung 3 addresses interventions for potential donors already willing to donate by providing the time to donate and the means to get to the donation site (e.g., time off for the donation, travel cost reimbursement). Rung 4 provides additional incentives and rewards donors that are already motivated to donate through incentives like loyalty programmes, additional health check results (post-donation) and free medication delivery. Furthermore, rung 5 provides incentives to encourage individuals who would not typically consider donating. Examples include vouchers and additional time off from work.

Some incentives can be assigned to more than just one rung; for example, lotteries and gifts can be used for both encouragement and as a reward for existing donors (rung 4) or to attract new donors (rung 5). Communication framing plays an important role in how these incentives are perceived by donors. Moreover, there are different uses for time off work, either for the time of donation (rung 3) or as additional time off work (rung 5), and for health checks, either as a pre-donation health check (rung 2) or additional health check results post-donation (rung 4). Lastly, rung 6 involves financial incentives personally benefiting the donor after donating, such as cash payments, tax deductions or pension benefits. In conclusion, we identify incentives along the entire intervention ladder, except for communication tools (rung 1), in our dataset.

Incentives in the EU and other countries

In total, we identify 26 countries (19 from the EU) that collect blood plasma. We first analyse incentives that are used in more than two different countries (see Table 1) and then individually discuss incentives used in less countries.

Starting with rung 6, our findings show that plasma donors can receive *financial compensation* in Austria, Czechia, Germany, Hungary, Latvia, Lithuania, Sweden, Canada and the United States, ranging from the equivalent of 10–35€ for European countries. In Germany, the amount of money usually varies between 17 and 30€. However, depending on the donation volume and the number of donations, donors receive up to 45€. Given that you can donate up to 100 times yearly in the United States, plasma donors can earn an additional 9500€ yearly from donating plasma. Along rung 5, we find *that redeemable vouchers* are only used in Belgium, Czechia, Germany and Hungary. While Germany provides vouchers for burger restaurants or the cinema, Belgium issues thank you tokens that can be exchanged for small *gifts* like towels. The monetary value of vouchers varies from 2.50 (e.g., in Belgium) up to 20€ in Italy. Moreover, donors receive additional time off from work (rung 5) in Latvia (up to 5 days) and North Macedonia (plasma collection in planning; 2 days). *Lotteries* (rung 4/5) are only used in seven European countries and the value of the lottery ranges from 10 to 100€ in Germany, whereas *gifts* (rung 4/5) are more commonly used in 14 countries. For example, Czechia gives out wine, Estonia focuses on coffee mugs and chocolate, donors in Finland get reflectors and Slovenia distributes New Year's gifts. The monetary value of the gifts ranges from 1 (e.g., in Belgium or Spain) up to 160€ in Denmark. In addition, some countries (e.g., Germany, Belgium and Denmark) offer referral programmes where a donor is recruited by another donor and both receive a reward. Referral programmes are not displayed as a separate category in Table 1, as they refer to the other incentives used to motivate plasma donations.

One of the most used incentives is the pre-donation health check (rung 2), which is part of a donor health programme or eligibility check. Some countries also provide additional *health check* results after the donation (rung 4), including measures like cholesterol and thyroid-stimulating hormone in Estonia at every first and tenth donation. Similarly, Austria and Germany offer additional measures once a year. Italy and Spain communicate all post-donation laboratory results automatically to their donors. *Loyalty programmes* (rung 4) are widespread but vary in design. For example, Austria, Hungary and Canada increase cash payments for loyal donors, whereas Czechia's loyalty programme includes additional health checks, vouchers and lotteries. Estonia offers additional health checks for every tenth plasma donation. Belgium combines both factors and awards 2.50€ vouchers and cinema tickets after every third donation. In England, France, Italy, Lithuania, Poland and Spain, loyal donors are rewarded with recognition through items like badges and certificates. High frequency donors (200 donations in 10 years) are eligible for an additional pension. Denmark and the Netherlands provide gifts on donation anniversaries.

Rung 3 incentives aim to overcome donor barriers. Belgium, France, Germany, the Netherlands, Poland and Sweden cover *travel* and *parking expenses* (in France and the Netherlands only upon request). Poland and Italy offer *time off* from work for the donation day.

Out of 26 plasma collecting countries, 23 countries offer *post-donation snacks* (rung 2), either to ensure donor health safety or as a reward incentive. Snacks range from sweets and refreshments to

well-stocked buffets of home-cooked food. Austria, Czechia, France, Hungary, Latvia and England offer variations of on-site *entertainment*, like free wi-fi, books, series or music.

In addition to the more common incentives listed in Table 1, we found some less widespread incentives. German and Swedish donors have the possibility to *donate the financial compensation received to charity organizations*. After the donation, Spain and Germany send *thank you messages* to donors, whereas Poland offers *free medication delivery*. Polish donors receive further *benefits within the healthcare system* if they reach a certain amount of donations and obtain the title of 'honorary donor'. Instead of healthcare benefits, Lithuania offers the possibility to receive a *state pension* after 200 plasma donations over 10 years. In terms of financial incentives, the United States offers *prepaid Mastercard cards*, whereas Czechia (partially) and Poland provide *tax deductions*.

Synthesis on country level

Pre-donation health checks (rung 2) and post-donation snacks (rung 2) are the most common incentives, implemented by more than 18 (health checks) and 23 (snacks) out of 26 countries with a plasma donation programme. Next, we created different country groups based on whether they (1) have a centralized or decentralized plasma collection model, (2) use any form of financial compensation and (3) the extent of their incentive portfolio. We define countries who offer incentives from rung 2 (recognition-based incentives) and a maximum of two other incentives as having a basic incentive strategy. Table 2 provides an overview of the resulting country groups.

Within the group of paying countries, Austria, United States, Latvia and Lithuania provide the basic level of incentives (e.g., pre-donation health checks and post-donation snacks), and Latvia and Lithuania have a centralized collection model, meaning the incentive strategy is the same within the country. In contrast, Canada, Czechia, Germany, Hungary and Sweden, with a decentralized model, use a wider range of incentives, including loyalty programmes and seasonal specials like offering ice cream. Germany implements nearly every identified incentive except for providing time off from work.

We identify four different groups among the countries that do not pay for plasma donations. Although Norway and Portugal operate a decentralized model with several different plasma donation organizations, these countries only offer basic incentives. The same rather low level of incentive diversity applies to centralized countries like Australia, England, France, North Macedonia, Luxembourg, Slovenia and Switzerland. However, in the decentralized markets of Italy, Slovakia and Spain, incentives are more advanced and broader in content; for example, loyalty programmes and a paid day off. In contrast to paying countries, we find several centralized countries with advanced incentive strategies such as Belgium, Denmark, Estonia, the Netherlands and Poland.

In conclusion, our extensive dataset indicates that countries using financial compensation within a centralized model tend to have less diversity in incentives when to encourage plasma donations. Conversely, we find more advanced incentive strategies within decentralized models, possibly due to the presence of different plasma donation organizations. However, we observe that in non-paying countries, even centralized models offer a wider range of non-monetary incentives—potentially due to the prohibition of financial compensation.

DISCUSSION

This study provides a systematic overview of different incentives that are used for plasma donations. All 26 investigated countries that are currently collecting plasma use incentives to promote donation behaviour.

TABLE 2 Country groups based on their incentive strategy and market situation.

	With financial compensations		Without financial compensations	
	Basic incentive strategy	**Advanced incentive strategy**	**Basic incentive strategy**	**Advanced incentive strategy**
Decentralized plasma collection model	Austria USA	Canada Czechia Germany Hungary Sweden	Norway Portugal	Italy Slovakia Spain
Centralized plasma collection model	Latvia Lithuania	-	Australia England France North Macedonia Luxembourg Slovenia Switzerland	Belgium Denmark Estonia The Netherlands Poland

Note: A basic incentive strategy refers to incentives from rung 2 (recognition-based incentives) and a maximum of two other incentives, whereas offering more incentives is considered an advanced incentive strategy.

Future avenues for blood establishments

We observe that post-donation snacks are—beyond donor health-related reasons—used to show gratitude for an altruistic donation (rung 2), which is implemented by 23 out of 26 active plasma collecting countries. For new blood establishments, a starting point could be to offer snacks to plasma donors pre- and post-donation, as studies show that certain nutrients may prevent fainting [18, 19]. Additionally, 18 of these countries offer pre-donation health checks, and 5 of these also offer additional health check results after the donation. Health checks may be worth considering, as they appear to be effective in both short- and long-term retention and the cost of providing health parameters is relatively low [20]. Beyond efficacy and return behaviour, further studies are needed to assess the potential risks of such a strategy for transfusion safety, as health checks may also encourage donors with potential risks.

Second, our data shows that interventions to overcome barriers (rung 3) are very important. Only a few countries grant time off from work, which is not easy to implement without government involvement. However, fostering cooperation with companies that offer the possibility to donate as part of their employee healthcare programme might be promising. In addition, it is worth considering that donation locations have free parking and are also easily accessible by public transportation. Blood establishments should evaluate whether the reimbursement of travel costs (if necessary, only upon request) could be an option to facilitate the access to plasma donation for new plasma donors. The incentives reviewed in this study may prove useful in reducing some extrinsic donation barriers. However, it also seems important to develop interventions aiming at reducing (potential) donors' intrinsic donation barriers, such as fear or misperceptions about plasma donation [21, 22]. Such interventions could focus on intrinsic motivations to donate, for example, by increasing donors' *warm glow* or sense of donor identity [23], as is partly done in Denmark, Germany and Sweden.

Regarding loyalty programmes, our country examples show that their designs vary. Blood establishments that do not currently reward loyal donors may consider testing the implementing of a systematic loyalty programme. This can be as simple as acknowledging regular donations by sending donors *thank you messages* and rewarding them with certificates or badges at certain milestones. More advanced programmes can financially reward donors or allow them to collect points that can be redeemed for gifts or vouchers that may already be part of the incentive strategy. Additional gifts or vouchers that are already used to incentivize can also be rewarded to donors who bring friends or recommend the blood establishment. There are several ways to test what donors prefer, especially with regard to gifts and vouchers. Partnering with local restaurants, shops or entertainment companies may help attract new donors.

Direct financial compensation (i.e., cash payments) are not offered in many countries and are often regulated by law. Beyond legal restrictions, the World Health Assembly resolution additionally urges member states to encourage voluntary non-remunerated donations given the elevated risks of disease transmission and the potential harm to donors' health from excessive blood donations linked to paid donations [24]. In addition, there is a regulatory debate in the EU that has resulted in the recommendation that no financial incentives or inducements should be offered to donors. However, compensation to donors for losses (costs) may be allowed [25]. Our country-level analysis reveals that countries which financially compensate donors only seem to offer few other incentives if they operate in decentralized models. Thus, the potential to attract new donors or existing donors on a regular basis through a more advanced incentive strategy could be explored as more than half of the countries only operate with basic incentives.

In addition to the incentives in our dataset, plasma collectors may also benefit from insights from other health-related fields, such as living organ donations in Israel that are rewarded with insurance reimbursement as well as social support services [26]. In general, cooperation with health insurance companies can be very promising. In many countries (e.g., Germany), health insurance companies offer the possibility to collect points for preventive healthcare behaviour, such as regular dentist appointments. As being healthy is a prerequisite for plasma donation, it could be added to the programme to attract new donors. Moreover, similar to the Hollywood Walk of Fame, a star is embedded in the sidewalk of a German city (Muenster) for every new blood donor [27]. This donor recognition approach could also be considered as a plasma donation incentive.

Beyond the scope of health management, blood establishments could also consider transferring strategies from other areas that depend on customer loyalty. Various innovative approaches are possible when partnering with entertainment companies, such as streaming providers or publishing houses, to offer exciting and attractive incentives. Tickets to concerts and sports events can also be attractive rewards for donors and have the advantage of the media hype that these events generate. In terms of community management, digital badges for plasma donors could easily be integrated into social media channels (e.g., LinkedIn), signalling their stance and connecting them with other plasma donors. In Austria, as well as Australia, contests between universities are set up and known as 'vampire clubs/cups.' Here, two competing groups try to donate as much blood as possible and are motivated to win some type of attractive reward [28, 29]. Recent research shows that using such competitions to motivate young plasma donors is a promising way to significantly increase plasma donations during the competition [29].

Future avenues for research

We used the classification of incentives introduced by Chell et al. [17], who primarily divide incentives for blood donation into *monetary* and *non-monetary* incentives. Our study contributes to the literature by extending this framework to plasma donation and adding new incentives. Moreover, we related the incentives found in our study to the rungs of Nuffield's intervention ladder. This classification allows for a more nuanced differentiation between altruistic and non-altruistic strategies. Using the intervention ladder, we can place the

incentives on a continuum (ladder 1–6), assessing whether, or to what extent, the donor's material, financial and personal well-being is neutralized or improved after their donation, given a specific incentive.

This research has limitations. Although we have systematically scanned the blood establishments in our target countries, we acknowledge that we may have not identified all blood establishments in the target countries. However, the main targets of this study were to (1) identify commonly used incentives across EU and non-EU countries, and (2) give a consolidated summary. As plasma supply differs between countries, plasma collectors can share information on their incentive strategies and benefit from each other. Our overview of incentives presents avenues for future practice. Future research is needed to measure the effects of these incentives on donor motivation and donations and to derive implications on what incentives work best given different market situations and portfolios of incentives within a country. In addition, it would be worth looking at what specifically works in terms of recruitment and/or retention as we are not able to make distinctions between the two in our current data. Moreover, future research could test the effectiveness of innovative incentives that are used in other contexts (e.g., health insurance programme). Countries could also test these incentives in a smaller setting and evaluate their effect on plasma donation in the field.

Finally, with this study, we aim to provide a starting point for international exchange and create a space to learn from each other and to jointly develop the best practices to achieve the common goal of strengthening plasma donation behaviour in an ethical perspective required by the particular nature of this chain of interhuman solidarity.

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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 openly available in Zentrum für nachhaltiges Forschungsdatenmanagement at https://www.fdr.uni-hamburg.de/record/13407, reference number 10.25592/uhhfdm.13407.

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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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REVIEW

Vox Sanguinis ISBT International Society of Blood Transfusion

Is drug interference still an issue for pretransfusion testing of patients on anti CD38 and other monoclonal antibody therapies?

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Abstract

Certain therapies that target CD markers on some blood cells can affect pretransfusion testing. Key examples are anti-CD38, CD47 monoclonal antibody (mAb) therapies such as daratumumab (DARA) and magrolimab, which have presented a challenge for transfusion medicine laboratories around the globe. Scientists have been faced with not only introducing a protocol to provide safe blood to patients but also investigating the most effective method to remove the pretransfusion pan-agglutinating interference caused. A number of papers in the last 5 years have reported on various methods to remove pretransfusion interference; however, most of these studies have been conducted only in a few countries. Most recent reviews on this topic have focused on techniques and reagents to remove pretransfusion interference, and dithiothreitol is currently the gold standard for removing DARA interference. However, it was clear from this review that while many laboratories have developed processes for addressing interference in pretransfusion testing, and DARA interference may not be a major issue, there are still laboratories around the world, that may not have adequately addressed this issue. In addition, the impact of mAb interference on widely used techniques such as flow cytometry is unclear.

Keywords

daratumumab, DTT, interference, pretransfusion, transfusion medicine laboratories

Highlights

- Monoclonal antibody (mAb) therapies can still present a challenge for pretransfusion testing in some settings.
- There is variability in approaches addressing pretransfusion testing interference across laboratories.
- A global approach to testing of blood from patients on mAb therapies is urgently required.

INTRODUCTION

Monoclonal antibody therapies and potential issues for transfusion medicine

Multiple myeloma (MM) is an incurable haematological condition that affects men and women between the ages of 60 and 70 years of age and after New Zealand, Australia has the second highest incidence rate of MM with 2405 people diagnosed between 2012 and 2020 [1, 2]. In 2015, the United States Food and Drug Administration approved the use of daratumumab (DARA), a monoclonal antibody (mAb) therapy used to treat MM patients that have relapsed or have refractory forms of the disease [3, 4]. This drug targets the CD38 antigen sites on the transmembrane protein, which is highly expressed on plasma cells and malignant myeloma cells. Due to this targeted approach, DARA can interfere with pretransfusion testing as CD38 is also known to be expressed on reagent red cells and can demonstrate as a weakly positive pan-agglutinating alloantibody in indirect antiglobulin tests (IATs) including antibody screens (ABSCs), antibody identification panels and IAT crossmatches. This interference can be observed for up to 6 months after the last dose is given to a patient [5]. This can be an issue for transfusion medicine laboratories (TMLs) in being able to provide blood to patients in a safe and timely manner [6, 7]. There is variability across the globe in how TMLs resolve the interference this therapies cause. In some countries, negating the interference is no longer an issue due to the established use of dithiothreitol (DTT) and other testing approaches. However, in some countries like Australia and New Zealand, there are ongoing issues dealing with DARA interference in pretransfusion testing. While there have been several reviews published on this topic in the last few years, none have included the Australian perspective. This systematic review is focused on investigating the quality of studies conducted to date and identifying remaining gaps.

Current Australian guidelines and procedures for mAb therapies

To aid TMLs in Australia and New Zealand in the provision of safe blood for patients on mAb therapies, the guidelines set by the Australian and New Zealand Society of Blood Transfusion [8] (ANZBT) for 2021 stipulate that prior to commencing mAb therapies like DARA or magrolimab, clinicians should communicate with the TML that patients are to begin with treatment with a specified mAb therapy and that a blood group, ABSC and either a genotype or phenotype consisting of antigen typing for Rhesus (Rh), Kell, Kidd, Duffy and MNS blood systems is to be performed (Figure 1). Following commencement of either anti- CD38 or anti- CD47 therapy, if a patient

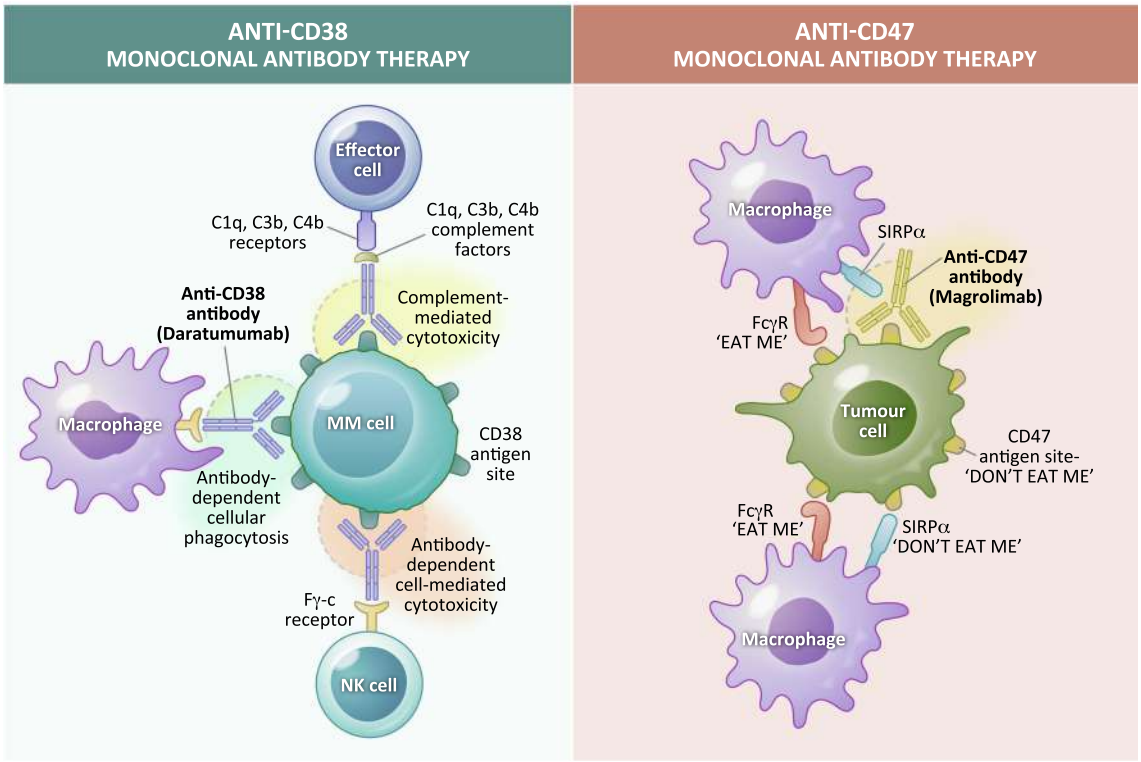

FIGURE 1 Monoclonal antibody therapies, such as daratumumab and magrolimab, work by attaching to specific antigens on cells. For example, daratumumab binds to CD38 antigen sites found on multiple myeloma cells to encourage antibody-dependent cell-mediated cytotoxicity and phagocytosis by macrophages and natural Killer cells or complement mediated cytotoxicity by complement factors. Magrolimab works by binding to CD47 antigen sites found on solid tumour cells. This antigen site is important as it signals to macrophages and others cells to not phagocytose the tumour cell, that is, 'don't eat me'. By binding to this antigen, magrolimab blocks SIRPα from this signal and allows for phagocytosis to occur.

requires transfusion of packed red blood cells (PRBCs), these red cells should be as close to antigen negative for the patient as possible, with Rh and Kell being of highest priority to MNS being of least priority. The guidelines also mention that laboratories may attempt to remove the interference with DTT or trypsin or they may elect to implement a protocol for transfusion extended matched PRBCs. In 2018, the Australian Red Cross Lifeblood service saw a 30% increase in demand for genotyping to be performed, with 23 out of 49 TMLs indicating that it was a requirement for mAb therapy [9]. There is currently no international standard for what TMLs should be doing to negate DARA and other mAb therapy interference and each country, including Australia, and individual laboratories may also have different approaches.

METHODS

Keywords used in the literature search

The following databases were searched for relevant papers: EBSCO (Health) and PubMed Central. These databases were selected as they are widely used, and it is acknowledged that some papers that are not readily available via these sites could have been missed and may be a limitation of this review.

Keywords used were 'daratumumab', 'blood bank', 'transfusion', 'transfusion medicine', 'anti-CD47', 'anti-CD38', 'magrolimab' and 'interference'. The result of 2047 articles were uploaded into EndNote Referencing software [10]. Once duplicate articles were removed, 108 articles, 1939 articles were screened by their title and abstract for relevance to the review. These articles were then further evaluated before a decision was made regarding inclusion or exclusion for this review. A total of 1917 articles were deemed irrelevant. To ensure the best practice for systemic reviews, the PRISMA process for systemic reviews was followed and includes inclusion and exclusion criteria, list of databases searched, full search strategies and data collection processes [11].

Inclusion and exclusion criteria

Inclusion criteria included the following keywords: 'blood banking', 'transfusion', 'interference', 'mitigating', 'CD47', 'CD38' or 'antibody screening'. From the full-text articles, further inclusion criteria included methods or technology described in detail on how either CD38 or CD47 interference was mitigated. These studies could also include comparison studies between reagents.

Exclusion criteria included keywords such as 'antibody incidence', 'red cell transfusion outcomes', 'clinical application', 'practical guidance', 'clinical efficacy', 'sustained response', 'treatment' or 'therapies'. Reviews and case studies were also not included.

RESULTS

What do previous studies tell us?

From a total of 2047 publications initially identified through the keyword search strategy, 22 papers were selected for inclusion in this review.

Based upon the 22 papers reviewed, 14 different reagents were identified as being used to remove interference. The oldest article that was published was in 2015 with the newest articles published in 2023 [12–14]. Number of samples tested with each reagent and how effective each reagent was in removing mAb therapy interference have been summarized in Table 1. While most articles focused on one method, articles described the use of several established reagents to negate both CD47 and CD38 mAb therapies, which included DTT, trypsin and papain [15].

Statistical analysis of the data was variable across the papers reviewed, with most studies reporting percentages, while 12 articles did not report undertaking any statistical analysis. Some approaches used include Cohen's kappa coefficient to compare two institutions' results and determine a substantial agreement between them [16]. One article utilized a t-test to compare the effectiveness of DTT versus polybrene [17]. The variability in the approach to data analysis observed is not surprising, as statistical of analysis of data may not have been appropriate due to small numbers of samples tested in some studies. For example, there were five articles that analysed less than five samples and 14 articles that tested more than 10 samples.

Spiking of samples was described in many studies. There was one study that only used antisera spiked samples and there were two studies that used no spiked samples in their studies [16, 18, 19]. The study that used the most samples tested 61 DARA patients compared with one study that used only four DARA samples [15, 16]. The spiking of the samples included either taking a sample from a patient with known alloantibodies and spiking it with DARA or taking patients' samples, who are on DARA treatment with no alloantibodies and spiking them with commercial antisera. For example, Chinoca Ziza, Paiva, Mota, Dezan, Schmidt, Brunetta et al. [20] tested 20 patient samples; however, three of their samples were spiked with commercial antisera including alloanti-D, -C, -Kell, -Jkb, -S and Kpb. Additionally, two studies used a DARA spiked sample that had alloantibodies for E and Kell [12, 21]. These samples were then tested with anti-DARA to remove the pretransfusion interference and validate their method to detect those alloantibodies.

The low number of samples and spiking of samples was commonly observed in many papers reviewed, possibly indicating a lack of access to suitable clinical samples. However, it is important to note that the study with the greatest number of samples (61) did not use any spiked samples [16]. It was one of nine articles that didn't analyse spiked samples to validate their chosen method.

DTT is considered the gold standard for removing anti-CD38 interference in pretransfusion interference; however, there are drawbacks with the use of DTT in that it is time-consuming, increases the

TABLE 1 Reagents used in various studies.

Reagent observed	Targeted mAb therapy (specific or general)	Number of samples (total)	Rate of success	References
DTT	Anti-CD38- general	200	92.0%	[13, 14, 17, 18, 20, 22, 23, 28–30, 43]
Anti-DARA	DARA- only	40	96.6%	[12, 21]
Papain	Anti-CD38- general	33	100%	[24]
Trypsin	Anti-CD38- general	72	99.2%	[13, 16]
BMAP	Anti-CD38- general	20	95%	[20]
LISS and/or PEG	Anti-CD38- general	33	69.6%	[26]
Daudi cells	Anti-CD38, Anti-CD47- general	19	Indeterminant[a]	[27, 37]
Fab Fragments	DARA- only	50	86%	[14, 19]
2-ME	Anti-CD38- general	37	Indeterminant[a]	[28]
Polybrene	Anti-CD38- general	58	Indeterminant[a]	[17, 28]
Gamma-clone anti-IgG (IgG4-deficient)	Hu5F9-G4- only	9	Indeterminant[a]	[15, 33, 44]
Allogenic PEG absorption	Anti-CD47- general	4	Indeterminant[a]	[33]
Soluble CD47	ALX148- only	0[b]	Indeterminant[a]	[33]
High-affinity SIRPα monomers	ALX148- only	0[b]	Indeterminant[a]	[33]

Abbreviations: BMAP, blockage MAb protocols; DARA, daratumumab; DTT, dithiothreitol; LISS, low ionic strength saline; mAb, monoclonal antibody; PEG, polyethylene glycol.

[a]For indeterminant, it is to be assumed that while there was no exact statistical analysis, researchers have stated that it was successful.

[b]It was noted where no patient samples were used, it may be deemed effective as it was effective for another anti-CD47 mAb therapy.

workload for staff as it is completely reliant on having to be performed manually, is dependent on experienced staff to be performed and causes the loss of the Kell antigen [22]. Although studies using a lower concentration of DTT, particularly in gel column agglutination technology (CAT), were able to preserve the Kell antigenicity, there is the theoretical problem that it may diminish the ability to detect IgM alloantibodies [23]. Other methods such as papain and trypsin reagents destroy antigens of clinically significant alloantibodies, such as Duffy or MNS blood group systems, and several other clinically insignificant alloantibodies like Chido, Rogers and Lutheran [24, 25]. Low ionic strength saline (LISS) and polyethylene glycol (PEG) have been observed to alleviate DARA interference up to 70% of the time [26].

Daudi B cell line, 2-ME and other anti-DARA reagents also have limitations [27, 28]. For example, anti-DARA is limited to only DARA formulations and would not work on patients receiving any other CD38 drugs like Isatuximab [12].

Preliminary research performed by Chapuy, Nicholson, Aguad, Chapuy, Laubach and Richardson [13] first described the use of DTT in tube to denature the CD38 antigen to negate the interference DARA causes. However, this also results in the Kell blood groups being denatured and, subsequently, it was advised that if this reagent was to be used, Kell negative blood is to be provided for patients. The following year, in an international study, Chapuy, Aguad, Nicholson, AuBuchon, Cohn and Delaney et al. [29] and the Biomedical Excellence for Safer Transfusion Collaborative sent 25 samples worldwide to TMLs, mainly large centres and RCRLs, to determine if their DTT method could consistently detect alloantibodies, including either alloanti-S or Duffy. In their study, all sites reported that they were able to correctly identify the alloantibodies in either gel CAT, solid phase or tube technology. Since then, researchers have been reviewing other reagents such as trypsin, papain, cord cells, recombinant anti-idiotype antibody (anti- DARA), polybrene, blockage MAb protocols, DARA Fab fragments, PEG and LISS [25].

Studies have also attempted to simplify and modify the use of DTT in CAT and tube to aid in the detection of Kell alloantibodies through weaker concentrations of DTT. Known as the Osaka method, researchers found a way to modify the DTT method to be able to identify Kell alloantibodies [30]. These researchers lowered the concentration of DTT, down to 0.01 mol/L, and used an automatic cell washer to ease the labour-intensive methodology. More recent studies performed by Izaguirre, Del Mar Luis-Hidalgo, Gonzalez and Castano [23], have also had successful outcomes creating their own modified versions of the Osaka method, that removed the DARA interference, detect Kell alloantibodies and make it easier for less experienced staff to be able to perform.

DISCUSSION

Mitigating mAb interference in pretransfusion interference

DTT in tube is the current gold standard in negating anti-CD38 mAb therapy interference for TMLs. With a 92.0% success rate, DTT can successfully and easily remove the interference while still being able

to detect all but one, clinically significant alloantibody. However, this method is seen as labour-intensive, with the requirement of reagent ABSC red cells to be treated with DTT prior to testing [31]. Additionally, it has been suggested that patients on DARA may have a lower risk of alloimmunization [32].

In contrast, negating anti-CD47 interference in pretransfusion testing has been seen as a bigger challenge due to the strong interference not only being observed in IAT ABSCs but also observed in blood grouping. As described by Kim, Yoon, Hustinx, Sim, Wan and Kim [33], while reagents and techniques such as Gamma-clone anti-IgG that is IgG4-deficient and allogenic PEG absorption aid to remove this interference in IAT ABSCs, these reagents and techniques do not work to negate all anti-CD47 therapy interferences (Table 1). For example, it was noted by the authors that while Gamma-clone anti-IgG, a preferred reagent for IAT ABSCs for patients that are on the anti-CD47 therapy, Hu5F9-G4, is ineffective for patients on the anti-CD47 therapy, ALX148. However, soluble CD47 may be an effective way to negate both anti-CD47 therapy interferences but more studies are required. Similarly other strategies have been suggested for removing anti-CD47 drug interference [34–36]. It is important to note that, Daudi cell stroma has been recognized to be able to remove both anti-CD47 and CD38 interference in pretransfusion testing [37].

Overall, TMLs still need to consider that there is innovative research being performed on anti-CD38 reagents and modifications of DTT to negate DARA interference. Reagents such as anti-DARA and DARA Fab fragments, while not yet commercially approved and distributed, have demonstrated that for routine laboratories, they will be less labour-intensive and require basic transfusion knowledge. In contrast, modifications of the DTT methods, such as Valencia or Osaka methods, show promising results, although further research with larger sample sizes is required to understand the full effectiveness of these methods. Despite the success of the DTT approach, issues still remain in the broad implementation of this method in some TMLs. For example, a 2019 survey conducted by the Australian Red Cross Lifeblood showed that five of 45 responding TMLs performed DTT or enzyme-treated cell panels as the first line of investigation off patients on anti-CD38 MAb therapies [9]. Additionally, they highlighted that 18 out of the 45 responding TMLs preferred to send patients' samples antibody investigation or perform molecular genotyping prior to a patient commencing MAb therapy.

DARA was listed on the Australian Pharmaceutical Benefit Scheme as of 2021 as a second line of treatment for patients with MM [38] followed by approval for a treatment for systemic light chain amyloidosis [39]. In late 2021, the ANZBT Guidelines released an amendment advising DTT treatment of reagent red cells [8]. Unfortunately, at this time in Australia, DTT has not been approved for use in a diagnostic clinical setting from the Australian Therapeutic Goods and Administration as it is considered to be 'for research use only'. This has meant that Australian TMLs have not been using DTT as widely as TMLs in other countries such as the United Kingdom, where there is approval to use DTT to negate DARA interference in the clinical laboratory setting [40].

Where to from here?

It is important to note that this review included studies from the United States, United Kingdom, Taiwan, Belgium, Germany, Brazil, Spain, Mexico, Japan, Canada, China, Korea and The Netherlands. In many of these countries, anti-CD38 mAb therapy interference with pretransfusion testing is no longer an issue. In some countries, for example, Ireland, TMLs that are not registered for antibody identification send any positive ABSCs to a registered TMLs, and therefore, the drug interference does not concern them [41]. However, to the best of the authors' knowledge in countries like Australia and New Zealand, DARA and other mAb therapies are still an issue to standardize testing in TMLs across the country. Anti-CD38 therapies are widely used across the globe; however, due to the lack of published studies from many other countries, it is unclear if there are other countries with similar issues with DARA interference in pretransfusion testing. In addition, while several studies have investigated DARA interference there are some remaining issues in that many of them do not include testing of weak or dosage presenting antibodies such as Duffy.

With many other anti-CD38 mAb therapies in the pipeline, more research is needed on these and other therapies such as anti-CD47 mAb therapies in relation to pretransfusion interference. In addition, DARA interference may not only be an issue for TMLs as this drug can interfere with other tests such as flow cytometry [42]. In future, multi-centre studies need to be considered, in order to overcome the challenge of not being able to access an adequate number of samples to test in many clinical facilities. Involving pharmaceutical companies who develop mAb therapies in discussions relating to this research is important. This will ensure enhanced awareness of potential mAb drug interference in pretransfusion testing and engage these companies in discussions on strategies to reduce this interference when new drugs are being developed. There is probably also a role for professional societies and blood banks in leading discussions and supporting clinical education, as well as developing clear guidelines relating to drug interference in pretransfusion testing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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ORIGINAL ARTICLE

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Immunoadsorption as a method of antibody donation during the COVID-19 pandemic

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Abstract

Background and Objectives: Initial therapeutic efforts to treat severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) included the use of plasma from convalescent donors containing anti-SARS-CoV-2 antibodies. High-neutralizing antibody titres are required for therapeutic efficacy. This study aims to show that immunoadsorption followed by tangential flow filtration can be used to obtain antibody concentrates with high-neutralizing capacities.

Materials and Methods: Eligible donors ($n = 10$, five males and three females) underwent immunoadsorption using adsorber columns specific for human antibodies. Glycine-washed out eluates of 1.5 L volume were further concentrated by tangential flow filtration using 30 kDa ultrafiltration membranes. The same membranes were applied for diafiltrations to exchange residual glycine for 0.9% normal saline.

Results: Antibody concentrates were obtained within 8 h from the start of donation and had 4.58 ± 1.95, 3.28 ± 1.28 and 2.02 ± 0.92 times higher total IgG, IgA and IgM concentrations, 3.29 ± 1.62 and 3.74 ± 0.6 times higher SARS-CoV-2 N and S antibody concentrations and 3.85 ± 1.71 times higher SARS-CoV-2 S-specific IgG concentrations compared to the donors' peripheral blood. The specific SARS-CoV-2 virus neutralization capacities increased in all but one concentrate. All antibody concentrates (50–70 mL final volume) passed microbiological tests, were free of hazardous glycine levels and could be stored at −80°C and 4°C for 1 year with 20 ± 3% antibody loss.

Conclusion: Immunoadsorption followed by tangential flow filtration is a feasible procedure to collect IgG, IgA and IgM as well as SARS-CoV-2 N- and S-specific antibody concentrates of low volume, free of albumin and coagulation factors. Whether these concentrates can be used as passive immunisation in infected patients remains to be elucidated.

Keywords

antibody donation, COVID-19 convalescence, immunoadsorption, virus neutralization

Highlights

- Combining immunoadsorption and tangential flow filtration is a feasible method to obtain antibodies from convalescent donors. We have successfully collected IgG, IgA and IgM antibodies as well as SARS-CoV-2 N- and S-specific antibodies from peripheral blood in all 10 donations. A median of 2586 ± 741 mg IgG, 271 ± 83 mg IgA, 138 ± 73 mg IgM have been obtained per donation.
- The resulting antibody concentrates had on average three to four times higher SARS-CoV-2-specific antibody concentrations compared to the peripheral blood of the donor and showed increased neutralization capacity in all but one sample.
- This is a safe method for manufacturing antibody concentrates of approximately 50 mL within 1 day that can be stored at 4°C and −80°C for over 2 years and are free of albumin and coagulation factors.

INTRODUCTION

Several treatments against COVID-19 are available to help patients with severe symptoms. One of them is the use of COVID-19 convalescent plasma (CCP) [1]. This approach relies on the hypothesis that antibodies are formed in sufficient amounts in the previously infected individuals to be donated as passive immunisation.

Studies on the effectiveness of CCP in treating severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have identified the importance of high neutralization titres [2–4].

In this study, we present a method of antibody donation that can obtain high neutralization titres, by using one-time plasmapheresis including immunoadsorption (IA) followed by tangential flow filtration (TFF) for the production of antibody concentrates (ACs) as an alternative to CCP. The ACs are of low volume and free of plasma components like albumin and coagulation factors that potentially could, if still present, lead to unwanted side-effects during transfusion. Side effects possibly related to volume overload, when CCP doses are increased, are also circumvented.

IA is an established alternative to plasma exchanges for the treatment of a wide range of autoimmune diseases and is considered to be safe for patients [5, 6]. Its efficacy is based on the removal of immunoglobulins from patients' plasma and simultaneously returning most of the antibody-cleared plasma to the patients whereby removed antibodies are usually discarded. Instead of throwing these antibodies out, we collected them from our convalescent donors during in-process elutions of the adsorbers [7].

TFF is commonly used in the downstream processes of pharmaceutical products, for example in the concentration of immunoglobulins, vaccines and recombinant proteins [8–10], using a porous membrane of defined pore size that traps the product in a loop while constantly removing liquids plus particles smaller than the defined pore size. In addition, it can be used to exchange liquids by continuously adding a new solution to the loop at the same rate the previous one is removed, a process called diafiltration. TFF has already been successfully applied in the downstream process of SARS-CoV-2 neutralizing antibodies (nAb-SARS-CoV-2) derived from cell cultures [11].

Our first two IA for the production of ACs with reusable adsorbers, specific for IgG (subclasses 1–4), IgA and IgM antibodies, were quite promising [12]. Here, we present the extension of our initial case report, with additional quantitative data and focus on (1) using one-time adsorbers, (2) assessing the concentration process in detail and (3) comparing two different storage conditions, 4°C versus −80°C.

MATERIALS AND METHODS

Sampling

In all donations, samples for analyses were drawn from the peripheral blood of the donors before and after the IA as well as in the final ACs directly after preparation. Once COVID-19 antibody assay availability was significantly improved, immunoglobulins and specific antibodies were also monitored in eluates, during concentration and diafiltration steps of the TFF, as well as on several time points during storage at 4°C and −80°C for 2 years (3, 6, 9, 12, 18 and 24 months).

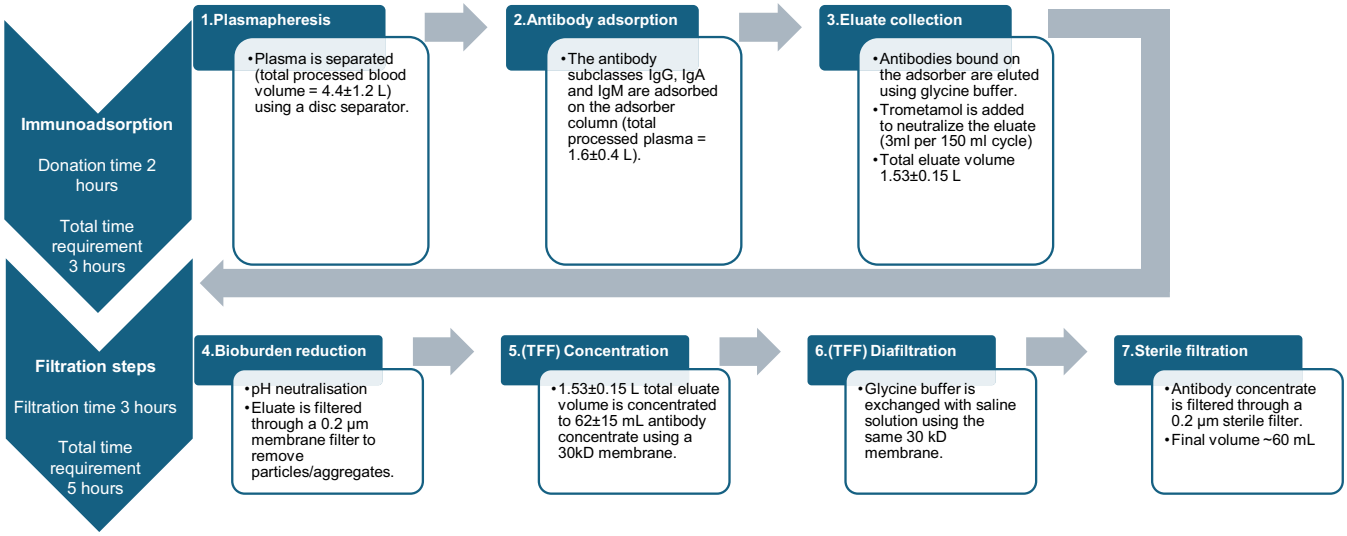

FIGURE 1 Schematic representation of the process steps during immunoadsorption and tangential flow filtration. Immunoadsorption is divided into three steps. Plasma donation (separates the antibody containing plasma from the remaining blood), antibody adsorption (binds the antibodies on the adsorber) and eluate collection (using a glycine buffer to elute the antibodies). Tangential flow filtration is divided into four steps. Bioburden reduction filtration (to remove particles larger than 0.2 µm), concentration (to concentrate the antibody solution), diafiltration (to exchange the glycine buffer with saline solution in water) and final sterile filtration (to remove contaminants).

Immunoadsorption

Five male and three female donors participated in this study after medical assessment, as previously described [7]. Informed consent was obtained and documented from all donors. Two of the donors (1 and 2) agreed to donate twice with approximately 1 year gap between the first and second donation. Each donor had recovered from a previously diagnosed SARS-CoV-2 infection prior to the antibody donation. Due to initial non-availability, the first three donors had not been vaccinated before IAs. IAs were performed as previously described [12] and were tolerated by all donors very well. A schematic presentation of each step of antibody donation and concentrate preparation can be found in Figure 1. Each donor was tested for serum protein, SARS-CoV-2 antibodies, immunoglobulins and blood count before inclusion in the study, before and after IA.

Tangential flow filtration

To remove excessive volume, column eluates obtained from the IA containing IgG-, IgA-, IgM- and COVID-specific antibodies were first filtered through a 0.2 µm membrane filter with 260 cm^2 filter area (Supor EAV, Cytiva Dreieich, Germany) to reduce the bioburden and then concentrated using a TFF system as previously described [7]. A total of 0.9% (w/v) NaCl solution in water, subsequently referred to as saline solution, was used to exchange the remaining glycine [12]. Finally, sterile filtration with 5.6 cm^2 filter area (Supor ECV, Cytiva Dreieich, Germany) was performed before aliquoting the ACs in 1.5 mL tubes. Samples were stored at 4°C and −80°C. A schematic representation of each step during TFF can be found in Figure 1.

Antibody determination and virus neutralization

The Tina-Quant IgG/IgA/IgM Gen.2 immunoassays (Roche Diagnostics GmbH, Mannheim, Germany) are immunoturbimetric assays and were used in combination with the Cobas 501 analyser to determine the IgG/IgA/IgM concentrations. The assays are based on goat-derived anti-human IgG that reacts by forming an antigen/antibody complex. Polyethylene glycol was then added to determine concentrations using a turbidimeter.

SARS-CoV-2 N-specific antibodies were determined using the quantitative Elecsys Anti-SARS-CoV-2 electrochemiluminescence immunoassay (ECLIA) (Roche Diagnostics GmbH, Mannheim, Germany) on the Cobas e801 analyser. Biotinylated SARS-CoV-2 specific recombinant antigen and ruthenylated SARS-CoV-2 specific recombinant antigen combine to form a sandwich complex. This complex represents the nucleocapsid (N) antigen of SARS-CoV-2. Sample is added to the mixture and the SARS-CoV-2 N specific antibodies present in the sample bind to the sandwich complex. This complex then subsequently binds to streptavidin-coated microparticles. Inside the measuring cell, the microparticles are magnetically captured onto the surface of the electrode. Chemiluminescent emissions are induced and measured using a photomultiplier. Results are reported as cut-off indices (COI) comparing the electrochemiluminescent signal with cut-off values previously determined via calibration. Calibration curves were added by the manufacturer in a later assay version and used from donation 3 onwards.

The Elecsys Anti-SARS-COV-2 S ECLIA (Roche Diagnostics GmbH, Mannheim, Germany) is a quantitative assay to determine antibodies against the receptor binding domain (RBD) of the spike (S)-protein (SARS-CoV-2 S-specific antibodies). This assay was used as soon as it became available starting from the third donation in

combination with the Cobas system. It is based on a recombinant protein that represents the RBD from the spike (S)-antigen resulting in a chemiluminescent sandwich ELISA analogous to the Sars-Cov2 N assay. A 2-point calibration curve is used to determine the concentration of SARS-CoV-2 S-specific antibodies, reported in IU/mL (1 IU/mL is equivalent to 1 binding antibody unit [BAU]/mL).

To detect IgG levels against the SARS-CoV-2 spike S1 subunit (S), the anti-SARS-CoV-2 QuantiVac-ELISA (Euroimmun, Luebeck, Germany) was applied, and the results are reported in BAU/mL. Values are plotted against a 6-point calibration curve using World Health Organisation reference serum.

To determine the antiviral efficacy, in-house full virus neutralization tests were performed as previously described using the Wuhan-Hu1 WT Isolate (GISAID Accession number: EPI_ISL_425126) [12].

The Elecsys anti-thyroglobulin (Tg) and anti-thyroid-peroxidase (TPO) immunoassays (Roche Diagnostics GmbH, Mannheim, Germany) were used to determine the anti-Tg and anti-TPO antibody concentrations in donation 5. Both antibodies are disease-triggering autoantibodies in Hashimoto thyroiditis [13]. Antibodies from the sample compete with antibodies from the reagents for limited binding sites. The mixture is analysed on the Cobas e801 analyser. The results are determined using a 2-point calibration curve and converted into IU/mL.

Glycine, albumin and protein analyses

Glycine concentrations were measured during the IA and in the AC using the Biochrom 30+ amino acid analyser (Biochrom GmbH, Berlin, Germany). This ion exchange chromatography is based on five lithium citrate buffers with post column derivatization using Ninhydrin. The measurements were normalized according to the glycine HCl solution used during the IA.

The Albumin Gen.2 and the Total Protein Gen.2 test (Roche Diagnostics GmbH, Mannheim, Germany) were used to determine the albumin and total protein concentrations according to the manufacturer's instructions.

Microbiological tests

Microbiological controls were performed according to the Institute for Transplant Diagnostics and Cell Therapeutics standard operational procedures equivalent to tests on cellular products as demanded in the national guidelines for the production and administration of haematopoietic stem cell products [14].

For aerobic and anaerobic bacterial cultures, test tubes were inoculated with 1 mL each per product on the day of production after the last filtration step and repeated after 2 years in storage. Bacterial colonies were determined on day 8 after inoculation. In case of positive bacterial contamination detected by Virtuo (BioMerieux), bacterial colonies are analysed on agar plates.

Statistics

Statistical analysis to calculate median, standard deviation and significance (paired *t*-test) were performed using Microsoft Excel 365.

Ethics statement

The Ethics Committee of the University Clinic Duesseldorf has reviewed this study and voted positive (study number 2022-1132). Informed consent was obtained and documented from all donors.

RESULTS

IgG, IgA and IgM concentrations and absolute values

The median antibody concentrations of IgG, IgA and IgM in donors' peripheral blood prior to donations were 947 ± 211 mg/dL, 141 ± 40 mg/dL and 118 ± 82 mg/dL, respectively (Table 1). Antibody serum concentrations in the peripheral blood decreased after IA on average by 29 ± 7% (IgG), 27 ± 7% (IgA) and 23 ± 6% (IgM). Final concentrations in the AC compared with the peripheral blood increased 4.6 ± 2.0 times for IgG, 3.3 ± 1.3 times for IgA and 2.0 ± 0.9 times for IgM. A median of 2586 ± 741 mg IgG, 271 ± 83 mg IgA, 138 ± 73 mg IgM have been obtained per donation.

SARS-CoV-2 antibody concentrations and absolute values

SARS-CoV-2 nucleotide (N)-specific antibodies in donors' sera prior to the IA ranged between 1.7 COI and 154 COI (Table 1). After IA, the SARS-CoV-2 N antibody serum concentrations decreased on average by 32 ± 15%. SARS-CoV-2 N antibodies in the final AC resulted in 1.3–6.8 times higher values compared with the peripheral blood.

In addition to the SARS-CoV-2 N antibodies, antibodies against the spike protein (S) were determined by two different test systems except donations 1 and 2 in the final AC ranging between 1773 and 180,000 IU/mL.

Once the test was available on a routine basis, SARS-CoV-2 S-specific IgG was measured between 597 and 8162 BAU/mL (Table 1) and decreased on average by 24% ± 11% after IA in peripheral blood of the donors. In the final AC, IgG specific for SARS-CoV-2 S lay 3.8 ± 1.8 times higher compared with the peripheral blood.

Comparing the neutralization titres from the peripheral blood of the donor to the AC, all but one titre (donation 4) had increased by at least one titre level (two-fold serial dilution) (Table 1). On average, an increase of 2 ± 1.3 titre levels was determined. Most prominent titre increases were seen in donation 5 (increased from 1:1280 to 1:10240) and donation 6 (from 1:1280 to 1:20480) (Table 1). Median absolute SARS-CoV-2 specific IgG content ranged from 36,134 to 1,203,482 BAU.

TABLE 1 Summary of antibody concentrations, neutralization titres, glycine rest and final product volume.

	Donor blood before IA					Donor blood after IA					Antibody concentrate						
	IgG, IgA, IgM	CoV2 (N)	CoV2 (S)	CoV2 (S IgG)	NT	IgG, IgA, IgM	CoV2 (N)	CoV2 (S)	CoV2 (S IgG)	NT	IgG, IgA, IgM	CoV2 (N)	CoV2 (S)	CoV2 (S IgG)	NT	Glycine	Volume
Donation	mg/dL	COI	IU/mL	BAU/mL	1/x	mg/dL	COI	IU/mL	BAU/mL	1/x	mg/dL	COI	IU/mL	BAU/mL	1/x	µmol	mL
1	899, 119, 84	30.5[a]	t.n.a.	t.n.a.	n.t.	600, 83, 66	t.n.a.	t.n.a.	t.n.a.	n.t.	5591, 523, 212	106[b]	t.n.a.	738[b]	160	11.7	49
2	879, 113, 111	42.5[a]	t.n.a.	t.n.a	20	558, 74, 89	25.2[a]	t.n.a.	t.n.a.	n.t.	7696, 603, 372	148[b]	t.n.a.	937[b]	80	67.5	61
3	1551, 197, 251	154	597	597	80	996, 127, 163	114	n.t.	442	80	4956, 456, 367	204	1773	623	160	12.6	58
1.1	919, 120, 85	1.7	>2500[c]	3535	2560	632, 90, 61	1.3	>2500[c]	2707	2560	6176, 661, 333	11.6	>2500[c]	25,058	10,240	1.63	44
4	1212, 175, 148	91	>2500[c]	3681	2560	946, 146, 120	73.6	>2500[c]	3224	1280	2534, 274, 140	177	>2500[c]	10,706	2560	13.1	101
5	962, 177, 325	53	>2500[c]	3280	1280	752, 144, 274	37.8	>2500[c]	3140	640	3628, 444, 430	171	49,046	15,600	10,240	18.5	72
2.1	931, 130, 124	14.6	>2500[c]	1882	640	700, 98, 97	11	>2500[c]	1224	n.t.	2753, 338, 164	45	53,227	6400	1280	12.5	63
6	1166, 152, 72	112	55,052	8162	1280	904, 117, 59	80	39,631	5564	1280	3904, 370, 116	140	180,000	20,398	20,480	11.1	59
7	1110, 87, 169	60	6304	1206	320	830, 67, 135	43	4655	802	320	4517, 248, 271	184	29,013	6021	2560	10.8	50
8	802, 217, 55	7	23,451	2558	1280	469, 136, 36	2	5622	678	640	3755, 721, 115	36,8	96,894	10,466	5210	12.2	61
Median	947, 141, 118	48	23,451	2919	1280	726*, 108*, 93*	38*	5622	1966	640	4211, 450, 242	144	51,137	8433	2560	12	60
Std. dev.	211, 40, 82	47	20,191	2174	906	168, 28, 66	37	16,265	1651	770	1516, 153, 112	65	57,535	8066	6249	17	15

Note: Shown are concentrations of IgG-, IgA-, IgM- and COVID-specific antibodies (Sars-CoV-2 N and S) and neutralization titres (NT) in the peripheral blood of donors before and after the immunoadsorption (IA) and in the final antibody concentrate.

a: Determined with a qualitative test, due to lack of better options. b: Determined on back up sample as soon as a quantitative test was available (6 months). c: Sample was not diluted. Data points not included in median; t.n.a.: test not available.; n.t.: not tested.

*Significant difference between before and after IA.

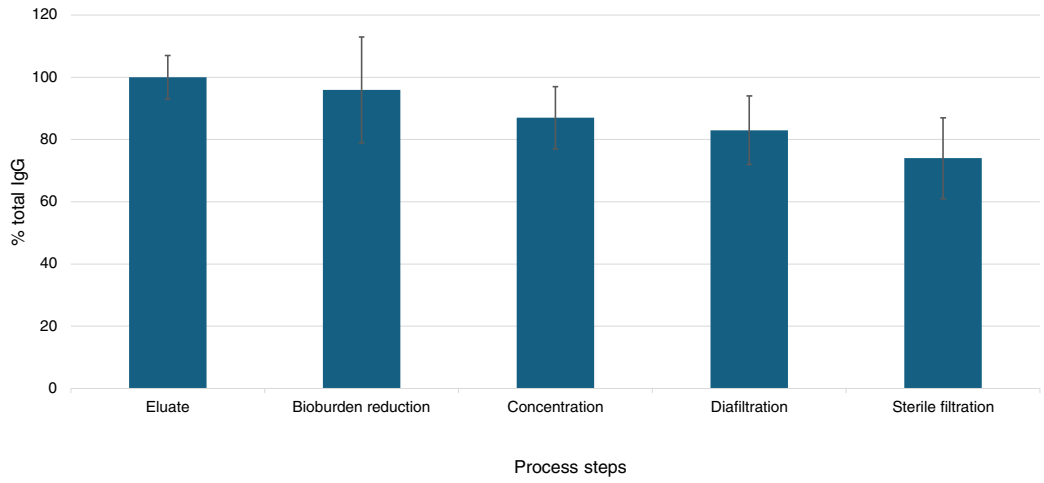

FIGURE 2 Recovery of IgG at every filtration step. Average IgG recovery, shown as a percentage of the baseline, were monitored during every step of the process (after bioburden reduction, concentration, diafiltration and sterile filtration) in donations 2.1, 5, 6, 9 and 10. Total IgG content of the eluate served as baseline 100% and was calculated using the IgG concentration in mg/dL multiplied by the volume at that stage. Recovery for donation 5 at the bioburden step was not measured.

Monitoring TFF

In five donations (2.1, 5, 6, 9 and 10), product loss was monitored during TFF. Small amount of IgG was lost during each step of the process (Figure 2). On average, 13.0% ± 7.7% were lost during concentration, 4.2% ± 3.9% during diafiltration and 8.6% ± 5.2% during sterile filtration. The highest loss of IgG was found in donation 2.1 with 25% during the concentration step. The cumulated IgG loss over all process steps was 26% ± 8%.

Safety and storage

Glycine is necessary for the removal of bound antibodies from adsorber matrices allowing a quick in-process recovery of matrix-bound anti-human IgG for following adsorber cycles. Using diafiltration, glycine was successfully removed from our ACs and replaced by saline solution. In every AC, the final glycine concentration was below 1% of the original value (Table 1), and final microbiological tests showed that all ACs passed microbiological tests after donation.

The end volume of every AC was separated into 1.5 mL tubes and then stored at 4°C and −80°C. ACs 1 and 2 from the first two donations were stored for 2 years already. Looking at SARS-CoV-2 N specific antibodies, samples frozen at −80°C compared with those stored at 4°C show a slightly better outcome in frozen specimen (donation 1 at 176 COI [−80°C] compared with 133 COI [4°C] and donation 2 at 228 COI [−80°C] compared with 217 COI [4°C] [Table SI]).

Comparison of Ig flex and Ig omni 1

Antibody concentrates 1 and 2 were produced by using Ig flex columns for IA. Interestingly, the values of SARS-CoV-2 N specific antibodies started to increase during storage at −80°C for 12 months in the first two ACs adsorbed with Ig flex. The SARS-CoV-2 N specific antibodies from donation 1 almost doubled from 106 to 196 COI and increased from 148 to 246 COI in the AC from donation 2. Consecutively, in the second year of storage, the values decreased to 158 COI (donation 1) and 174 COI (donation 2) after 24 months of storage. This was different in those ACs collected using the Ig omni 1 adsorbers. Though not all the donations using Ig omni 1 adsorbers have been stored for more than 1 year yet, SARS-CoV-2 N- and S-specific antibodies remained stable after 6 and 12 months (Table SI).

SARS-CoV-2 S-specific antibodies show a similar trend under storage conditions. Freezing at −80°C preserved slightly more antibodies for donation 1 compared to storage at 4°C with 453 IU/mL (−80°C) compared to 350 IU/mL (4°C) after 18 months. Concentrations of donation 2 were equal in samples stored at −80°C and 4°C (783 IU/mL) after 18 months of storage (Table SII). The values for the first two donations also increased during the second year of storage from 426 to 449 IU/mL (donation 1) and 730 to 783 IU/mL (donation 2). Donations of ACs obtained by Ig omni 1 adsorbers did not show concentration changes during storage.

Hashimoto thyroiditis

Donor 4 turned out to have a positive history of autoimmune Hashimoto thyroiditis, which had not been communicated during the enrolment in this study but was detected afterwards by coincidence. Of course, anti-Tg and anti-TPO antibodies were also collected and concentrated by our method. All antibodies and immunoglobulins remained stable with the exception of anti-Tg which doubled during 1 year of storage (Figure S1).

DISCUSSION

IA is an effective therapeutic approach to reduce circulating autoantibodies [5, 15]. To our knowledge, we are the first to use IA to obtain

viral-specific antibodies with the objective to achieve an AC for potential therapeutic use. Using IA followed by TFF, we succeeded in the production of 10 ACs from convalescent donors. The results show that the ACs contain IgG, IgA and IgM as well as the SARS-CoV-2 N and S-specific antibodies in high concentrations. In addition, we were able to show that the antibodies' neutralizing capacities for the SARS-CoV-2 virus was not lost, but instead increased in all but one sample; the neutralization capacity in that particular sample remained stable.

In contrast to CCP therapy, ACs obtained from IA are concentrated to a volume of 50 mL and do not contain plasma components, like albumin and coagulation factors. Though no severe side effects were reported in trials using CCP [16], our low-volume ACs could potentially be applied in patients with heart or renal decompensation, who had been excluded from CCP trials.

We tested two different adsorber pairs, namely the Ig flex used for donation 1 and 2 and—as production of the Ig flex was discontinued—changed to Ig omni 1 in the following procedures. Ig flex uses polyclonal sheep anti-human IgG and binds to multiple epitopes on the Fc and F(ab) fragments. Ig omni 1 columns are coated with recombinant camelid anti-human immunoglobulins and bind only to the constant region of the light chains. Ig omni 1 and Ig flex are similar in removing IgG and IgA, whereas the design of Ig flex suggests a preference for IgG accompanying a lesser efficiency in IgM removal [17].

Considering that 75% removal of IgG was observed during one-time IA on other studies and can be increased to 95% when repeated on two consecutive days [15], the median 29 ± 7% IgG removal in this study is comparatively low. Increasing the blood volume during antibody donation using plasmapheresis is possible and could further increase the output. Afterwards, patients' IgG levels recovered to 75% of the pre-treatment values 2 months after IA with a low risk for infectious complications without immunoglobulin substitutions [18]. Likewise, no clinical diagnosis of SARS-CoV-2 reinfection was reported to the investigators after donation.

Antibody concentrates gained by Ig omni 1 seemed to be more stable, whereas ACs produced with Ig flex demonstrated continuous increases in SARS-CoV-2 specific antibodies during storage, while total amounts of immunoglobulins (IgG, IgA and IgM) remained stable at the same time. One explanation could be that plasmatic sheep antibodies used in Ig flex initially blocked SARS-CoV-2 binding sites and were partially removed upon storage. Another explanation could be conformational changes of these eluted antibodies by freeze-thawing. This might also explain the increase of anti-thyreoglobulin, as observed in one donor.

In contrast, camelid-coated Ig omni 1 adsorbers resulted in stable COVID-19 ACs. This stands in line with findings on CCPs, in which antibody concentrations and neutralization titres remained stable for 42 days at 4°C as well as after freezing and thawing [19].

In the beginning of the COVID outbreak in 2019, initial studies using CCP revealed that patients treated with high (≥1:250) neutralization titres have a low but significant 1% (95% CI = 0.24, 1.78) absolute reduction in 7-day mortality and 1.5% (95% CI = 0.43, 2.67) absolute reduction in 28-day mortality [20].

As stated by Focosi et al., in order to achieve a measurable (two-fold) increase in the SARS-CoV-2 antibody titre of patients, one 200 mL plasma unit has to contain an approximately 10 times higher antibody titre compared with the patients' blood, because it will be diluted in about 2.5 L of patient plasma volume thereby possibly reducing therapeutic efficacy [4]. Increasing the amount of plasma-transfused means more volume load and coagulation factors co-administered to the patient, with potentially unwanted side effects like a transfusion-related circulatory overload, allergic, febrile or other reactions of incompatibility. In contrast, IA returns these coagulation factors to the donors during the process of donation while still preserving or even increasing the antibody neutralization capacities.

Our ACs had neutralization titres of ≥1/10240 in 3 of 10 donations. Comparing with titres in the peripheral blood of donors to titres in the final AC, we can see a 2 ± 1.3 increase in titration endpoints including an increase of four titration levels in donation 6 as the highest increase. SARS-CoV-2 S-specific IgG content increased by 3.8 ± 1.8 times and up to 7.1 times in donation 1.1. This means that ACs manufactured by our method resulted in high titre levels independent of the initial blood values, thereby widening the range of suitable donors to those with low neutralization capacities [21].

Apart from CCP, monoclonal antibodies (mAbs) against COVID-19 supported patients' recovery; however, the new variants SARS-CoV-2 BQ and XBB have evasive properties against mAbs, so that the COVID-19 Treatment Guidelines Panel from the National Institutes of Health does not recommend the use of mAbs in its last update from 6 March 2023 [22]. In contrast, ACs obtained with our approach are derived from convalescent donors and can therefore be efficient against the newest variants as soon as a suitable donor is identified.

Studies have shown that the neutralization effect of CCPs can decrease down to the point of not being detectable if the CCP dose from an older variant is used in a patient suffering from a new variant [23]. It is therefore important to obtain antibodies as quickly as possible from the region of transmission for an efficient treatment [20]. The development of COVID-19 monoclonal antibodies (mAbs) is an extensive and time-consuming process. For example, it took 16 months and an emergency use authorization to get the first mAbs (Sotrovimab manufactured by GSK) ready to use [24]. This developmental process paired with a risk of failing regulatory requirements culminates in high prices per dose for mAbs. In contrast, our ACs can be produced within 1 day, as soon as a donor is available. IA is performed within 3 h, while the necessary preparation for the following downstream processing can be done in parallel. Bioburden filtration, concentration, diafiltration and sterile filtration took less than 5 h to finalise ACs production. Taking into account the fast development of new virus variants our approach provides new specific antibodies quicker than the development of monoclonal antibodies. Considering EU pharmaceutical legislation, our ACs may be considered as an Investigational Medicinal Product and not a Blood Product, depending on jurisdiction. This does imply clinical trials for dosing, safety, comparative efficacy and so forth similar to the development of monoclonal antibodies against viral infections. The possibility of compassionate use in urgent clinical situations remains as a last onsite option after ethical approval.

To assess the safety of the donated AC, all concentrates passed microbiological tests. Only the first donation was found to be positive for *Staphylococcus hominis* after 2 years of storage at −80°C, most likely due to handling contamination during test bottle inoculation. A retained sample from the same donation was retested and was found to be negative for aerobic microbes. Bound IgG, IgA and IgM as well as SARS-CoV-2 N- and S-specific antibodies were washed out from the adsorbers using a high molecular glycine solution. The latter was effectively removed and exchanged with saline solution during the diafiltration process leaving only small amounts of non-biohazardous residuals. The glycine buffer needed to be removed because of its high osmolarity (200 mmol/L), although it is termed as non-biohazardous (EG 1272/2008). The no adverse event level lies at 2 g/kg body weight. A total of 100 mL glycine buffer contains 1.5 g glycine, meaning that a person with an average body weight of 70 kg would have to be infused with 9.3 L of glycine buffer in order to develop potential side effects. As seen in one donor concealing his Hashimoto thyroiditis, unwanted autoimmune antibodies were also concentrated. However, we doubt that co-infusion of highly concentrated beneficial antiviral antibodies together with disease-triggering antibodies will lead to strong or permanent side effects, since autoimmunity cannot be transferred by blood donations. Nevertheless, this event calls for a comprehensive pre-donation scrutiny.

Pathogen inactivation has not been assessed in our ACs, but we want to point out that the process already contains two steps, which help to reduce pathogens. First, the adsorbers specifically bind to anti-human Fc of human immunoglobulins and not to virus membranes potentially present in plasma. Second, the glycin-HCL buffer used for eluting bound immunoglobulins from the adsorbers at each collection cycle is performed at low pH comparable to an acid elimination of potentially infectious particles. Once large-scale production from pooled eluates will be anticipated, additional pathogen inactivation procedures might come into practice.

In conclusion, IA combined with TFF proved to be a feasible method for the manufacturing of IgG, IgA and IgM as well as SARS-CoV-2 N and S ACs. These could be produced within 1 day, passed microbiological tests and were free of hazardous glycine levels. Final antibody neutralization titres were found to be as high or even higher than in the peripheral blood. However, we recognize that unwanted autoimmune antibodies will be concentrated to the same extent as the intended ones calling for careful donor screenings beforehand. Tests on clinical efficacy, for example, in animal models remain to be performed. With new viral replicants like those of H5N1 birds' influenza on the horizon, our strategy of on-site delivery of ACs could serve as a model in future pandemics.

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J.R. performed the research and wrote the first draft of the manuscript; J.R. and A.M. designed the research study; S.R.B. contributed essential reagents, tools, collection design and operational support; L.M., P.N.O. and D.H. performed the analytical methods on the samples; A.M., J.C.F and J.S. supervised the research and reviewed and edited the manuscript. Open Access funding enabled and organized by Projekt DEAL.

[Correction added on 25 May 2024, after first online publication: In addition to minor grammatical errors throughout the paper, the last author's ORCiD, Correspondence affiliation, Figures 1 and 2, Table 1, Reference 14, the 2nd last sentence of Materials and Methods: Immunoadsorption and the 1st sentence of paragraph 2 of the Acknowledgements were corrected.].

CONFLICT OF INTEREST STATEMENT

J.R. is employed by Cytiva; S.R.B. is an employee of Miltenyi Biotec. L.M., P.N.O., J.F., D.H., J.S. and A.M. have nothing to disclose.

DATA AVAILABILITY STATEMENT

Data and methods presented in this study are available in Transfusion Apheresis Science at DOI: 10.1016/j.transci.2021.103193 and in Transfusionsmedizin 2022; 12: 1–6 (ISSN 2191-8805) at DOI: 10.1055/a-1720-8203.

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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

The effect of near-infrared low-level light on the in vitro quality of platelets during storage

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Abstract

Background and Objectives: Near-infrared (NIR) light has been successfully applied to improve the quality of mouse platelets during storage. Because it is suspected that the mitochondria contain the primary photon acceptor, we hypothesized that human platelets for transfusion may be affected similarly and could benefit from NIR light treatment.

Materials and Methods: The optimal light dose was determined using portions of platelet concentrates (PCs) in PAS-E. A pool-and-split design was used to prepare PCs in PAS-E or plasma ($n = 6$). On day 1, one unit of both pairs was illuminated with 830 nm light (light-emitting diodes, 15 J/cm^2). PCs were stored at 22°C and sampled regularly for analysis. Data were compared with their corresponding controls with a paired two-sided *t*-test.

Results: Illuminated platelets in PAS-E were less activated with significantly lower CD62P expression (day 8: 10.8 ± 1.8 vs. 12.2 ± 2.6, $p < 0.05$) and lower Annexin A5 binding (day 8: 11.8 ± 1.9 vs. 13.1 ± 2.4, ns). They produced significantly less lactate resulting in a higher pH (days 6–10). ATP content and mitochondrial membrane potential were not affected. Although these trends were also observed for PCs in plasma, the differences did not reach statistical significance as compared with the control group.

Conclusion: Our study demonstrates that the glycolysis rate of human platelets can be modulated through the use of NIR, possibly through mitochondrial aerobic metabolism, but this requires confirmation. If NIR illumination can be further optimized, it may potentially become a useful tool in situations in which glycolysis and platelet activation are exacerbated.

Keywords

near infrared, platelet concentrates, storage lesion

Highlights

- Glycolysis rates in stored human platelets can be modulated by near-infrared light-emitting diodes with wavelength 830 nm at low doses of 15 J/cm^2.

- Small but significant changes in in vitro quality parameters were observed in platelets stored in PAS-E up to day 10.
- Glycolysis rates of platelets stored in plasma were more variable than in PAS-E and the effects of light treatment were larger and also positive, but sometimes negative.

INTRODUCTION

Treatment of cells and tissues with low doses of red or near-infrared (NIR) light is under investigation since the late 1960s. Soon after the discovery of its stimulating effects on hair growth and wound healing in mice, low-level NIR light therapy has been applied to human tissues and on patients [1]. Low-level light refers to (infra)red light with wavelength ranging from 600 to 1100 nm, and low power (<500 mW) and energy density (<50 J/cm^2) [2]. Currently, there are several clinical applications like reduction of inflammation and treatment of chronic joint disorders, wounds and neurological disorders [1]. The discovery that not only lasers but also light-emitting diodes (LEDs) can be used has contributed considerably to expansion of the technique [1].

Although some literature exists, relatively little is known about the effects of NIR treatment on blood and blood cells. In red blood cells, NIR is absorbed by haemoglobin and induces dissociation of oxyhemoglobin to deoxyhaemoglobin, resulting in a shift to an echinocytic cell shape [3]. Aside from this, a protective effect of NIR against ozone-induced red cell damage and damage during extracorporeal flow was observed [4, 5]. In another study, exposure of stored red cells to 830 nm laser illumination improved the deformability of the red cells, as concluded from higher filtration rates [6].

Treatment with 750–1100 nm light was helpful in protecting pig platelets (PLTs) from harmful effects during extracorporeal circulation. When blood was illuminated during flow through the heart-lung machine, PLT counts decreased less and PLT aggregation, expressed as area-under-the-curve, was similar or less intense as compared with controls [7]. Effects of NIR on mouse PLTs were investigated after whole-body illumination of mice with anti-CD41 antibody-induced immune thrombocytopenia, for 30 min a day, with 830 nm LEDs. This resulted in higher PLT counts, and also shorter tail bleeding times were observed due to LED-dependent enhancement of megakaryocyte mitochondrial biogenesis and activity and preservation of PLT mitochondrial functions [8].

To our knowledge, Zhang et al. [2] were the first to apply NIR with the aim of improving the storage of mouse PLTs. After illumination with low doses (0.2–20 J/cm^2) of 830 nm light by LEDs, prior to storage, they found lower lactate concentrations, higher pH, and inhibition of reactive oxygen species (ROS), while sustaining mitochondrial membrane potential (MMP), adenosine triphosphate (ATP) levels and morphology in the stored PLTs. Compared with controls, illumination also sustained aggregation capacity and in vivo survival of stored PLTs, while PLTs were not additionally activated, as suggested by similar CD62P expression and enhanced agonist-induced aggregation. The authors conclude that this additive-free and noninvasive technique has the potential to prolong the shelf life of PLT concentrates (PCs) for therapeutic use beyond the current 5 or 7 days.

Despite the long history of research, the biochemical mechanism of photobiomodulation by NIR treatment is not fully understood. Upregulation of oxygen consumption, and, for example, higher ATP [9] and nicotinamide adenine dinucleotide levels after illumination point towards the mitochondria as the primary target of NIR. Matching of action and absorption spectra, that is, comparison of the light absorbed and light that induced a biological response, has led to cytochrome c oxidase, Complex IV of the mitochondrial inner membrane, as the potential chromophore. Based on the observation that nitric oxide (NO) is simultaneously released from cells, two mechanisms are proposed. One is the dissociation of NO from cytochrome c oxidase. Because NO acts as a respiration inhibitor, the release of NO results in an increase in O_2 consumption and ATP generation. The other proposed mechanism is the production of NO by cytochrome c oxidase, which can act as a nitrite reductase, especially under hypoxic conditions [10, 11]. Except for increased O_2 consumption, treatment with (infra)red light may also result in a positive or negative change in ROS, which are important factors in cellular signalling [10].

We aimed to investigate the effects of NIR treatment on human PLTs, exploring low doses of 830 nm LED light on PLTs during prolonged storage of up to 10 days. After different pilot studies using mini PCs or paediatric volumes to find the optimal light intensity and illumination frequency during the storage period, results are presented of paired studies comparing illuminated PCs with controls, using standard PCs for adults in plasma or in PLT additive solution type E (PAS-E).

MATERIALS AND METHODS

Pilot studies to determine optimal NIR doses

As explained by Chung et al. [1], the light must be of sufficient power and duration to cause the desired response, otherwise, there will be no response or the response may be inhibited. Furthermore, for practical reasons, the illumination time should be short and, because PLTs are stored between narrow temperature limits, heat effects should be avoided. So, pilot studies were designed with feasible illumination times, and energy densities that were partly based on the experiments with mouse-PLTs of Zhang et al [2].

Whole blood (500 mL) was collected in citrate–phosphate-dextrose, and after overnight hold at room temperature and separation, the buffy coat (BC) was used to prepare PCs. Consent from the donor to use their BC was part of the donor questionnaire. Five AB0

compatible BC were pooled with 300 mL of PAS-E (T-PAS+, Terumo BCT, Lakewood, CO, USA) for preparation of a leukoreduced PC, using a soft spin (5.70 × 10^6 ACE, Sorvall RC12BP, Thermo Fisher Scientific, Asheville, NC, USA) and an inline filter (C5000, Fresenius, Bad Homburg, Germany).

To investigate different light doses, paired experiments were performed with aliquots of 20 mL in 150 mL containers (P4159 [PVC-DEHP] or 3FREQ06A0E [PVC-TOTM], Fresenius), obtained from one PC. The experimental setup of one of these experiments is outlined in Table S1. Also, PCs, split into 4 units of 70–90 mL in 600 mL containers (P4201, Fresenius), were used. Details about one such experiment are given in the Methods section in Supporting Information S1.

Except for controls, PCs were treated with NIR light of 830 nm on day 1. Two light sources were used, a mini light (Ø 12 cm, 12 LEDs, Infrared Mini 830, Red Light Man Ltd, Manchester, UK) or a body light (114 × 17 cm, 200 LEDs, Infrared 830 Bodylight, Red Light Man Ltd). NIR treatment was performed with the light beam directed downwards and PC units on the desk, without agitation. According to specifications, distances of 30, 20 and 10 cm between the light source and PC were used for the mini light, which resulted in power densities of 50, 100 and 200 mW/cm^2, respectively. Distances of 40 and 9 cm were applied when using the body light, resulting in power densities of 100 and 200 mW/cm^2. Temperature effects were checked during the illumination of adult dose PCs using a non-contact infrared thermometer (62 mini IR, Fluke, Everett, WA, USA).

Illumination of PC stored in PAS-E or plasma

The effect of the optimal NIR treatment dose was further investigated on adult dose PCs. To perform a 4-armed paired study, 20 BCs had to be pooled. However, in this study, 22 AB0-compatible BCs were pooled, to compensate for the loss of PLTs in containers and tubing. Subsequently, the BC pool was equally divided into four PC pooling systems (C5000, Fresenius). Two of the BC pools were diluted with 300 mL PAS-E and centrifuged as described above. Two were diluted with 300 mL plasma, obtained from two of the 22 donations, and centrifuged at 1250×*g* for 4.5 min (Sorvall RC12BP). After harvesting PLTs through the inline leukoreduction filter, PC pairs in PAS-E or plasma, respectively, were pooled and split again to minimize differences in volume and PLT count. After 30–60 min, one of the pairs was illuminated with 15 J/cm^2 (5 min) while the other served as a control. This time, PCs were agitated during illumination because the mini light was used, with the aim to cover a greater area of the 1300 mL storage container with the light beam of the mini light. PCs were stored on a flatbed shaker at 20–24°C and sampled for in vitro analysis on days 1, 6, 8 and 10 as described below. This four-armed study was performed six times, based on the expected outcome [2].

The experiment was partly repeated with pairs of PCs in PAS-E ($n = 3$) that were illuminated using the body light, enabling simultaneous and non-agitated exposure of three PCs.

In vitro analysis of platelet concentrates

On each sampling day, PCs were weighed for determination of the volume, and the swirling effect was judged on a scale from 0 (no swirl) to 3 (excellent swirl). Small samples (3.5 mL) were taken aseptically from the storage bag using a swan-lock adapter (Codan ref. 16.4200, Lensahn, Germany) and syringe. Immediately after sampling, the syringe was presented to the blood gas analyser (Radiometer ABL90, Radiometer, Copenhagen, Denmark) to determine pH, pO_2, pCO_2, glucose and lactate at 37°C. PLT counts were determined from an EDTA tube and mean PLT volume (MPV) from a dry tube, using a blood cell analyser (Sysmex XT2000i, Kobe, Japan). Extracts of adult dose PCs in PAS-E were analysed with high-performance liquid chromatography (HPLC) to assess the level of nucleotides, as described earlier in detail [12]. The PC samples (120 µL) were diluted in phosphate-buffered saline, centrifuged for 1 min at 16,873 × *g*, and after removing the supernatant, the pellet was extracted with 300 µL ice-cold 0.4 N perchloric acid and stayed on ice for 15–30 min. After a second centrifugation run, the extract was neutralized with 5 M K_2CO_3 and stored at −30°C until analysis with HPLC.

Flow cytometric assays

Expression of the PLT activation marker CD62P on the plasma membrane and externalization of phosphatidylserine (PS) as a marker for apoptosis, was assessed using flow cytometric assays as described previously [13].

A measure of the MMP was obtained with the fluorescent dye JC-1, also as described [13]. The final analysis of all assays was done with an FACSCanto II flow cytometer (BD Biosciences).

Statistical analysis

Illuminated PCs were compared with their corresponding control using a paired, two-sided *t*-test (Microsoft Excel, Redmond, WA, USA). The heat map was constructed by calculating the difference in JC-1 ratio, pO_2, pCO_2, lactate production, MPV and CD62P expression between each illuminated mini PC and the corresponding control, and normalizing this difference to a scale of 2, related to the maximum difference observed: $\Delta \times 2/\Delta_{max}$. A higher JC-1 ratio, lower pO_2, higher pCO_2, lower lactate production, lower MPV and CD62P expression were considered to be positive features, and the colour represents the mean ($n = 3$).

RESULTS

Optimal light doses

To determine the optimal light dose, we performed experiments with 20 mL PC aliquots in which we used a fixed power density of 50 mW/cm^2 and varied the illumination time and thus energy density (see Table S1). When comparing the illuminated mini PCs with the

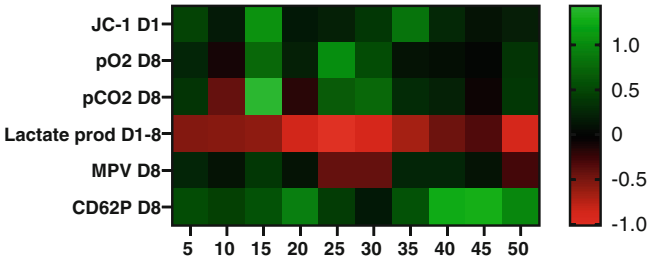

FIGURE 1 Heat map of differences of illuminated PCs versus controls, normalized to −2 or +2, of mitochondrial membrane potential (JC-1 ratio) on day 1 and storage variables on day 8. A higher JC-1 ratio, lower pO_2, higher pCO_2, lower lactate production, lower mean platelet volume (MPV) and CD62P expression were considered to be positive features, and the colour represents the mean ($n = 3$).

controls on day 1, we observed a higher JC-1 ratio of mitochondria in illuminated PLTs in 22 of 30 mini PCs. A more precise evaluation of JC-1 ratio on day 1 and relevant variables after the 7-day storage period resulted in the heat map depicted in Figure 1. At some power densities, lower oxygen tension combined with higher carbon dioxide tension was observed (Figure 1), especially when 15 J/cm^2 had been applied, indicating more active metabolism by mitochondria on day 8. In this pilot, this effect was accompanied by a small increase in lactate production.

Because from other pilot studies in mini PCs, illumination at 10 or 15 J/cm^2 was most promising, light doses of 5, 10 or 15 J/cm^2 were applied in an experiment with split-PCs of 70–90 mL for fine-tuning. Although differences between the groups were not statistically significant (Analysis of Variance), it was found that the largest positive effects were obtained with 15 J/cm^2 (Table S2). Remarkably, effects on the glycolysis rate remained neutral at all three different light doses in PLTs with an average glucose consumption (0.06 mmol/day/10^{11} PLTs), while PLTs with elevated glucose consumption (0.10 mmol/day/10^{11} PLTs) were strongly affected by the NIR treatment (Table S2).

In addition, we tested whether repeated illumination could further improve PLT quality. We performed several studies with 20 mL aliquots and varied the illumination frequency, starting illumination on day 1 or 2 of storage with subsequent illumination intervals of 24 or 48 h (Table S3). Additional pilot experiments were performed with 15 and 90 J/cm^2, but no additional beneficial effects on the in vitro quality of PLTs as compared with the single illumination strategy could be identified, and in some cases, even worse PLT quality was observed (data not shown). Temperature effects were checked at a critical condition with a short distance (10 cm) between the light source and the PC with an illumination time of 7.5 min (90 J/cm^2), which only resulted in a temperature increase of <1°C beyond the limits of 20–24°C that is required for room temperature storage of PCs.

PC in PAS-E or plasma after a single illumination on day 1

Next, we sought to understand whether the storage solution (plasma or PAS) impacts the platelet sensitivity to the NIR light treatment. Using the previously established optimal light dose of 15 J/cm^2, we found significant changes in PLTs suspended in PAS-E from day 6 or thereafter (Table 1 and Figure 2). The data of PCs in PAS-E, illuminated with the body light ($n = 3$), were combined with data of the paired study illuminated with the mini light ($n = 6$), because experimental conditions were identical. Both pH and glucose levels in the Illuminated PCs in PAS-E were found to be somewhat higher, while significantly lower lactate levels were observed, indicating a decrease in glycolysis rate due to illumination. The maximum decrease in the glycolysis rate over 7 days of storage was 7.4%. Furthermore, lower CD62P expression and storage-induced swelling (MPV) were observed. A lower MMP (JC-1 ratio, $p < 0.05$) was measured on day 6 in the illuminated PCs (Table 1 and Figure 2). No significant differences were observed in the Annexin A5 binding (PS exposure); however, after the exclusion of an outlying pair of PCs (increased, instead of decreased glycolysis rate after illumination, PC5 in Figure 3a,b), Annexin A5 binding of illuminated PLTs on day 8 was significantly lower compared with controls. ATP, adenosine diphosphate and adenosine monophosphate levels were not found to be significantly different between the two groups (Table S4).

PCs in plasma showed a similar trend, though not reaching a statistical difference when compared with the controls (Table 1). Looking in detail at the paired study, glycolysis rates of PLTs in plasma were more variable than in PAS-E, and the effects of the light treatment were larger (Figure 3). In plasma, the maximum decrease in glycolysis rate over 7 days of storage was 10.6%. Those PLTs in plasma which showed decreased glycolysis rate due to illumination (Figure 3c,d), were also significantly less activated (days 6–10) and had lower Annexin A5 binding (day 8), while PCs had lower oxygen tension (days 8–10), indicating better maintenance of oxygen consumption at the end of storage. All PCs in PAS-E or plasma, whether illuminated or not, showed an excellent swirling score of 3 up to day 10.

DISCUSSION

For the first time, we show that the in vitro quality of stored human PLTs can be modulated by treatment with NIR. After illumination, PLTs in PAS-E showed decreased glycolysis rates and were subject to lower levels of activation and apoptosis, indicative of better maintenance of PLT quality during storage. In agreement with Zhang et al. [2], we found that one single dose of illumination <20 J/cm^2 on day 1 gave the best results. An indication from experiments with mini PCs, where exposure to 90 J/cm^2 could also lead to promising results, was not confirmed.

Similar effects of NIR as described for mouse PLTs were found [2], but the differences in our study were generally smaller. This is possibly due to intrinsic differences between mouse and human PLTs, or to the sub-optimal mouse PLT storage conditions. When basic storage conditions are not optimal, the window of opportunity becomes larger. In contrast, human PCs are stored in a highly optimized storage container and solution, which renders the margin for further quality improvement smaller. As such, implementation of an illumination

TABLE 1 Storage variables of adult-dose PCs in PAS-E or plasma, illuminated on day 1 with 830 nm NIR light at energy density 15 J/cm^2 versus controls (mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001 vs. corresponding controls).

Variable	Day	PC in PAS-E control $n = 9$	PC in PAS-E NIR treated $n = 9$	PC in plasma control $n = 6$	PC in plasma NIR treated $n = 6$
Volume, mL	1	366 ± 11	366 ± 12	370 ± 8	369 ± 9
PLT, $\times 10^9$	1	355 ± 43	356 ± 43	356 ± 38	351 ± 35
pH, 37°C	1	7.06 ± 0.01	7.06 ± 0.01	7.05 ± 0.02	7.05 ± 0.02
	6	7.16 ± 0.04	7.17 ± 0.04*	7.19 ± 0.04	7.21 ± 0.05
	8	7.15 ± 0.05	7.16 ± 0.04***	7.12 ± 0.05	7.14 ± 0.06
	10	7.11 ± 0.06	7.14 ± 0.05***	7.03 ± 0.05	7.04 ± 0.06
Bicarbonate, mM	1	8.4 ± 0.4	8.4 ± 0.4	18.5 ± 0.7	18.7 ± 0.6
	6	7.0 ± 0.7	7.2 ± 0.6*	11.1 ± 0.8	11.5 ± 0.9
	8	6.6 ± 0.9	6.9 ± 0.8	8.9 ± 1.0	9.2 ± 0.9
	10	6.1 ± 1.1	6.5 ± 1.0*	6.7 ± 1.8	7.0 ± 0.7
pO_2, mmHg	1	127 ± 5	124 ± 6	116 ± 6	110 ± 9
	6	71 ± 20	67 ± 19	79 ± 11	79 ± 12
	8	69 ± 24	67 ± 22	83 ± 11	79 ± 10
	10	78 ± 19	75 ± 18	86 ± 13	82 ± 11
Glucose, mM	1	7.6 ± 0.3	7.6 ± 0.3	18.2 ± 0.6	18.1 ± 0.6
	6	5.3 ± 0.5	5.3 ± 0.4	14.4 ± 0.6	14.7 ± 0.7
	8	4.2 ± 0.7	4.3 ± 0.6*	13.2 ± 0.6	13.3 ± 0.6
	10	2.9 ± 0.8	3.0 ± 0.7*	11.8 ± 0.7	11.9 ± 0.4
Lactate, mM	1	2.6 ± 0.2	2.6 ± 0.2	5.3 ± 0.4	5.3 ± 0.4
	6	7.3 ± 1.0	7.1 ± 1.0**	11.2 ± 0.8	10.8 ± 1.0
	8	9.4 ± 1.2	9.2 ± 1.2**	14.0 ± 1.1	13.6 ± 0.9
	10	11.7 ± 1.4	11.4 ± 1.4**	17.0 ± 1.4	16.6 ± 1.5
MPV, fL	1	9.7 ± 0.2	9.7 ± 0.2	10.0 ± 0.1	10.0 ± 0.2
	6	9.4 ± 0.3	9.4 ± 0.2	9.6 ± 0.2	9.6 ± 0.2
	8	9.5 ± 0.2	9.4 ± 0.2*	9.7 ± 0.2	9.7 ± 0.2
	10	9.6 ± 0.2	9.5 ± 0.2*	9.8 ± 0.2	9.8 ± 0.2
CD62P-expr, % pos. cells	1	1.1 ± 0.4	1.0 ± 0.4	0.8 ± 0.3	0.9 ± 0.3*
	6	7.9 ± 2.2	7.0 ± 1.7*	6.3 ± 1.4	5.7 ± 1.6
	8	12.2 ± 2.6	10.8 ± 1.8*	8.4 ± 2.0	8.1 ± 1.9
	10	12.1 ± 2.9	10.5 ± 2.3**	9.2 ± 1.3	9.1 ± 1.3
Annexin A5, % pos. cells	1	3.2 ± 0.9	2.9 ± 0.9	3.4 ± 0.9	4.0 ± 0.6
	6	9.8 ± 2.1	9.4 ± 3.1	14.1 ± 1.6	12.4 ± 2.0
	8	13.1 ± 2.4	11.8 ± 1.9	17.5 ± 2.3	16.8 ± 1.6
	10	16.4 ± 3.6	14.8 ± 2.4	21.2 ± 3.4	21.3 ± 2.6
JC-1 ratio	1	4.6 ± 0.8	4.5 ± 0.7	3.7 ± 0.4	4.0 ± 0.5*
	6	3.3 ± 1.1	2.9 ± 1.2*	3.4 ± 0.6	3.4 ± 0.5
	8	3.2 ± 1.2	3.2 ± 1.3	3.7 ± 0.9	3.6 ± 0.9
	10	3.0 ± 1.0	2.8 ± 1.0	3.5 ± 0.6	3.5 ± 0.6

Note: Illumination was performed with the mini light at power density 50 mW/cm^2 during 5 min for $n = 6$ of the paired study or with the body light at power density 100 mW/cm^2 during 2.5 min for $n = 3$ PC in PAS-E.
Abbreviations: MPV, mean platelet volume; NIR, near infrared; PC, platelet concentrates; PLT, platelets.

strategy may be most advantageous when storage conditions are less optimal, as is, for example, the case when applying pathogen reduction technology, or for donors with elevated glucose metabolism [14, 15]. However, further studies in this area are needed, including functional tests like aggregation behaviour and cytokine release that may be affected [16]. Be it as it may, it remains an open question how (improved) NIR illumination of PCs and its effects on in vitro quality translate to in vivo recovery after transfusion.

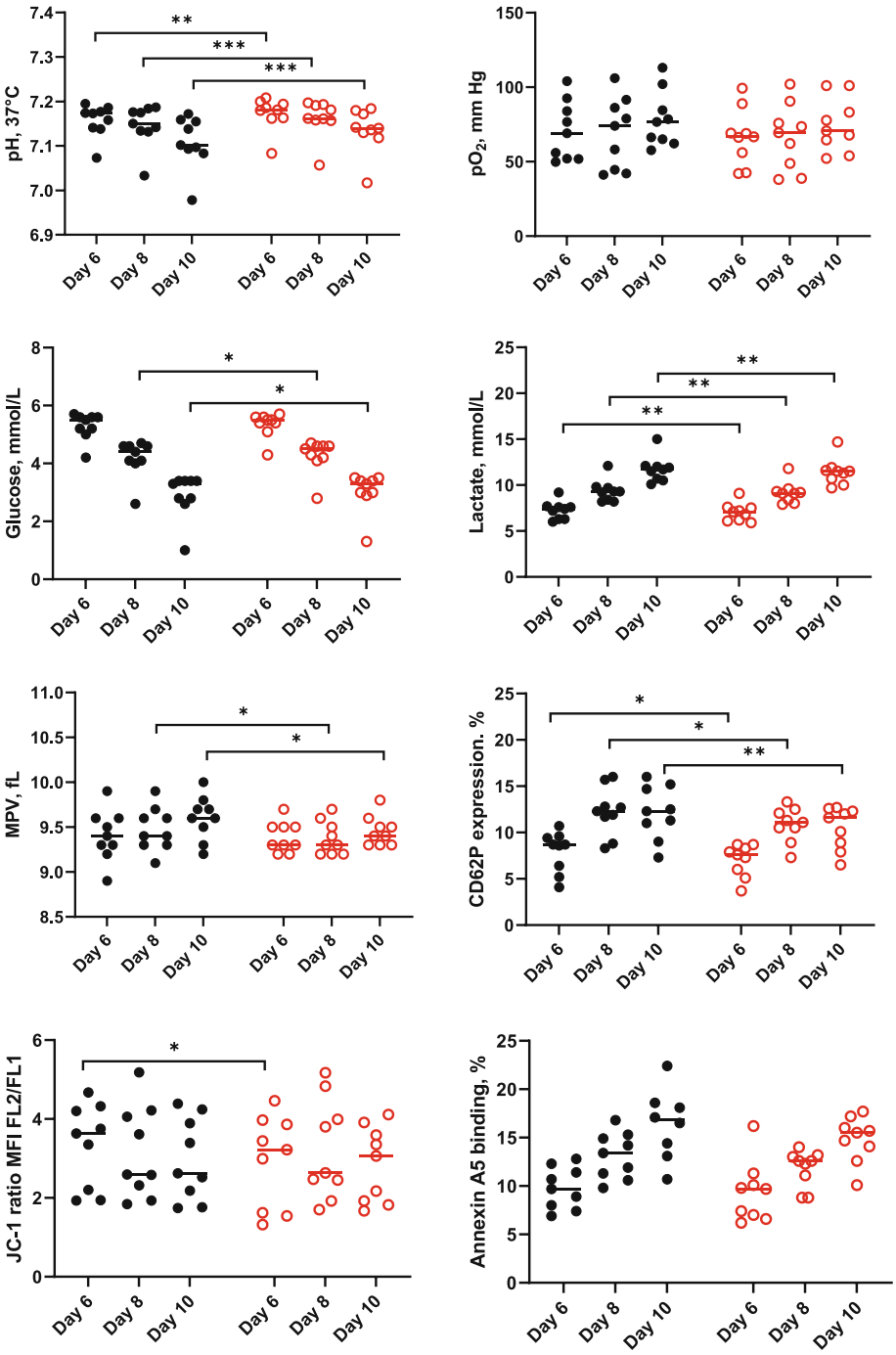

FIGURE 2 Storage variables of adult-dose PCs in PAS-E, combined data from PCs illuminated with the mini light ($n = 6$) and PCs illuminated with the body light ($n = 3$) on day 1 with 830 nm near-infrared light at energy density 15 J/cm^2 (○) versus controls (●); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. MPV, mean platelet volume.

Also, as revealed by the studies presented here, PLTs from different donors may respond differently to the same light dose. In the experiments with aliquots, the high glucose-consuming PLTs were far more sensitive to the applied light treatment than the low glucose-consuming PLTs. Glucose consumption and lactate production are known donor-dependent factors, and a positive association between MPV and donor health issues was established earlier [12, 15]. PLTs stored in plasma with relatively low glycolytic rates and apparently highly functional mitochondria (2 of 6, Figure 3c,d) were generally affected negatively when illuminated with 15 J/cm^2, in contrast to PLTs with higher glycolytic rates (4 of 6). Although no differences in MMP (JC-1 ratio) were detected, it is reasonable that the impact of NIR on mitochondria of these PLTs was positive, based on the lower lactate levels, oxygen tension, CD62P expression and PS exposure. In the paired units with PAS-E, the PLTs with low glycolytic rates in plasma were slightly positively affected, so it may be

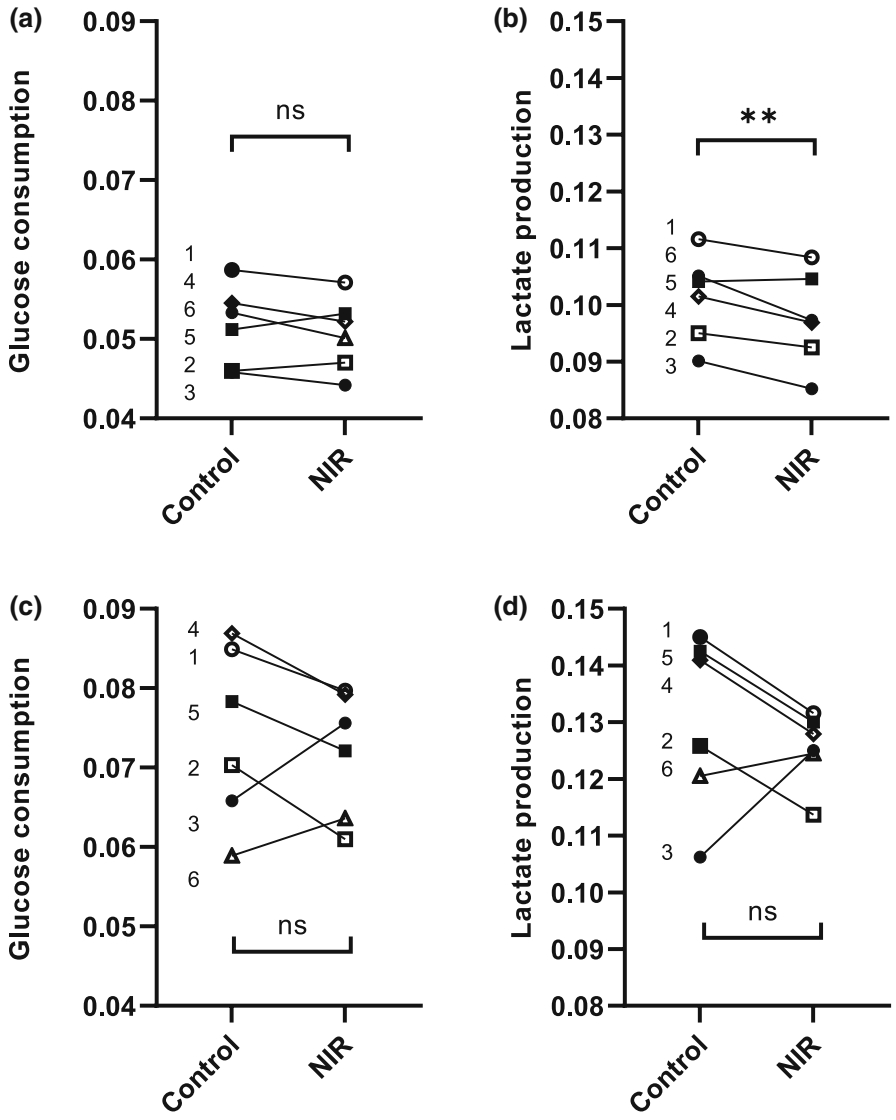

FIGURE 3 Glucose consumption (a: PAS-E and c: plasma) and lactate production (b: PAS-E and d: plasma) of platelet concentrates of the paired study illuminated with the mini light versus controls ($n = 6$), calculated for storage between days 1 and 8 in mmol/day/10^{11} platelets. Each number represents a paired experiment (○, 1; □, 2; ●, 3; ◇, 4; ■, 5 and Δ, 6). NIR, near infrared.

concluded that unknown storage medium factors, such as the high concentrations of potassium and magnesium in PAS-E, also play a role in the illumination process and mechanism. This phenomenon of donor variation was not observed in the mouse experiments because PLTs from one genetically identical mouse strain were used [2].

The technical and physical characteristics of the system that is used to illuminate (i.e., type and plastic of the bag, thickness of the fluid layer and agitation speed) require further optimization to increase the positive effects. Adult dose PC in PAS-E were affected in a similar way with the mini light while agitated as with the body light while non-agitated, and so agitation (speed) and other variables should be optimized, as was done for UV light [17]. For example, little is known about the optimal configuration of the NIR beam, and the optimal material and surface area of the container used in the illumination process. To detect the proportion of light that is absorbed, scattered, reflected and/or transmitted by the PLTs, measurements with a luminometer are necessary. Moreover, unknown storage medium factors include not only those that contribute to the glycolysis rate of the PLTs but also the role of plasma proteins, turbidity of the plasma (fraction), and contamination level with red cells.

The relatively simple, additive-free procedure, as well as its non-invasive character, are clearly strong points of PLT NIR treatment. However, the procedure may require an extra handling step. Potentially though, NIR light may simply be incorporated into the storage incubators and be subject to programmed illumination, avoiding such additional handling. PLT NIR light treatment will require a certain degree of monetary investment. However, as shown in this manuscript, NIR treatment can induce positive effects in PLTs. If further optimized, the potential benefits for the patient receiving NIR-treated platelets may far outstrip the required investments.

Taken together, our data show that, as proof of principle, glycolytic rates can be modulated through non-invasive NIR illumination, which may significantly improve PC quality, especially those PC that are of relatively lower quality at baseline. Further research should be

directed towards the optimization of the system, identification of factors that can predict efficacy in specific donors, as well as attaining a more thorough molecular understanding of the underlying mechanism.

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I.J.B. and L.A.E.d.L.-L. performed the experiments and analysed the data. I.J.B., P.F. v.d.M. and T.R.L.K. designed the study and wrote the manuscript. The manuscript was reviewed by A.E.d.L.-L.

CONFLICT OF INTEREST STATEMENT

There are 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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Extracellular vesicles derived from stored red blood cell suspensions enhance invasion and migration of lung cancer cells by miR1246 and miR150-3p

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Abstract

Background and Objectives: Aged red blood cell (RBC) transfusions in lung cancer patients are often related to cancer recurrence and shorter lifespans. Extracellular vesicles (EVs) accumulated in stored RBC suspensions may be one of the important influential factors. This study aims to investigate how EVs derived from RBC suspensions affect the progress of lung cancer through the most enriched microRNAs (miRNAs) previously reported in our research.

Study Design and Methods: EVs derived from stored RBC suspensions in Weeks 1, 3 and 5 were harvested via ultracentrifugation. Lung adenocarcinoma H1975 cells were co-cultured with EVs and transfected with miR1246 and miR150-3p mimics to evaluate alterations in their proliferation, invasion and migration abilities in vitro. Proteomics and bioinformatics were performed to predict the signalling pathway related to invasion and migration of H1975, which were verified by western blotting (WB) and flow cytometry.

Results: EVs derived from stored RBC suspensions in Weeks 3 and 5 could significantly enhance the invasion and migration ability of H1975 cells and also increase the expression of miR1246 and miR150-3p. After transfection with miR1246 and miR150-3p mimics, invasion, migration and proliferation of H1975 cells were obviously enhanced. Proteomics analysis demonstrated that EVs co-cultivation and miRNA transfection groups were both enriched in cell adhesion molecules. WB and cytometry indicated that integrin beta-1 (ITGB1) and Rap1b were increased.

Conclusions: EVs derived from stored RBC suspensions can enhance invasion and migration ability of lung cancer cells via the most accumulated miR1246 and miR150-3p, which may increase the expression of ITGB1 through Rap1 signalling pathway.

Keywords

extracellular vesicles, invasion, lung cancer, migration, miRNA, RBC suspensions

Zhanrui Cheng and Yujie Kong are co-first authors.

Highlights

- Extracellular vesicles (EVs) derived from stored red blood cell suspensions could enhance invasion and migration of lung cancer cells.
- miR1246 and miR150-3p were delivered into lung cancer cells via EVs and promoted invasion and migration.
- miR1246 and miR150-3p may increase the expression of ITGB1 through the Rap1 signalling pathway.

INTRODUCTION

Lung cancer stands as the primary cause of cancer-induced fatalities globally [1]. Lung cancer patients often suffer from anaemia and frequently require red blood cell (RBC) transfusions [2]. Tartter's study in 1984 [3] was the first to reveal that blood transfusion during the perioperative period might result in the reoccurrence and metastasis of lung cancer in patients who have undergone surgeries. Subsequently, the same conclusion was confirmed by a meta-analysis incorporating 23 cohort studies [4]. In addition, the storage duration of RBCs suspensions may be strongly associated with the severity of the prognosis in tumour patients [5].

With the progress of research, it is now widely accepted that residual white blood cells, platelets, haemolytic components and extracellular vesicles (EVs) derived from suspended RBCs may influence the tumour progress [6, 7]. EVs are particles expelled from cells, enclosed by a lipid bilayer and lack the capacity for autonomous replication [8]. More evidence suggests that EVs secreted by host cells or cancer cells participate in the occurrence, growth, invasion and migration of tumours [9–11]. RBCs, as the most common cell type in the body, can produce a large number of EVs daily, and it has been discovered that RBC–EVs can act on neutrophils to exert effects [12]. Therefore, the EVs continuously produced from suspended RBCs during storage may potentially have a direct effect on the prognoses of lung cancer [13].

MicroRNA (miRNA), as the most abundant non-coding small RNA in EVs, can exert its function by inhibiting the translation of target mRNA and is an essential molecule in regulating gene expression [14]. Studies have confirmed that EV-associated miRNAs participate in the proliferation, migration and epithelial–mesenchymal transformation (EMT) of lung cancer cells [15, 16]. Our research group's previous studies have demonstrated that during the storage period of RBC suspension, the EV-associated miRNAs with the highest expression levels were miRNA1246 and miRNA150-3p [17]. We speculate whether EVs play a transport role in delivering miRNAs to lung cancer cells. Following this, we aim to investigate the mechanism of these two miRNAs, which is beneficial for the blood transfusion management of lung cancer patients and may enhance the efficacy and safety of clinical blood transfusions.

MATERIALS AND METHODS

Study samples

Non-leukoreduced RBC suspensions with anticoagulants (aciacid citrate dextrose solution, formula A) sourced from the Central Blood Bank of Deyang City in Sichuan, China. A total of 12 RBC suspensions were used in this study: 3 validations for the cell migration, 3 validations for the cell invasion and proliferation, three validations for the proteomics and 3 validations for the western blotting (WB) and flow cytometry (5 bags were randomly selected for nanoparticle tracking analysis [NTA] and protein concentration detection through bicinchoninic acid assay [BCA]); the specifications uniformly adhere to a 2 U standard, equating to 400 mL. The research protocol received the approval from the ethics committee of the Institute of Blood Transfusion, Chinese Academy of Medical Sciences.

RBC suspension storage, EV isolation and purification

EVs were isolated as described previously [17]. The RBC suspensions were divided into 100 mL portions in 100 mL transfer bags and stored at 4°C. EV extraction was performed on these aliquots in Weeks 1, 3 and 5 during storage. Initially, the aliquots were centrifuged at 3000*g* for 20 min to obtain supernatant, from which EVs was isolated through ultracentrifugation. First, the RBC supernatant was centrifuged at 300*g* for 10 min to separate the cells, and the supernatant was centrifuged again at 2000*g* for 10 min to separate the dead cells. This supernatant was centrifuged at 10,000*g* for 30 min at 4°C to isolate cell debris and two final 70 min EV separation stages at 100,000*g* at 4°C. The EV precipitate was then re-suspended in 200 µL of phosphate-buffered saline (PBS). To eliminate large EVs, 0.22-µm centrifugal filters (Millipore, USA) were utilized after the first centrifugation at 100,000*g*.

EV identification

EV characteristics in RBC supernatants were determined through NTA, transmission electron microscopy (TEM) and WB. NTA was conducted using NanoFCM's N30E instrument, diluting vesicles in PBS and analysing them under light scattering. The size and concentration data were obtained through Brownian motion analysis applying the Stokes–Einstein equation. For TEM, EVs were fixed on electron microscope fixative and placed on copper meshes for overnight fixation at 4°C before being observed under a Hitachi TEM. WB involved processing EVs with radio-immunoprecipitation assay (RIPA) buffer, determining protein concentration through the BCA and verifying protein markers TSG101 (Abcam, UK) and CD9 (Abclonal, China) through absorbance measurements in a PerkinElmer plate reader, with Calnexin (SAB, USA) as a

negative control and HEPG2 cell lysates as controls. Blot images were captured with a CLINX ChemiScope Mini 3000.

RNA isolation and complementary DNA preparation

The total RNA was extracted using the TransZol Up (TRAN, China). The quantity and quality of the total extracted RNA were measured by using the Agilent 4200 platform and the Quit 2.0 Fluorometer (Thermo Fisher Scientific, USA). Next RNA was reverse transcribed to generate complementary DNA using the ReverTra Ace Quantitative Polymerase Chain Reaction (qPCR) RT Master Mix (TOYOBO, Japan) for subsequent reverse transcription qPCR (RT-qPCR).

Cell culture and transfection

The H1975 cell line, characteristic of non-small cell lung cancer (NSCLC) and lung adenocarcinoma, was procured from Procell (Wuhan, China). Cultured in Dulbecco's Modified Eagle Medium with 10% foetal bovine serum (FBS), cells were incubated at 37°C, 5% CO_2. To induce miR1246 or miR150-3p overexpression, H1975 cells were transfected with 10 μM miR1246 or miR150-3p mimics from RiboBio (Guangzhou, China), using Lipofectamine 3000 (Invitrogen, USA) following the manufacturer's protocol.

Co-cultivation of cells with EVs

We re-suspended EVs extracted from 100 mL of the RBC suspensions in 800 μL of PBS for subsequent experiments. To ensure that the concentration of EVs in ex vivo co-culture is consistent with the concentration of EVs in the RBC suspension, we added 20 μL of the EV suspension to each well, which contained 2.5 mL culture medium in a six-well plate (Corning, USA). The cultivation conditions for EVs ex vivo were referenced from Shekari's study [18]. After 24 h, the cells were used for qPCR analysis, and after 48 h, they were employed for WB and flow cytometry assays.

Cell proliferation, migration and invasion assays

H1975 proliferation was assessed using Cell Counting Kit-8 (CCK-8) (Proteintech, Wuhan); 100 μL of cell suspension was seeded in a 96-well Corning plate for pre-culture, followed by the addition of 10 μL CCK-8 solution and absorbance measurement at 450 nm after 4 h using a plate reader. For migration assays, H1975 cells were cultured in dishes, scratched, washed with PBS, cultured in serum-free medium and observed after 24 h. For invasion assays, a Matrigel (BD, USA) pre-coated transwell insert (LABSELECT, China) was used; cells were seeded in the upper chamber with serum-free medium, lower chamber with 10% FBS media and incubated for 48 h. Post-incubation, non-migratory cells were removed, and the migrating cells were fixed, stained and counted.

RT-qPCR assay

RT-qPCR was performed following the manufacture's PerfectStart Green qPCR kit (TRAN, China) protocol with the miRNA-specific forward primer and the universal reverse primer (RiboBio, Guangzhou, China). The small nuclear RNA U6 was used as an internal control to normalize the expression of miR1246 and miR150-3p.

Data independent acquisition proteomics

The sample was lysed in DB buffer and sonicated, then centrifuged at 12,000*g* for 15 min at 4°C. The supernatant was treated with dithiothreitol (DTT), then iodoacetamide and followed by an ice-bath. After measuring the concentration of the protein extraction, the protein was precipitated and recollected. Following quality testing and trypsin digestion, ultra high performance liquid chromatography-mass spectrometry data independent acquisition (UHPLC–MS DIA) analysis was conducted. DIA-neural networks (DIA-NN) software processed the data with a 99% confidence filter for peptide spectrum matches and differentially expressed proteins (DEPs) under 1%. Functional annotation was performed using InterProScan for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) for pathway analysis. DEPs were analysed with volcano plots, heat maps and pathway enrichment to reveal expression patterns and biological insights. Protein quantification results were statistically analysed using the *t*-test, and differentially expressed proteins were defined as those with significant quantitative differences between the experimental and control groups ($p < 0.05$, fold change >1.5).

Bioinformatics analysis of miR1246 and miR150-3p

The target genes of miR1246 and miR150-3p were predicted by miRanda database (http://www.microrna.org/) and Pictar database (http://www.pictar.org/). To analyse the target genes, a GO analysis was conducted with a statistical significance level of $p < 0.05$ used to identify enriched GO categories. In addition, pathway analysis was performed to identify significant pathways associated with the differentially expressed genes using the KEGG database (http://www.genome.jp/kegg/).

Western blot assay

Cells were lysed using RIPA buffer with protease inhibitors. Protein concentrations were equilibrated to 1 μg/μL with a BCA kit. Each 24 μL protein sample run on 10% sodium doecyl sulfate-polyacrylamide gel electrophoresis, was transferred to polyvinylidene fluoride membranes, blocked with 5% bovine albumin in tris-buffered saline with Tween 20 for 2 h, then probed with primary antibodies Rap1b (Proteintech, 1:2000) and β-actin (Proteintech, 1:10,000) overnight at 4°C. After incubation with secondary antibodies (anti-rabbit

immunoglobulin G [IgG], horseradish peroxidase [HRP]-linked antibody, Cell Signaling Technology, 1:5000), chemiluminescent detection was performed using ECL HRP Substrate and ChemiDoc MP system. Strip grayscale values were analysed with Image J.

Flow cytometry

H1975 cell density was adjusted to 5×10^6 cells/mL. Then, 100 μL of cell suspension was added to the flow cytometry tube (BD Falcon, USA) followed by phycoerythrin-labelled CD29 (integrin beta-1 [ITGB1]) flow cytometry antibody (Biolegend, USA) and incubated in the dark at 4°C for 30 min. Afterward, centrifugation was performed to wash off the excess dye, and the tubes were analysed on the flow cytometer (BD FACSVia, USA).

Statistical analysis

All results are shown as mean values with standard deviation. For statistical comparisons, *t*-tests and one-way analysis of variance were used as required, utilizing GraphPad Prism software version 7.0. A *p*-value of less than 0.05 was deemed to indicate statistical significance.

Ethics approval and consent to participate

The research was approved by the ethics committee of the Institute of Blood Transfusion, Chinese Academy of Medical Sciences.

RESULTS

Examination and characterization of EVs obtained from RBC suspensions

TEM and NTA revealed that isolated vesicles from RBC suspensions in Week 5 had cup-shaped lipid bilayer membranes which is the typical EVs shape (Figure 1a), and the average diameter was 50–150 nm (Figure 1b). Next, WB analysis demonstrated the expression of TSG101 and CD9 in the isolated EVs, as well as the negative detection of Calnexin (Figure 1c).

Extended storage time of RBC suspensions leads to increased production of EVs

Randomly selected five groups of EV samples in Weeks 1, 3 and 5 were tested for particle size and concentration using NTA, with

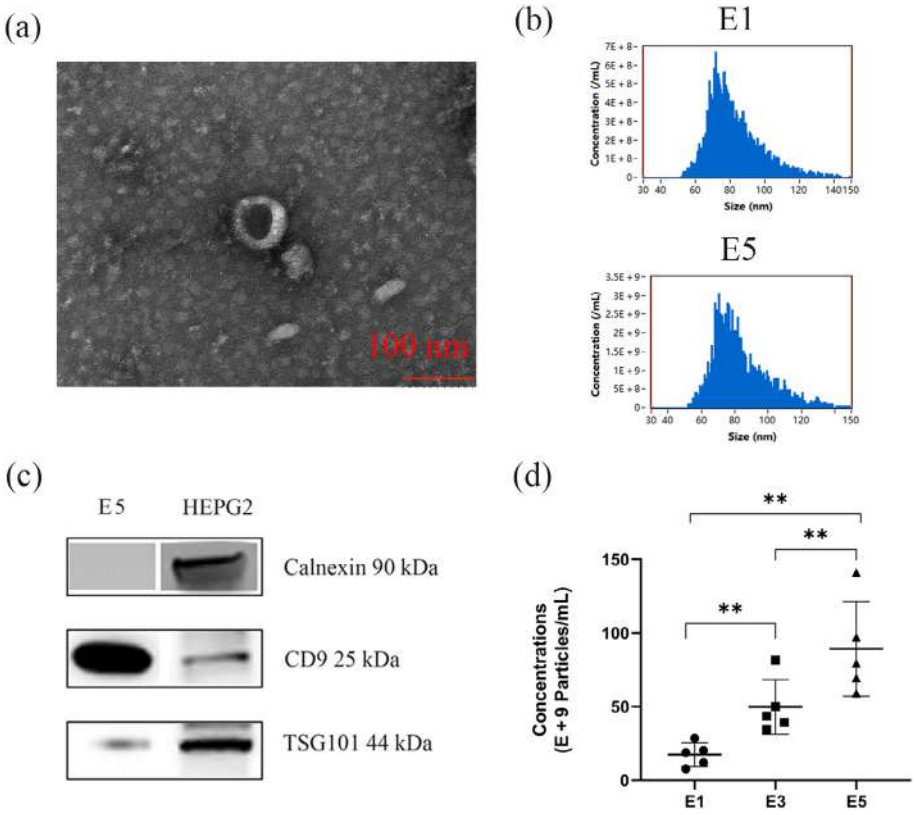

FIGURE 1 Characterization of extracellular vesicles (EVs) isolated from red blood cell suspensions. (a) Transmission electron microscopy (TEM) for the morphology of EVs in Week 5. Scale bar, 100 nm. (b) Nanoparticle tracking analysis (NTA) for the particle size distribution and concentration of EVs in Weeks 1 and 5. (c) Western blotting for the specific immunological markers TSG101 and CD9 of the EVs in Week 5 and hepatocellular carcinoma cell line 2 (HEPG2). (d) Concentration of EVs derived from RBC suspensions at different storage times ($n = 5$) (E1: EVs in Week 1; E5: EVs in Week 5).

results shown in Table 1 and Figure 1d. The average size of EV particles was between 60 and 90 nm. As the storage time of RBC suspensions increased, the concentration of EVs isolated from the supernatant gradually increased. There was a statistically significant difference in the concentration of EVs between Weeks 5 and 1, as well as between Weeks 5 and 3, indicating that the production of EVs gradually increases with the extended storage time of RBC suspensions. Similarly, the protein within EVs is detected using the BCA method, and ratios (particles/μg protein) show that all sample values are greater than 1.5×10^9 P/μg, meeting the purity requirements [19] (Table 1).

EVs from stored RBC suspensions increase the migration and invasion of lung cancer cells

EVs from RBC suspensions in Week 1 slightly increased the migration of H1975, with the EVs from Week 5 having the most noticeable impact; however, no statistically significant differences exist between Weeks 1 and 3, or between Weeks 3 and 5 (Figure 2a). Correspondingly, the invasion assays indicated the similar results, EVs in Weeks 3 and 5 doubled the invasion number of H1975 cells compared with those stored for 1 Week (Figure 2b). EVs in Weeks 1, 3 and 5 showed no statistical difference at optical density 450 (OD450), which indicated

TABLE 1 Concentration and protein content of extracellular vesicles (EVs) derived from red blood cell suspensions at different storage times.

	Concentrations (E+9 particles/mL)			Protein (μg/mL)			Ratio (particles/μg protein)		
Sample	E1	E3	E5	E1	E3	E5	E1	E3	E5
1	28.6	81.5	141	6.35	6.35	6.35	4.50	3.46	3.59
2	20.2	50.1	97.2	5.31	5.31	5.31	3.80	2.81	2.44
3	11.9	43.5	79.4	3.98	3.98	3.98	2.99	3.64	2.84
4	7.6	39.3	59.2	1.37	1.37	1.37	5.55	4.63	3.76
5	18.8	34.6	69.5	2.94	2.94	2.94	6.39	4.73	3.41

Note: $n = 5$; E1: EVs in Week 1; E3: EVs in Week 3; E5: EVs in Week 5.

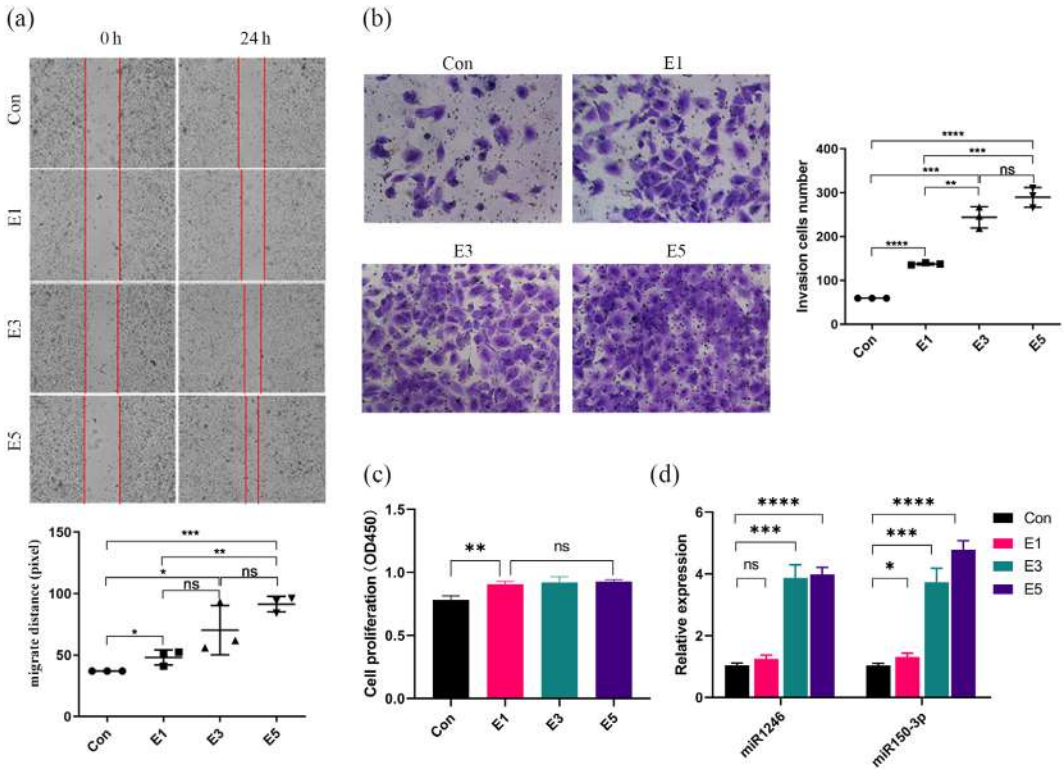

FIGURE 2 Extracellular vesicles (EVs) from red blood cell suspensions enhance the migration and invasion of H1975. Cell migration was assessed by scratch test (a) and cell invasion was assessed by transwell invasion assay (b) in H1975 cells. (c) Effect of EVs on H1975 cell proliferation was determined using Cell Counting Kit-8. (d) Reverse transcription qPCR (RT-qPCR) test for the change in contents of miR1246 and miR150-3p in H1975 cells, the cycle threshold (CT) values for U6 across the various samples are showed in Table S2. Experiments were performed in triplicate, and data are represented as mean ± standard deviation. $n = 3$; Con: phosphate-buffered saline; E1: EVs in Week 1; E3: EVs in Week 3; E5: EVs in Week 5; ns: not significant, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

that EVs have no influence on the proliferation of lung cancer cells (Figure 2c). Compared with the data in Week 1, EVs in Week 3 and Week 5 approximately resulted in a fourfold increase in the miR1246 and miR150-3p within H1975 cells after co-cultivation (Figure 2d).

miR1246 and miR150-3p increase lung cancer cells migration, invasion and proliferation

We transduced miR1246 or miR150-3p mimics into H1975 to investigate the functional significance of miR1246 and miR150-3p in lung cancer. After transducing, the expression of miR1246 and miR150-3p have sharply risen to validate the transfection efficiency (Figure 3a). We found that the levels of proliferation of cells overexpressing miR1246 or miR150-3p both obviously increased compared with the control group (Figure 3b). In addition, overexpression of miR1246 or miR150-3p cells showed a sharp increase in migration (Figure 3d) and invasion ability (Figure 3c), which indicated that miR1246 and miR150-3p promoted metastasis of lung cancer cells by increasing the migration, invasion and proliferation.

Proteomic analysis of lung cancer cells co-cultivated with EVs

Cells co-cultivated with Weeks 1 and 5 EVs were selected for proteomic analysis. A total of 8857 proteins were identified (Table S1), 145 proteins were differentially expressed between cells co-cultivated with EVs in Weeks 5 and 1 (88 were upregulated and 57 were downregulated) (Figure 4a).

GO enrichment analysis showed that the differentially expressed proteins were associated with metabolic process, single-organism metabolic process and oxidation–reduction process (biological processes [BP]). The proteins of Week 5 group were enriched in ion binding (molecular functions [MF]) (Figure 4b). KEGG pathway analysis showed that the mainly enriched pathway was cell adhesion molecules (CAMs) (Path ID: 04514) which is closely related to cancer cell invasion and metastasis (Figure 4c).

Proteomic analysis of lung cancer cells after transfection with miR1246 or miR150-3p

Proteomic analysis was also performed on cells that were individually transfected with these two types of miRNAs. Overall, 396 proteins were differentially expressed between transfected cells with miR1246 and control, and 534 proteins were differentially expressed following miR150-3p transfected (Figure 5a). The C-means cluster analysis (Figure 5b) and hierarchical clustering analysis (Figure 5c) revealed that miR1246 and miR150-3p could induce some dysregulated proteins.

GO enrichment analysis showed that the differentially expressed proteins of these two transfected groups were both associated with the response to stimulus (BP). In addition, the involvement of these

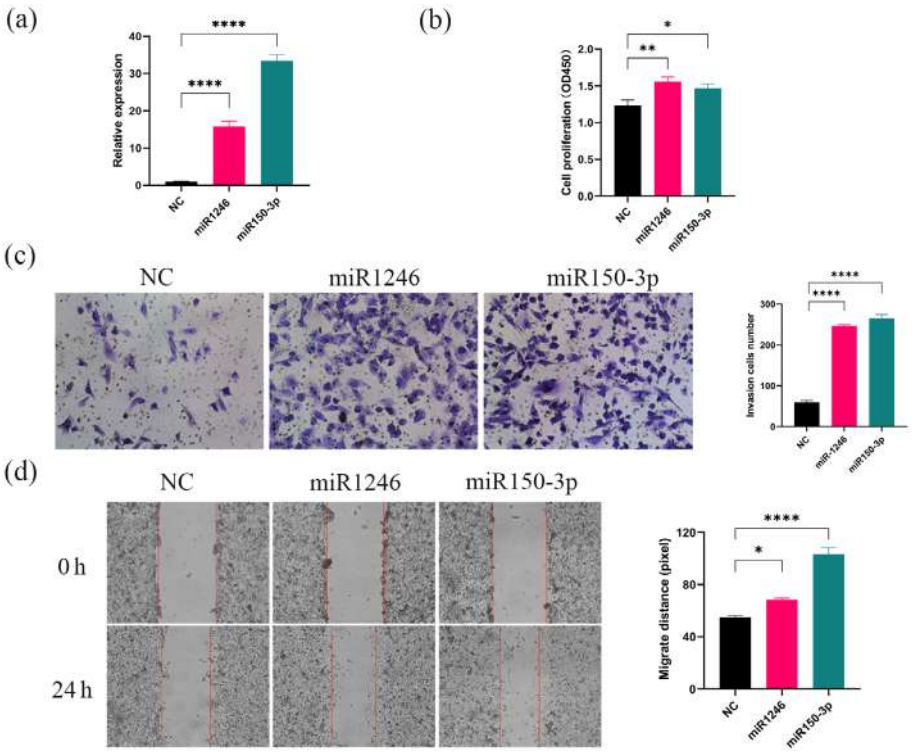

FIGURE 3 miR1246 and miR150-3p enhances the proliferation, migration and invasion of H1975 cells. (a) Transfection efficiency of miR1246 or miR150-3p in H1975 cells. (b) The cell viability of H1975 cells after transfection. The migration (c) and invasion (d) ability of H1975 cells after transfection. Experiments were performed in triplicate, and data are represented as mean ± standard deviation ($n = 3$; NC: micrON mimic negative control; miR1246: miR1246 mimic; miR150-3p: miR150-3p mimic; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

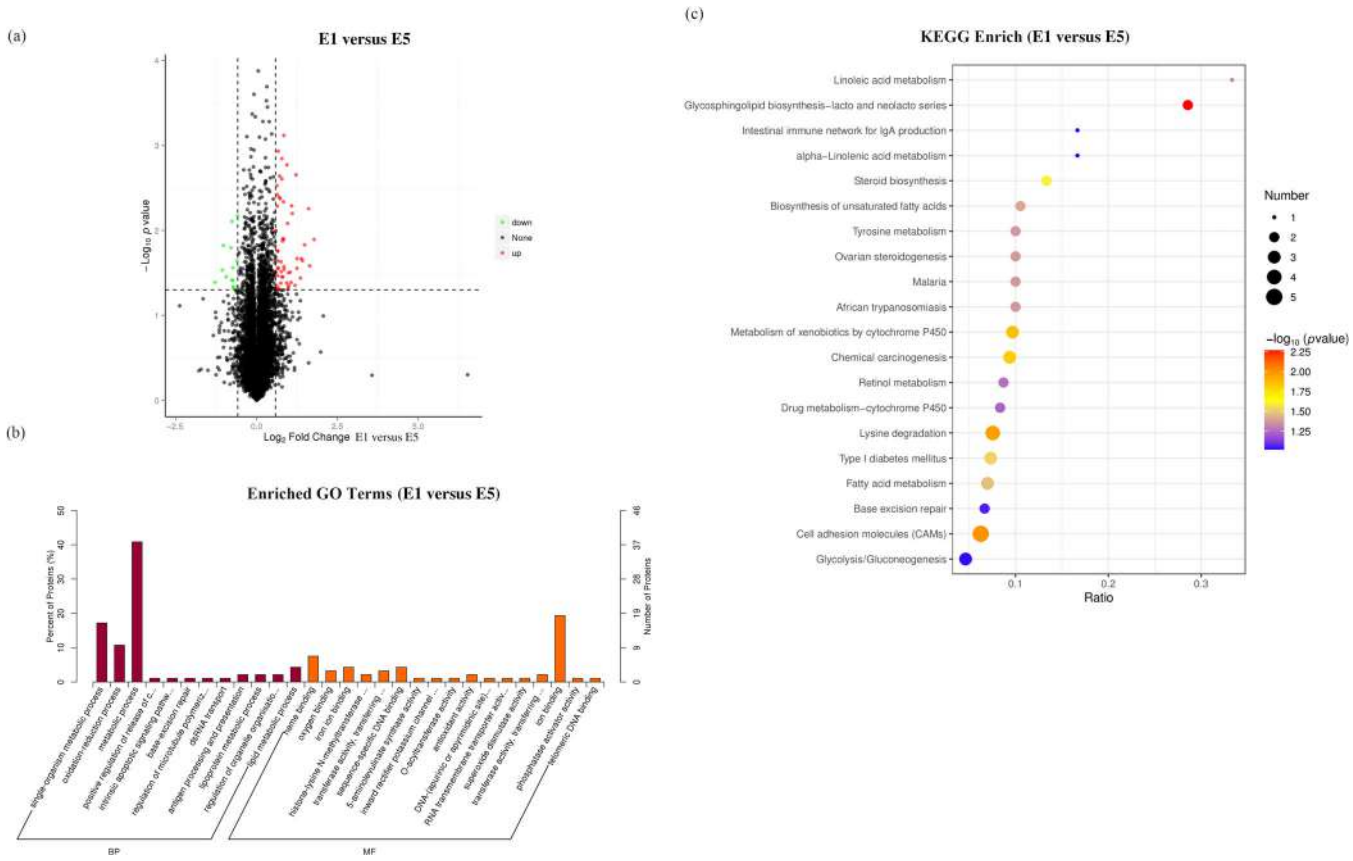

FIGURE 4 DIA proteomics was performed to evaluate the differential proteins after co-cultivation with extracellular vesicles (EVs) in Weeks 1 and 5 in H1975. (a) Volcano plots of protein expression changes. (b) Gene Ontology enrichment of differential proteins after co-cultivation with EVs. (c) Kyoto Encyclopedia of Genes and Genomes enrichment of differential proteins after co-cultivation with EVs ($n = 3$; E1: EVs in Week 1; E5: EVs in Week 5). BP, biologicla processes; GO, gene ontology; MF, molecular functions.

two groups extends to cellular structures like membrane, integral component of membrane and membrane part (cellular components [CC]). The differentially expressed proteins of transfected miR1246 group were enriched in peptidase activity. Meanwhile, the transfected miR150-3p group were enriched in metal ion binding (Figure 5d). KEGG pathway analysis shows that the significant pathway in these two transfected groups was the Herpes simplex infection (Path ID: 05168) and Kaposi's sarcoma-associated herpesvirus infection (Path ID: 05167) (Figure 5e). Other pathways were significantly enriched, including antigen processing and presentation (Path ID: 04612) and CAMs (Path ID: 04514).

Exosomal miR1246 and miR150-3p affect lung cancer cells through the pathway of cell adhesion molecules

Using a Venn diagram, we selected two pathways that were simultaneously enriched in both the co-cultivation and miR1246 transfection groups (top 20 KEGG enrichment, Tables S3 and S4): CAMs (Path ID: 04514) and Type I diabetes mellitus (Path ID: 04940) (Figure 6a). Using the same method, we found that the same results were enriched in the miR150-3p transfection group (Figure 6a). CAMs are closely related to the invasiveness and migration capabilities of tumour cells, and integrins are an important component of the CAM family [20]. ITGB1 is the largest subgroup within the integrin family, which has been described as tumour progressor in various tumour [21]. After co-cultivation of with EVs, these cells indicated a significant increase in the expression of the membrane protein ITGB1 (Figure 6b). Compared with Week 1, the expression levels of ITGB1 increased the most at Week 5. Overexpression of miR1246 or miR150-3p in H1975 cells also caused an increase in the levels of ITGB1 (Figure 6b). Rap1b is the core gene of Rap1 signalling, which could regulate ITGB1 [22]. The pathway was also activated after co-cultivation with EVs and transfection with miR1246 or miR150-3p, especially between EVs in Weeks 3 and 5 (Figure 6c).

Further prediction of the target genes of miR1246 and miR150-3p

miRanda and Pictar databases were used to predict targeting genes of these two miRNAs, suggesting 820 non-repeated genes.

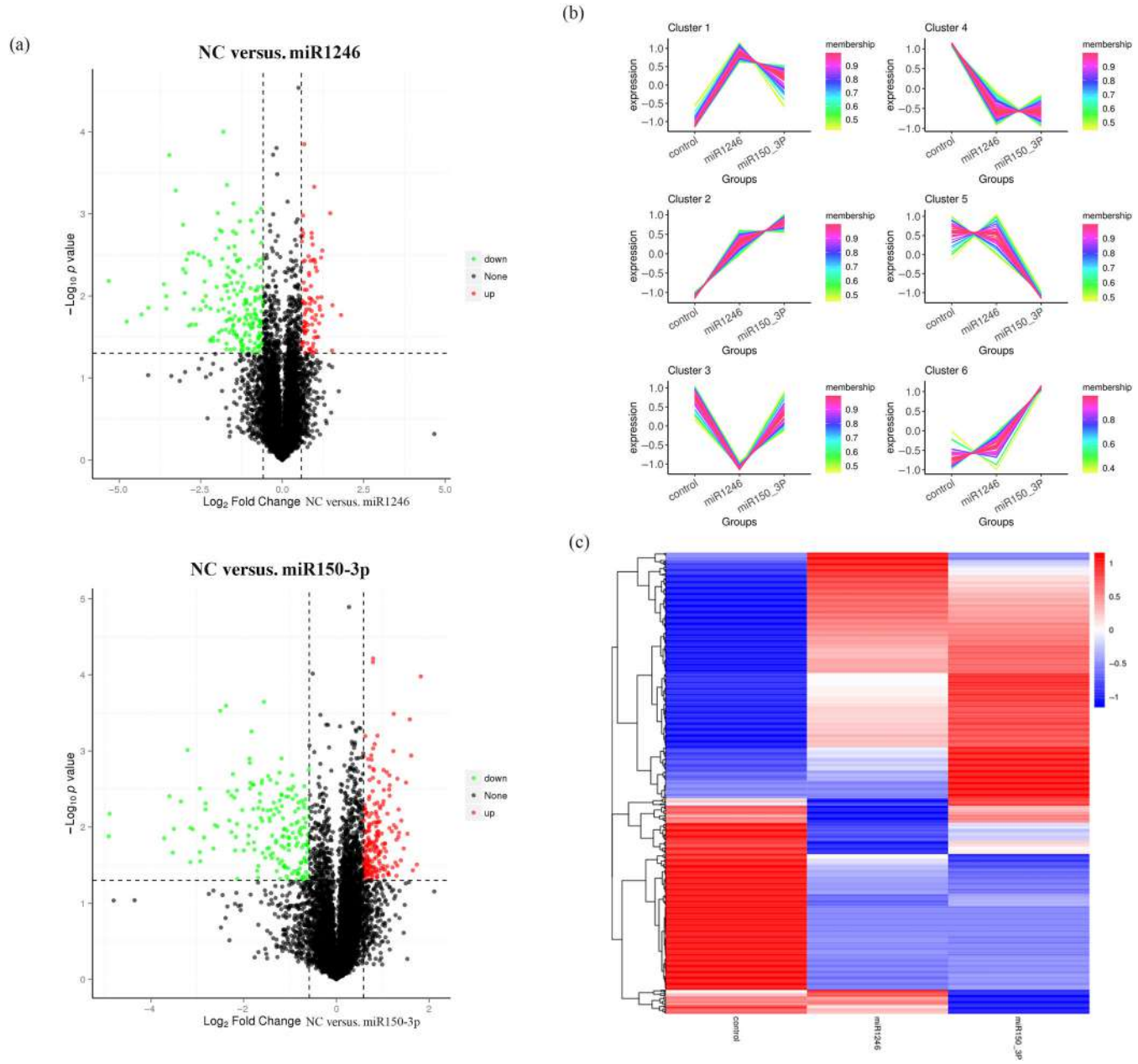

FIGURE 5 DIA proteomics was performed to evaluate the differential proteins after miR1246 or miR150-3p transfection in H1975. (a) Volcano plots of protein expression changes. (b) C-means cluster diagram of differential proteins. (c) Hierarchical clustering analysis of differential proteins after transfection. (d) Gene Ontology enrichment of differential proteins after transfection. (e) Kyoto Encyclopedia of Genes and Genomes enrichment of differential proteins after transfection ($n = 3$). BP, biologicla processes; CC, cellular components; GO, gene ontology; HTLV, human T cell leukemia virus; MF, molecular functions; NOD, nucleotide-bindig oligomerization domain.

Insights from the GO enrichment analysis suggest that these genes are predominantly engaged in the modulation of transporter activity and in the alteration of proteins through alkylation (BP). In addition, their involvement extends to cellular structures like the leading edge of the cell (CC), and they are implicated in functionalities such as the activity of DNA-binding transcription activator activity (MF) (Figure 7a). KEGG pathway analysis showed that the most significant pathway was the cAMP signalling pathway (Path ID: 04024) (Figure 7b).

DISCUSSION

The clinical phenomenon that transfusing stored RBCs is linked to a higher incidence of recurrence and death among lung cancer patients is not yet fully understood. A study in 2010 [23] analysed the different expression profiles of miRNAs related to the apoptosis pathway in RBCs stored for different time. Considering the protective effect of EVs on extracellular miRNA, our preliminary research [17] found the expression profiles of miRNAs in EVs. And, the current research has

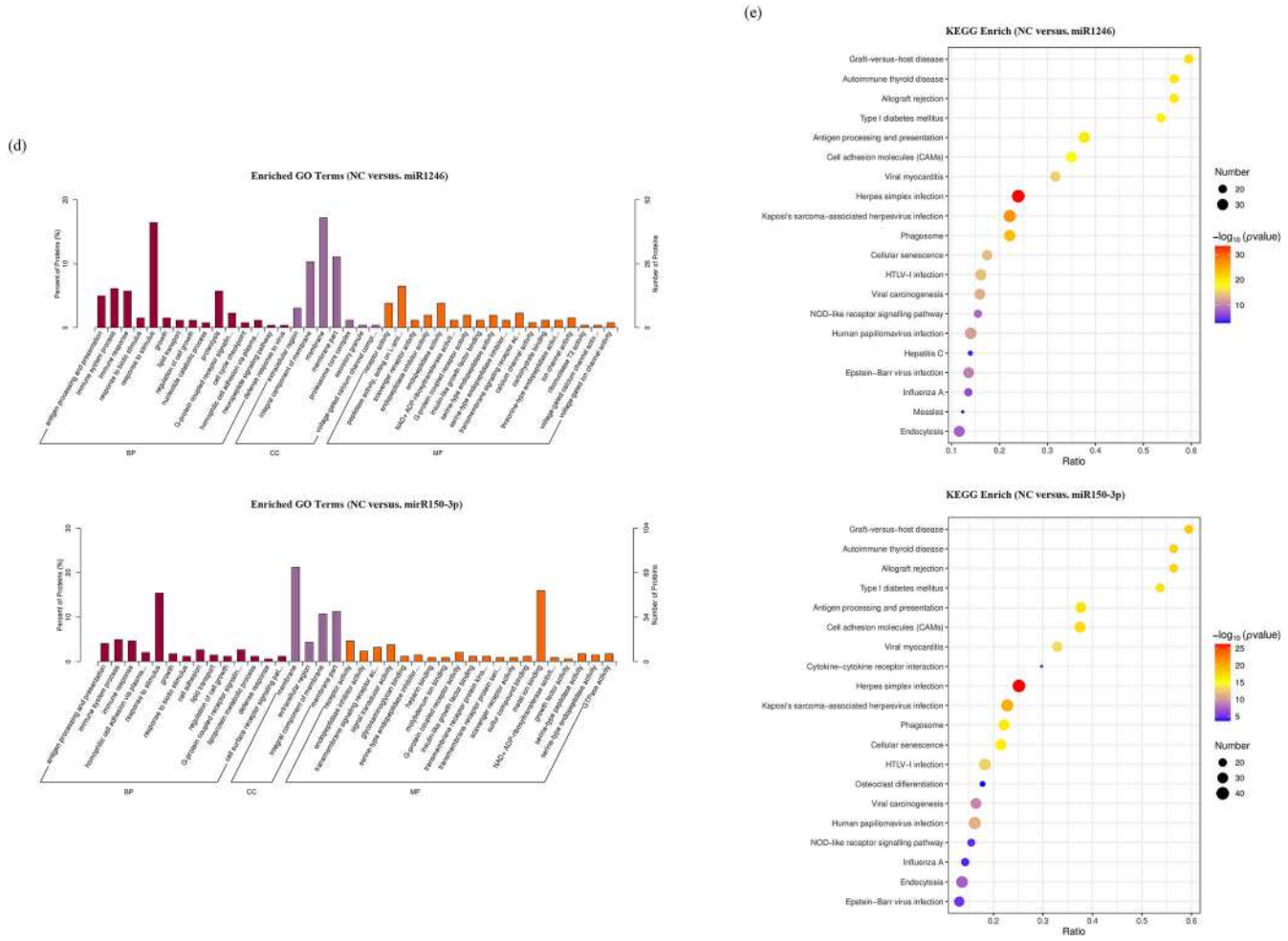

FIGURE 5 (Continued)

further uncovered that EVs originating from stored RBC suspensions could promote the metastasis of lung cancer and activate the Rap1b signalling pathway to regulate the ITGB1, mainly taking advantage of the miR1246 and miR150-3p.

During the standard storage conditions of RBCs, abundant biochemical alterations occur [24, 25] and the membrane of RBCs undergoes oxidation [26]. A possible consequence of RBCs membrane failure might be the emission of injurious bioactive micro-vesicles that accumulate in the supernatant [27]. Straa's study found that supernatants derived from stored RBC bags have higher concentrations of EVs compared with fresh RBC bags, which can stimulate the secretion of more inflammatory factors in whole blood [28]. As the results showed in Table 1 and Figure 1d, the number of EVs released by RBCs increases with prolonged storage time, accompanied by a rise in miR1246 and miR150-3p. Our research indicates EVs derived from RBC suspensions with different storage times all have an impact on the invasive and metastatic abilities of lung cancer cells, with the difference between those from Weeks 1 and 5 being the most significant (Figure 2).

In addition, we found the content of miR1246 and miR150-3p in lung cancer cells increased by fourfold after co-cultivation with EVs derived from Week 5, which may explain the regulatory role of functional miRNAs from EVs derived from RBC suspension (Figure 2d). miR1246 is closely associated with the invasion and migration of numerous tumours, such as colorectal cancer [29], oral squamous cell carcinoma [30] and hepatocellular carcinoma [31].A study in 2020 indicated that miR150-3p may be a decoy of *SRCIN1*, a tumour suppressor gene in the progression of NSCLC [32]. The conclusions from the aforementioned studies align with our research findings (Figure 3), thus supporting the notion that miR1246 and miR150-3p can enhance the metastasis of lung cancer. The transfection group could increase the proliferation of lung cancer cells, but the same trend was not observed after co-cultivation with EVs. On one hand, the fold change of miR1246 and miR150-3p in lung cancer cells in the co-cultivation group was lower than that in the transfection group; on the other hand, EVs contain a variety of substances that can interfere cell proliferation [33], which may mask some of the effects of miR1246 and miR150-3p.

After studying the effects of these two miRNAs on lung cancer cells, we conducted intersection analysis of KEGG after co-cultivation with EVs and transfection, resulting in the identification of a significant pathway—CAMs. CAMs constitute a diverse array of proteins

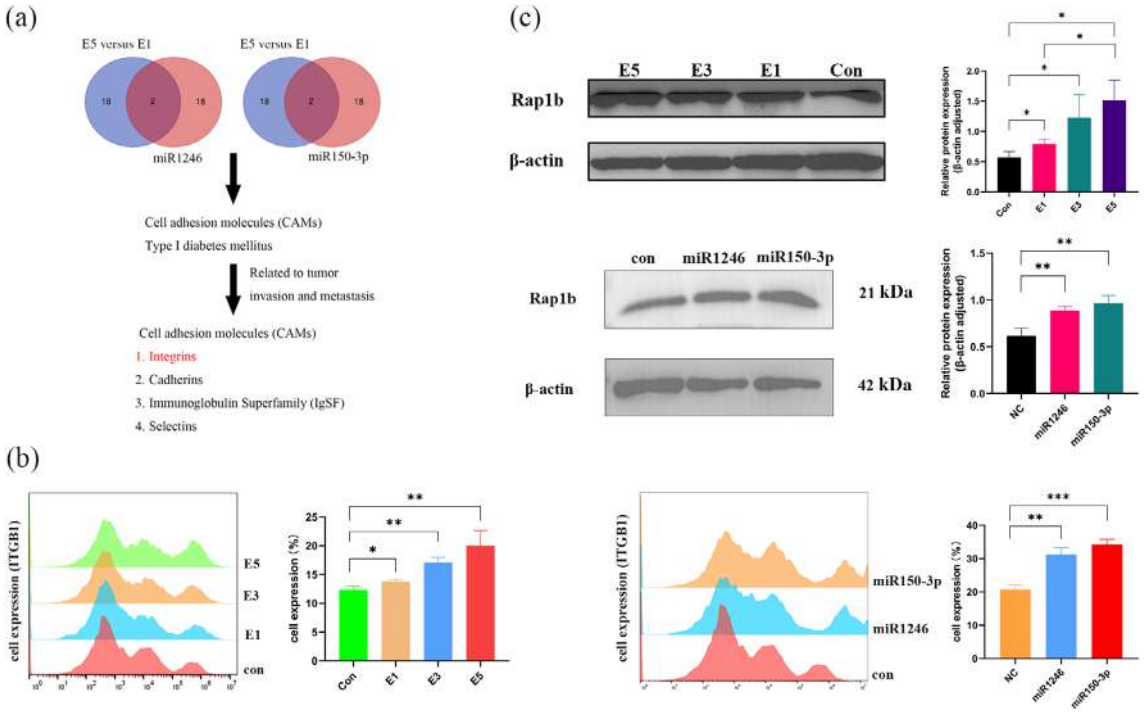

FIGURE 6 Integrin beta-1 (ITGB1) was selected by co-cultivated and transfected proteome results (a) Combined analysis of Kyoto Encyclopedia of Genes and Genomes within co-cultivated and transfected proteome results using a Venn diagram. Flow cytometry (b) and western blotting (c) for the confirmation of ITGB1 after co-cultivation with extracellular vesicles (EVs) and transfection with miR1246 or miR150-3p. Experiments were performed in triplicate and data are represented as mean ± standard deviation. ($n = 3$; Con: PBS; E1: EVs in Week 1; E3: EVs in Week 3; E5: EVs in Week 5; NC: micrON mimic negative control; miR1246: miR1246 mimic; miR150-3p: miR150-3p mimic; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

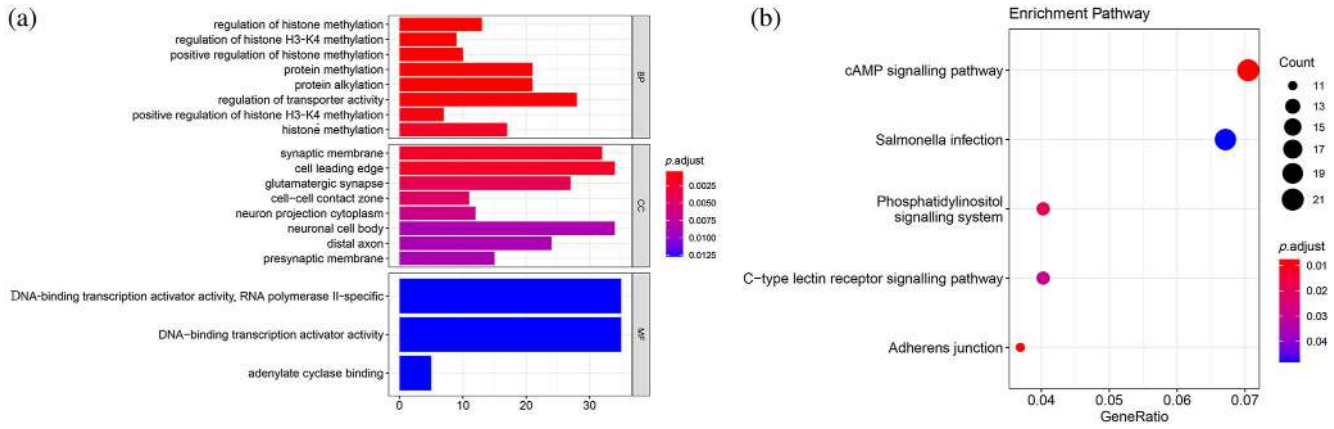

FIGURE 7 Prediction of the functions and signal pathways of target genes for miR1246 and miR150-3p by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrich analysis. (a) GO enrichment of predicted target genes. (b) KEGG enrichment of predicted target genes. BP, biologicla processes; cAMP, cyclic adenosine monophosphate; CC, cellular components; MF, molecular functions.

located on the cellular membrane, which operate by engaging receptor–ligand interactions to effectuate cellular adherence intercellularly, as well as between cells and the extracellular matrix (ECM). Structurally, CAMs are classified into four predominant families: the integrin family, the selectin family, the Ig superfamily and the cadherin family. Integrins are a class of type I transmembrane proteins that play a crucial role in mediating cell/cell and cell/ECM adhesion. ITGB1 is the largest subgroup within the integrin family, primarily responsible for mediating adhesion between cells and the ECM [34]. In 2021, Chang et al. [35] discovered that ITGB1 can further promote the development of lung adenocarcinoma by forming a positive feedback loop with ITGB1/Wnt/β-Catenin/MYC.

The prerequisites for tumour cell invasion are cellular adhesion and spreading [36] and invasion is also a fundamental step in tumour

metastasis [37]. Rap1b signalling is a key regulatory pathway involved in cell adhesion and movement [38]. Recent studies have shown that Rap1b regulates the migration and invasion ability of cancer cells. In colorectal tumours, the activation of Rap1b suppresses cell/cell adhesion while strengthening cell/ECM adhesion, thereby promoting cell invasion and distant metastasis [39]. One similar study indicated that the miR200b/c could target Rap1b directly and negatively regulated its expression, the upregulation of miR200b/c-Rap1b axis constrained papillary thyroid carcinoma cell proliferation, invasion, migration and EMT [40]. By modulating the expression levels and affinity of ITGB1, Rap1b plays an important regulatory role in intracellular signalling pathways [41]. In our study, we observed that RBC suspensions-derived EVs could enhance the expression of Rap1b and ITGB1 in lung cancer cells. In addition, the results after transfection with miR1246 or miR150-3p were consistent with this finding (Figure 6b,c).

In addition, bioinformatic predictions via KEGG suggest that the cAMP pathway is highly likely to be an enriched pathway for target genes. Furthermore, coupled with transfection and cellular validation results, we have identified that the cAMP pathway is one of the upstream pathways of the Rap1 signalling pathway. The second messenger cAMP can not only influence protein kinase A system but also bind and activate exchange protein directly activated by cAMP, which in turn affects downstream Rap1 signalling [42, 43]. Consequently, it is postulated that the target genes of miR1246 and miR150-3p may exert their influence on lung cancer cell invasion and migration through the cAMP-Rap1b-ITGB1 signalling axis.

Given that our samples were derived from non-leukoreduced RBC suspensions, residual leukocyte-derived EVs would also have a certain impact. In addition, EVs contain numerous molecules, including, but not limited to, miRNAs [44]. Specific proteins, lipids and nucleic acids within the EVs can also impact the cells, so what we observe is a cumulative effect [45]. Therefore, the increase in miRNA levels after co-culturing could also be due to the cells secreting a portion of miRNA under the influence of the EVs, rather than solely the transfer of miRNA from the EVs into the cells. Meanwhile, the limited number of samples is a limitation, and further experiments will consider increasing the sample size.

In conclusion, this study provides evidence that EVs derived from stored RBC suspensions may activate ITGB1 through miR1246 and miR150-3p, thereby enhancing the invasion and migration ability of lung cancer cells. This deepens our understanding of negative prognoses associated with transfusions and provides a molecular basis for blocking it, which can contribute to blood transfusion safety.

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Z.C., Y.K., L.T. and Z.L. contributed to conceptualization; Z.C. and Y.K. curated the data; Z.C. performed formal analysis; L.T. and Z.L. acquired the funding; L.T. and Z.L. conducted the investigation; Z.C., Y.K. and H.X. designed the methodology; Z.C., Y.K. and H.X. contributed to project administration; Z.C. performed software analyses; Y.K., L.X., L.T. and Z.L. supervised the study; L.X. and Z.L. validated the results; L.X., L.T. and Z.L. contributed to visualization; Z.C. and Y.K. wrote the original draft; H.X., L.X., L.T. and Z.L wrote, 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 on 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

A novel syphilis *Treponema pallidum* lipoprotein peptide antigen diagnostic assay using red cell kodecytes in routine blood centre column agglutination testing platforms

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Abstract

Background and Objectives: The detection of treponemal antibodies, which are used to make a diagnosis of syphilis, is important both for diagnostic purposes and as a mandatory blood donor test in most countries. We evaluated the feasibility of using Kode Technology to make syphilis peptide red cell kodecytes for use in column agglutination serologic platforms.

Materials and Methods: Candidate Kode Technology function-spacer-lipid (FSL) constructs were made for the *Treponema pallidum* lipoprotein (TmpA) of *T. pallidum*, using the peptide and FSL selection algorithms, and then used to make kodecytes. Developmental kodecytes were evaluated against a large range of syphilis antibody reactive and non-reactive samples in column agglutination platforms and compared against established methodologies. Overall, 150 reactive and 2072 non-reactive Syphicheck assay (a modified *T. pallidum* particle agglutination) blood donor samples were used to evaluate the agreement rate of the developed kodecyte assay.

Results: From three FSL-peptide candidate constructs, one was found to be the most suitable for diagnostics. Of 150 Syphicheck assay reactive samples, 146 were TmpA–kodecyte reactive (97.3% agreement), compared with 58.0% with the rapid plasmin reagin (RPR) assay for the same samples. Against the 2072 expected syphilis non-reactive samples the agreement rate for TmpA–kodecytes was 98.8%.

Conclusion: TmpA–kodecytes are viable for use as cost-effective serologic reagent red cells for the detection of treponemal antibodies to diagnose syphilis with a high level of specificity in blood centres. This kodecyte methodology also potentially

allows for introduction of the reverse-algorithm testing into low-volume laboratories, by utilizing existing transfusion laboratory infrastructure.

Keywords
blood safety, infectious disease, novel diagnostic assay, syphilis resurgence, *Treponema pallidum*

Highlights

- TmpA–kodecytes are modified reagent red cells suitable for the detection of treponemal antibodies in routine column agglutination testing platforms.
- TmpA–kodecytes have a much higher level of agreement (97.3%) for modified *T. pallidum* particle agglutination-defined reactive samples, in contrast to 58.0% by rapid plasma reagin testing.
- The cost of the function-spacer-lipid-TmpA1 constructs per assay volume of TmpA–kodecytes is 1 US cent.

INTRODUCTION

Syphilis is caused by the spirochete *Treponema pallidum* and is a sexually transmitted disease [1]. Today, although the incidence of transfusion-transmitted syphilis is extremely rare, primarily due to the shift to the use of refrigerated blood and blood components, the possibility for transfusion-transmitted syphilis may still remain for several days after 72 h of cold storage [2–4]. In addition, it is anticipated that the blood donors donated in the acute stage of their infection have a high potential to transmit syphilis by transfusion because they might have active and infectious treponemas in their blood, which often remain undiagnosed on immuno-serology-based screening assays. Furthermore, the US Food and Drug Administration and the World Health Organization (WHO) state that syphilis testing should be a mandatory requirement for blood donation [5], particularly as it can be a surrogate marker for high-risk behaviour associated with other non-tested infectious agents [2, 3].

Syphilis is surging globally, with substantial increases noted in several regions, including the United States, Europe, Japan, Australia and China [6]. Syphilis prevalence estimates are calculated for consensus positive infections (which include screen reactive, confirmed-positive infections based on total antibodies) and active infections (a subset of consensus positive that are also rapid plasma reagin [RPR] positive). Recently, the Transfusion Transmissible Infections Monitoring System Program in the United States reported a marked increase in syphilis prevalence in donations, which is considered to be a reflection of overall increasing trends in the general population (from 15.9/100,000 in 2012 to 51.5/100,000 in 2021) [3]. This resurgence is also seen in low- or middle-income countries (LMICs), with >6 million new cases occurring annually and causing foetal and neonatal morbidity and mortality [7].

The laboratory diagnosis of syphilis is complex and no single diagnostic assay is able to accurately determine both latent and active infections [8–12], although expectedly the higher cost treponemal antigen-based diagnostics assay outperform the lower cost non-treponemal assays [11]. Because many LMICs cannot afford the more expensive treponemal antigen-based assays for primary screening, they use an algorithm approach, and first screen with a low-cost non-treponemal assay (e.g., RPR) and then confirm reactive samples with the higher cost treponemal assay(s) [11, 12]. Many transfusion services also use a combination of data to determine who has an active or recent infection, including their previous donation testing results and the clinical history provided. In contrast, most high-income countries (and some LMICs where a low-cost treponemal assay is available) first use the more sensitive but more expensive treponemal-based assay for primary screening, followed by confirmation of reactive samples with either (or both) a different treponemal assay and/or non-treponemal assay (reverse-algorithm) [11, 12]. Despite this, not all treponemal antigen-based assays are equal, and a range of technological variations exist including enzyme immunoassays (EIAs), *T. pallidum* particle agglutination (TPPA) and its hemagglutination variation, the TPHA, and lateral flow point of care (POC) assays, each with their own limitations in sensitivity and specificity and affordability [8–12]. Thus, there remains a need, especially in LMICs, for low-cost, high-sensitivity, easy-to-use assays for the detection of treponemal antibodies that are used to diagnose syphilis, ideally those suitable for use with existing laboratory resources.

Recently, the Kode Technology platform [13] has been shown to be suitable for the detection of SARS-CoV-2 antibodies using existing blood transfusion services laboratory infrastructure [14–17]. With the peptide sequence of the syphilis treponemal spirochete *T. pallidum* well established [7, 8], using the same principles as for SARS-CoV2–kodecyte development, we investigated the opportunity to create *T. pallidum* lipoprotein (TmpA)–kodecytes for use on existing routine blood antibody screening column agglutination testing (CAT) diagnostic platforms.

MATERIALS AND METHODS

Reactive plasma samples ($n = 150$) were obtained from blood donors donated blood at Tata Medical Center, Kolkata—initially identified

with the Syphicheck modified TPPA device (Syphicheck-WB, Viola Diagnostic Systems, India) [18] and/or confirmed reactive with the EIA-OCD, (VITROS System Ortho Clinical Diagnostics, Pencoed, UK) and/or RPR (Carbogen, Coral Clinical System, India) assays. Non-reactive (NR) plasma samples ($n = 2072$) were from healthy blood donors and obtained from the Tata Medical Centre (Kolkata, India) and confirmed NR with the Syphicheck, RPR and EIA-OCD methods. Syphicheck-WB is a two-site double recombinant antigen (47 kDa and 17 kDa) sandwich immunoassay for the detection of total (IgM and IgG) antibodies to *T. pallidum* [18]. Although Syphicheck-WB is not a quantitative assay, it has been found to give positive results up to a dilution of 64 when qualitatively validated by testing different dilutions of plasma. The cut-off in EIA-OCD is 1.0 for reactivity. Ethical committee approval was waived by the institutional review board as donor details were not captured, only declassified blood samples were used and consent was obtained from each blood donor for the syphilis testing according to the national policy.

TmpA function-spacer-lipid constructs and kodecytes

The selection and design of synthetic peptide-based function-spacer-lipid (FSL) constructs is complex (see Appendix S1) [13–15]. In brief, the published syphilis peptide sequences for *T. pallidum* were used to find candidate non-glycosylated peptide epitopes suitable for construction as FSL constructs [13–15]. Following extensive serologic evaluations of these three candidates, TmpA1 were selected as the best candidate for field trials (see Appendix S1) [14]. Terminology for describing FSL constructs and the resultant kodecytes are as described in detail elsewhere [13–15, 19].

In brief, preparation of kodecytes [13] involved mixing a solution of FSL construct (e.g., 2.5 µmol/L) with washed packed group O red cells, incubation at 37°C for 2 h and storage in red cell stabilizer (ID-CellStab, DiaMed GmBH, Switzerland) solution at 4°C. No washing is required at any stage (and stored kodecytes were not washed prior to use). Kodecytes were rested overnight before use, and used within 21 days. A detailed protocol for the preparation of TmpA–kodecytes is available (see Appendix S1).

TmpA–kodecyte assays

TmpA serology was undertaken in routine CAT platforms with methodologies and scoring systems as recommended by the manufacturer, including the use of the grades 4+, 3+, 2+, 1+ and *w* to indicate weak positive reaction. Example CAT reactions are shown in Figure 1. Samples were tested in both the Bio-Rad ID-system with Coombs anti-IgG card (CAT-BIO; Bio-Rad Laboratories, DiaMed GmBH, Switzerland) and the Ortho BioVue system (CAT-OCD; anti-IgG cards; Ortho Clinical Diagnostics, Pencoed, UK). As part of the validation evaluations, unmodified group O cells (the same cell as used to make the kodecyte) were also used as controls, in addition to known positive and negative controls.

RESULTS

TmpA–kodecyte agreement rate (NR samples)

Against 2072 Syphicheck-defined NR blood donor samples, 24 (1.16%) were reactive with TmpA–kodecytes (Table 1). Of these reactive NR samples, most had reactivity grades 2+ or greater, and all were unreactive with unmodified cells, indicating the reaction was due to IgG/IgM directed against the TmpA1 peptide on the kodecyte.

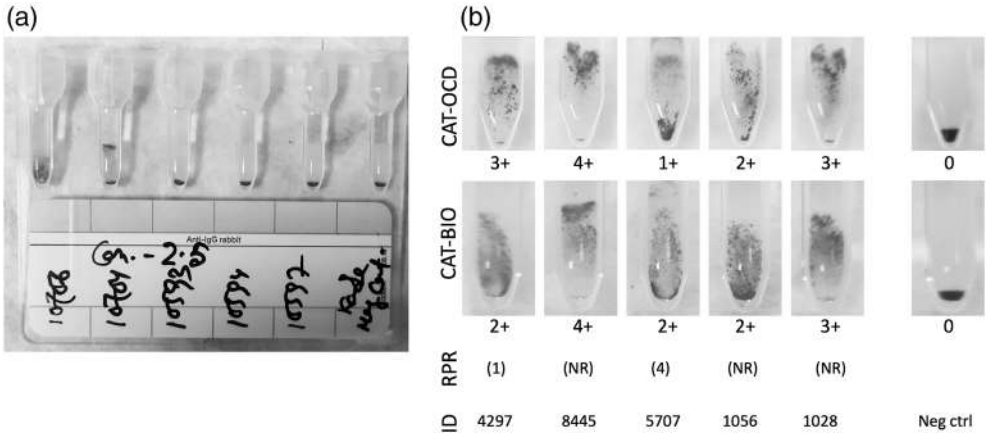

FIGURE 1 (a) Selected example of column agglutination testing (CAT)-BIO reactions of *Treponema pallidum* lipoprotein (TmpA)–kodecytes against Syphicheck (and enzyme immunoassay [EIA]-OCD) non-reactive (NR) donor samples. The false-positive reactions seen in lanes 1 and 2 were observed with 1.16% of NR samples. Lanes 3–5 show typical negative reactions against TmpA–kodecytes, whereas the negative reaction in lane 6 is against with unmodified cells (the same cell as used to make the kodecytes). (b) Selected examples comparing CAT-OCD versus CAT-BIO reactions with TmpA–kodecytes against Syphicheck (and EIA-OCD)-reactive donor samples. The serologic score is indicated as is the reciprocal of the rapid plasmin reagin (RPR) titre which is shown in brackets; this figure has been created by assembling individual images from different gel cards, scaling them to similar size and conversion to black and white.

TABLE 1 TmpA–kodecyte results for 2072 non-reactive (NR) donor samples.

NR samples (Syphicheck, RPR and EIA-OCD)				
			TmpA–kodecyte serologic grade	
Result	*n*	%	CAT-BIO	CAT-OCD
NR	2048	98.84%	0	0
Reactive	3		1+	1+
Reactive	1		2+	1+
Reactive	19		2+	2+
Reactive	1		4+	4+
Reactive (total)	24	1.16%	+	+

Abbreviations: CAT, column agglutination testing; EIA, enzyme immunoassays; RPR, rapid plasmin reagin; TmpA, *Treponema pallidum* lipoprotein.

TABLE 2 Correlation between CAT-BIO and CAT-OCD serologic grades against 150 Syphicheck-reactive donor samples.

Platform serologic grades against TmpA–kodecytes						
	CAT-BIO (grades)					
CAT-OCD (grades)	4+	3+	2+	1+	*w*	0
4+	10	(1)		(1)		
3+		47	(3)			
2+			63	(1)		
1+				19	(1)	
w						
0						4[a]

Note: Non-identical grades are indicated in brackets.
Abbreviations: CAT, column agglutination testing; TmpA, *Treponema pallidum* lipoprotein.
[a]2/4 were 1+ reactive with 5.0 μM TMPA1–kodecytes.

TmpA–kodecyte agreement rate (reactive samples)

Of 150 samples that were identified as reactive with the Syphicheck diagnostic as well as with EIA-OCD, 146 (98.8%) tested reactive against TmpA–kodecytes (Table 2). Of the four samples testing reactive with Syphicheck but NR with TmpA–kodecytes, all were NR by RPR (see Appendix S1) and one was borderline reactive by EIA-OCD. Overall, the correlation in grades between the CAT-BIO and CAT-OCD platforms was excellent with 149/150 (99.3%) of grades being within 1 grade of consensus (Table 2). Of the 7/150 samples (4.7%) that were outside of identical grades, all except one were only one grade lower in grade in the CAT-BIO platform. The one outlier (with identical results in repeat testing) had a 4+ grade in the CAT-OCD platform and 1+ grade in the CAT-BIO platform (Table 2). The reason for this large variation in reaction grade between platforms could not be explained.

The reactive rate for the RPR assay against these same samples was 58.0% (87/150 samples were reactive, see Appendix S1). The correlation of the TmpA–kodecyte grades with RPR titre was generally poor (see Appendix S1).

DISCUSSION

In blood centres, the mandatory screening for syphilis antibodies is done using immuno-serology-based methods [8–12]. The cost of the diagnostic assay is a significant factor in the decision of what platform is used, especially for LMICs.

There are essentially two categories of syphilis serological diagnostic methods (for the detection of syphilis antibodies). The first is the non-treponemal methods which use a cardiolipin, lecithin and cholesterol complex to detect anti-lipoidal antibodies (a consequence of syphilis infection); these assays include the venereal disease research laboratory (VDRL) and RPR tests [1], which have sensitivity in the range of 61%–78% for detecting latent syphilis and 78%–86% for detecting early syphilis infection [20]. However, a serological diagnosis of syphilis cannot be made using a single assay such as RPR. It must be confirmed with the use of either the traditional or reverse sequence algorithm. The second category of serologic assay, of which there are four main variations, uses treponemal antigens (natural or recombinant) to detect treponeme-specific antibodies with sensitivity in the range of 82%–100% across the spectrum of the disease [1, 8, 12, 18] and includes a variety of direct and indirect immunoassays [8–12]. The primary basis of these methods is to use *T. pallidum* antigen and labelled secondary antibodies to generate a quantitative result with sensitivity and specificity of >95% (reviewed in detail elsewhere) [8]. The third serological method variant is the *T. pallidum* particle agglutination (TPPA) assay. In this assay, *T. pallidum* antigen is coated onto coloured gelatine particles which in the presence of serum containing specific antibody will aggregate, which is interpreted as reactive. The TPPA test has a relatively high specificity rate of >97%, although it has a lower sensitivity rate for primary and latent syphilis (>86%) [9, 10]. The fourth serologic TPPA-like method, commonly used in LMICs due to their low cost, are the rapid POC diagnostic tests which use treponemal-specific antigens either in strips and/or on carrier particles [18, 21]. Although the sensitivity and specificity of these POC assays are reported to be very high in clinical laboratories [18, 21], there is some evidence that they do not perform as well (with sensitivity and specificity as low as 50%) in less regulated clinic settings [22].

In contrast to the above described treponemal antigen serologic assays, the TmpA–kodecyte assay does not use natural or recombinant antigens, but instead uses a comparatively small synthetic 15 amino acid peptide. This alternative approach is achieved by first identifying potential non-glycosylated epitopes of *T. pallidum* using a range of predictive algorithms and online tools [14, 15], followed by refinement to be compatible with FSL conjugation chemistry, and then optimization using serology for sensitivity and specificity (see Appendix S1). From the initial >20 potential candidates predicted, three were made into FSL constructs and after extensive serological testing FSL-TmpA1 including against reference standards and clinical samples was selected as the best [14]. In the developmental phase of this project [14], against 211 clinical syphilis samples (EIA-confirmed reactive), it was found that TmpA-10–kodecytes (10 μmol/L) had 98.6% agreement rate, in contrast to TPPA which had 89% and RPR which had 61% [14]. External WHO/CDC syphilis quality control samples (100% agreement rate) further validated the sensitivity of the TmpA–kodecytes, as did the NR results with 157 samples reactive for non-syphilis pathogens and autoantibodies [14].

The detection of treponemal antibodies may indicate a recent, past or successfully treated infection. An initial syphilis seroreactive donor is considered to have an active infection if they further test positive on a RPR test. A positive result for reagin indicates a recent infection at 3–6 weeks after a risk exposure. In this study, the Syphicheck assay was chosen as the diagnostic assay for comparison as it is the dominant TPPA assay used in India, with validated sensitivity and specificity [21]. The novel TmpA–kodecytes-based assay might be used as an alternative to the Syphicheck assay and has the potential to become the primary screening assay in a reverse sequence algorithm. Subsequently, the status of an active infection can be confirmed by the RPR test. In this study, reactive samples were primarily selected to be reactive by the Syphicheck assay, a process which creates a sensitivity selection bias [9]. As the Syphicheck assay is reported to have a sensitivity of 95.3% [21], theoretically 4.7% of true-positive samples will be NR with the Syphicheck assay, but as they were not selected for analysis, their reactivity against TmpA–kodecytes is unknown. With respect to the agreement rate of the 150 donor samples identified as reactive with the Syphicheck diagnostic, 97.3% were also tested reactive with TmpA–kodecytes, in contrast to 58.0% by RPR.

The agreement rate of the TmpA–kodecyte assay against 2072 NR donor samples was 98.84%, meaning that there was a reactive (false-positive) rate of 1.16% compared with the Syphicheck false-positive rate which ranges from 0.16% to 0.34% (see Appendix S1). Although the TmpA–kodecyte false-positive rate was higher than optimal, the rate of 1.2% was considered acceptable for the TmpA–kodecyte assay to be used for primary screening together with confirmation using another treponemal antigen diagnostic (e.g., TPPA/EIA). Even when including the cost of the CAT platforms, compared with other assays [10], the TmpA–kodecyte assay is a low-cost assay (e.g., 0.5 mg of FSL-TmpA1 at a cost of USD 1000 will enable more than 100,000 CAT assays as 2.5 μmol/L kodecytes) [15, 22] and is compatible with high-throughput screening. Therefore, the development of this new TmpA–kodecyte assay may be useful for blood centres in LMICs that are already using CAT platforms for pre-transfusion testing.

Approximately, 90% of the new syphilis cases reported every year occur in developing countries where there is often a lack of resources necessary to purchase and perform treponemal-based tests [23]. In addition to utilization for routine blood donor screening having access to a low-cost treponemal-based assay, particularly one which utilizes existing laboratory infrastructure may have an impact of syphilis diagnosis in these regions. Sample testing of blood donors offers a unique opportunity to screen healthy individuals for the presence of antibodies to infections on a population level [24]. This is a role that blood establishments can play in informing public health policy [22], and this type of serosurveillance could be expanded in the future for emerging infections [24]. Additionally, although blood services typically do not undertake screening for syphilis antibodies in samples other than their donor cohort, with the availability of a low-cost, high-throughput treponemal diagnostic like the TmpA–kodecyte assay, there is a possibility they could provide this service.

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CONFLICT OF INTEREST STATEMENT

Stephen M. Henry and Nicolai V. Bovin are employees and stockholders in Kode Biotech, the patent owner of Kode biosurface engineering technology. All other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings are available from the corresponding authors 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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West Nile and Usutu viruses are efficiently inactivated in platelet concentrates by UVC light using the THERAFLEX UV-Platelets system

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Abstract

Background and Objectives: West Nile virus (WNV) and Usutu virus (USUV) are mosquito-borne flaviviruses (Flaviviridae) that originated in Africa, have expanded their geographical range during the last decades and caused documented infections in Europe in the last years. Acute WNV and USUV infections have been detected in asymptomatic blood donors by nucleic acid testing. Thus, inactivation of both viral pathogens before blood transfusion is necessary to ensure blood product safety. This study aimed to investigate the efficacy of the THERAFLEX UV-Platelets system to inactivate WNV and USUV in platelet concentrates (PCs).

Materials and Methods: Plasma-reduced PCs were spiked with the virus suspension. Spiked PC samples were taken after spiking (load and hold sample) and after UVC illumination on the Macotronic UV illumination machine with different light doses (0.05, 0.1, 0.15 and 0.2 (standard) J/cm^2). Virus loads of WNV and USUV before and after illumination were measured by titration.

Results: Infectivity assays showed that UVC illumination inactivated WNV and USUV in a dose-dependent manner. At a UVC dose of 0.2 J/cm^2, the WNV titre was reduced by a $\log_{10}$ factor of 3.59 ± 0.43 for NY99 (lineage 1) and 4.40 ± 0.29 for

Ute Gravemann and Mathias Boelke contributed equally to this study.

strain ED-I-33/18 (lineage 2). USUV titres were reduced at the same UVC dose by a $\log_{10}$ factor of 5.20 ± 0.70.

Conclusions: Our results demonstrate that the THERAFLEX UV-Platelets procedure is an effective technology to inactivate WNV and USUV in contaminated PCs.

Keywords

blood product safety, THERAFLEX UV-Platelets system, Usutu virus, West Nile virus

Highlights

- The THERAFLEX UV-Platelets system reduces the infectivity of West Nile virus by 3.59–4.40 log10, depending on the virus isolate.
- This system efficiently reduces the infectivity of Usutu virus by 5.20 log10.
- Reduction of virus infectivity contributes to blood product safety.

INTRODUCTION

West Nile virus (WNV) and Usutu virus (USUV) are mosquito-borne flaviviruses of the Japanese-encephalitis serocomplex (family Flaviviridae, genus *Flavivirus*) [1] with increasing prevalence across Europe. WNV and USUV are maintained in enzootic cycles involving ornithophilic mosquitoes and birds, but both can infect humans and other vertebrates. About 80% of WNV infections in humans are asymptomatic. While West Nile fever (WNF) is the most common clinical presentation, immunocompromised patients are at risk to develop West Nile neurological disease (WNND) [2–4]. As for WNV, the most USUV infections are asymptomatic. However, USUV can cause similar symptoms as WNV, including fever and headache but also neurological implications, such as tremor and hyperreflexia, in immunocompromised patients [5].

USUV was first introduced into Europe in 1996, leading to a large outbreak and a massive die-off of common blackbirds (*Turdus merula*) in Tuscany, Italy [6]. Thereafter, the virus spread to several European countries and was first detected in Germany in mosquitos collected from Baden–Württemberg in 2010 [7]. Since then, the virus has spread across Germany with major outbreaks in common blackbird populations during 2011–2015 [8, 9]. USUV infections in blood donors were reported across Europe, for example, in Germany, Austria, Italy and the Netherlands [10–13].

WNV was first isolated from a patient blood sample in Uganda in 1937 [14], and since then has been detected in mosquitos in Eurasia [15], Australia [16] and the Americas [17]. Especially in the United States, WNV has spread rapidly. Starting from two cases of encephalitis reported to the New York City Department of Health and Mental Hygiene in 1999 [18], it had spread across the country to the West Coast by 2004 and was reported from all 48 continental states in 2012 [19]. Between 2002 and 2007, peak epidemics were reported with around 10,000 cases annually. From 2013 to 2018, a large number of cases, with more than 2000 cases annually, were reported [20]. In Germany, WNV was first detected in 12 zoo and wild birds and 2 horses in 2018 [21]. In the ensuing years (2019–2022), 229 WNV cases in birds, 94 in horses [22, 23] and 48 in humans were confirmed [24, 25].

Increased prevalence in Germany and travel-associated WNV infections as well as increased numbers of USUV infections raise concerns that the viruses may also be transmitted via blood donation. Cases of transfusion-transmitted infection (TTI) of WNV has been described in the United States [26]. There, 23 recipients of non-virus-inactivated blood donations were infected with WNV between 1999 and 2003 [26], and even after implementation of mandatory WNV testing, an additional 9 cases of TTI occurred [27, 28]. Furthermore, recipients of blood transfusions are more prone to develop severe infections [26, 28]. Of the 32 reported TTI cases, 59% (19) developed WNND, the most severe outcome of a WNV infection [26, 28]. Thus, blood donor history screening and laboratory testing may not be sufficient to eliminate the risk of transfusion-transmitted WNV infection.

To reduce the risk of virus transmission via blood products, several technologies for pathogen inactivation (PI) have been developed. The most prominent way to reduce the replication capacity of many pathogens including viruses, bacteria and protozoan parasites is the inactivation of pathogen genomes using UV light and/or photosensitizing components. Many blood centres in Europe, the United States, Canada, Asia and the Middle East pursue such methods for the treatment of plasma and platelet concentrates (PCs) [29, 30]. The THERAFLEX UV-Platelets system (MacoPharma) is one such system developed for the treatment of platelets (PLTs), which requires only illumination with ultraviolet C (UVC) light, without the addition of a photosensitizing compound. The THERAFLEX UV-Platelets system has been used to inactivate a large number of pathogens including several medically important arboviruses such as dengue 1–4 and chikungunya virus [31], but there are no reports regarding its ability to inactivate WNV and USUV.

MATERIALS AND METHODS

Selection of donors

All donations were from eligible, non-remunerated voluntary blood donors who were selected in accordance with current German

guidelines [32]. Only regular blood donors who fulfilled the requirements for blood donation and had given their informed consent were included in the study. Use of PCs for research purposes does not require ethical approval according to local/national guidelines.

PC preparation

Whole blood units of 500 mL were collected in 70-mL CPD anticoagulant solution (Day 0) and were kept at room temperature (22 ± 2°C) overnight. After centrifugation (4000*g*, 10 min; Roto Silenta 63 RS, Hettich), buffy coats (BCs) were separated. Pools of four BCs were mixed with 280 mL of PLT additive solution SSP+ (MacoPharma) and centrifuged (527*g*, 7.5 min). Plasma-reduced PCs were separated, leukodepleted by filtration (CompoStop Flex 2F, Fresenius) and stored under agitation at 22 ± 2°C until treatment.

Testing for anti-WNV antibodies

Antibodies against WNV can interfere with the virus titre determined by virus titration. PCs used in this study were therefore tested for anti-WNV antibodies using a luciferase immunoprecipitation system (LIPS) assay. WNV NS1 antigen was expressed as a secreted fusion protein with a nano-luciferase (NLuc). PC supernatants were tested for reactivity against the NS1-NLuc fusion protein and NLuc alone (which served as a background control). Only anti-WNV antibody-negative PC units with luminescence signals at or below background levels were included in the study.

Virus propagation/cell culture

Virus propagation and cell culture were done under biosafety levels 2 and 3 requirements according to the local standard operating procedures (SOPs). WNV-NY99 (Genbank Accession # MZ605381), WNV strain ED-I-33/18 (Genbank Accession # MH924836.1) and USUV strain HAN/SN/2018 (Genbank Accession # MT580899.1) were propagated on C6/36 cells (*Aedes albopictus*; CVCL Z230, Zellbank FLI Riems). C6/36 cells were cultivated in Schneider's Drosophila Medium supplemented with 10% FBS, 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate solution and 1% penicillin/streptomycin, at 25°C. Titrations were performed on BHK-21 cells (CCLV-RIE 164, Zellbank FLI Riems), which were cultivated using DMEM with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin at 37°C and 5% CO_2.

Viral inactivation

The PC (315 mL) was spiked with the virus suspension (35 mL 10% v/v) and mixed gently. The virus-spiked PC was transferred to the illumination bag and treated with the THERAFLEX UV-Platelets system following manufacturer's instructions. Samples were taken at different process steps according to Table 1. The load sample was titrated immediately after sampling. The hold sample was stored at room temperature until the end of the experiment and was then titrated. The virus titres were determined by endpoint titration and large-volume plating according to Table 1. Each treatment was done in three replicates (three different bags of PCs).

TABLE 1 Sampling scheme ($N = 3$ each).

Sample no.	Sample	Endpoint titration	Large-volume plating
1	Virus suspension	x	
2	Negative control	x	
3	After spiking (load)	x	
4	0.05 J/cm^2	x	
5	0.1 J/cm^2	x	
6	0.15 J/cm^2	x	x
7	0.2 J/cm^2	x	x
8	Hold	x	

Determination of virus titres and reduction factors

Potential cytotoxic effects of the PC on BHK-21 cells were ruled out before the virus inactivation study. Virus titres of virus-spiked PC were determined by endpoint titration in microtitre plate assays (1:10 serial dilutions, eight parallel samples per dilution, starting dilution 1:100) on BHK-21 cells.

To improve the detection limit of a sample (2 $\log_{10}$ $TCID_{50}$/mL), a large volume of a non-toxic concentration of the sample was incubated with BHK-21 cells (large-volume plating, Table 1, detection limit 1.4 $\log_{10}$ $TCID_{50}$/mL) [33]. For this, 220 µl of virus-spiked PC was mixed with 21.8 mL of a medium containing 2% FBS, and 100 µl of this mixture was added to the wells of a 96-well plate containing BHK-21 cells.

Plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 5 days, the cell layers were inspected microscopically for virus-induced changes in morphology (cytopathic effect, CPE). The virus titre that caused a positive result in 50% of the infected cultures ($TCID_{50}$) was calculated according to the method of Spearman and Kärber [34, 35]. The detection limit and virus titres from large-volume plating were calculated following current guidelines [33]. The number of infectious particles (PFU) per millilitre was calculated from the $TCID_{50}$/mL using a factor of 0.69. The effectiveness of each inactivation process step was calculated as a logarithmic reduction factor (R) using the equation

$$R = \log_{10} A_0 - \log_{10} A_n,$$

where A_0 is the total viral load after spiking and A_n is the total viral load in the treated sample. The overall R was expressed as the sum of Rs for all steps.

RESULTS

We tested the THERAFLEX UV-Platelets system at different doses (0.05–0.2 J/cm^2) with PC spiked with the WNV strain NY99, the German isolate ED-I-33/18 or the USUV strain HAN/SN/2018 and observed a dose-dependent inactivation. The standard dose of 0.2 J/cm^2 resulted in a 4.40 ± 0.29 log reduction of the WNV German strain ED-I-33/18 (Table 2), a 3.59 ± 0.43 log reduction of WNV NY99 (Table 3) and a 5.20 ± 0.70 log reduction of USUV titres (Table 4). For WNV, a combination of regular $TCID_{50}$ and large-volume plating was used to assess the virus reduction because of very low residual virus titres at the standard UVC dose. The hold sample indicated only minor losses of virus infectivity for WNV NY99 (0.13 ± 0.05 log reduction), WNV ED-I-33/18 (0.22 ± 0.33 log reduction) and USUV (0.40 ± 0.30 log reduction). This minor reduction in the hold sample indicates that all other reductions of virus titres observed in the samples treated with the THERAFLEX UV-Platelets system are due to the UVC illumination and not to the intrinsic virus inactivation by the blood product.

DISCUSSION

Global warming and increased travel activities favour the emergence of arbovirus vectors, which in turn leads to the increased geographical distribution of the vectors and the viruses they transmit. This has implications for public health and for the transfusion medicine community. Especially, WNV and the serologically indistinguishable USUV have emerged worldwide since 2000. The rapid spread in the United States since 1999 [17] and the emergence in Europe, including Germany, in the past years [22] have led to an increased risk for blood transfusion recipients to be infected with those viruses [24, 27, 28]. In the absence of other measures, rigorous donor testing is essential because there have been already 19 WNV infections confirmed by discriminatory nucleic acid testing (NAT) and sequencing in blood donors during 2020–2021 [24]. In addition, as a result of increased numbers of WNV outbreaks in Europe, higher numbers of travel-associated WNV infections are to be expected. Since notification of arboviral infections became mandatory according to the German 'Protection against infection law (IfSG)' in 2016, 19 cases of travel-

TABLE 2 Reduction of West Nile Virus strain ED-I-33/18 (lineage 2 German isolate) titres after THERAFLEX UV-Platelets treatment assessed using $TCID_{50}$ titration.

	1		2		3		Mean (+SD)	Mean (+SD)
Bag	Log_{10} $TCID_{50}$/mL	Log_{10} reduction factor	Log_{10} $TCID_{50}$/mL	Log_{10} reduction factor	Log_{10} $TCID_{50}$/mL	Log_{10} reduction factor	log_{10} $TCID_{50}$/mL	log_{10} reduction factor
Virus suspension	8.29		8.42		8.44		8.33 ± 0.07	
After spiking (load)	7.29	0.00	6.50	0.00	7.29	0.00	7.03 ± 0.37	0.00 ± 0.00
0.05 J/cm^2	5.59	1.70	5.50	1.00	5.80	1.49	5.63 ± 0.13	1.4 ± 0.29
0.1 J/cm^2	4.80	2.49	4.71	1.79	4.67	2.62	4.73 ± 0.06	2.30 ± 0.37
0.15 J/cm^2	3.61	3.68	3.73	2.77	3.56	3.73	3.63 ± 0.07	3.39 ± 0.44
0.2 J/cm^2	2.96	4.33	2.42	4.08	2.51	4.78	2.63 ± 0.24	4.40 ± 0.29
Hold	7.00	0.29	6.71	−0.21	6.71	0.58	6.81 ± 0.14	0.22 ± 0.33

Note: The three replicates refer to three different bags of platelets.

TABLE 3 Reduction of West Nile Virus Strain NY99 (linage 1) titres after THERAFLEX UV-Platelets treatment assessed using $TCID_{50}$ titration and the large-volume plating method.

	1		2		3		Mean (+SD)	Mean (+SD)
Bag	Log_{10} $TCID_{50}$/mL	Log_{10} reduction factor	Log_{10} $TCID_{50}$/mL	Log_{10} reduction factor	Log_{10} $TCID_{50}$/mL	Log_{10} reduction factor	Log_{10} $TCID_{50}$/mL	Log_{10} reduction factor
Virus suspension	8.20		7.50		7.85		7.85 ± 0.29	
After spiking (load)	6.67	0.00	6.50	0.00	6.43	0.00	6.54 ± 0.10	0.00 ± 0.00
0.05 J/cm^2	5.50	1.17	5.41	1.09	5.34	1.09	5.42 ± 0.06	1.12 ± 0.04
0.1 J/cm^2	5.20	1.47	5.33	1.17	4.52	1.92	5.02 ± 0.36	1.52 ± 0.31
0.15 J/cm^2	4.20	2.47	4.20	2.30	3.26	3.18	3.88 ± 0.44	2.65 ± 0.38
0.2 J/cm^2	3.22	3.45	3.36	3.14	2.26	4.17	2.95 ± 0.49	3.59 ± 0.43
Hold	6.57	0.10	6.29	0.21	6.34	0.09	6.40 ± 0.12	0.13 ± 0.05

Note: The three replicates refer to three different bags of platelets. Results obtained via large-volume plating are marked in orange.

TABLE 4 Reduction of Usutu Virus strain HAN/SN/2018 titres after THERAFLEX UV-Platelets treatment assessed using $TCID_{50}$ titration.

	1		2		3		Mean (+SD)	Mean (+SD)
Bag	Log_{10} $TCID_{50}$/mL	Log_{10} reduction factor	Log_{10} $TCID_{50}$/mL	Log_{10} reduction factor	Log_{10} $TCID_{50}$/mL	Log_{10} reduction factor	log_{10} $TCID_{50}$/mL	Log_{10} reduction factor
Virus suspension	9.12		9.12		9.37		9.20 ± 0.10	
After spiking (load)	8.25	0.00	8.00	0.00	8.50	0.00	8.30 ± 0.20	0.00 ± 0.00
0.05 J/cm^2	6.75	1.50	6.88	1.12	7.12	1.38	6.90 ± 0.20	1.30 ± 0.20
0.1 J/cm^2	5.63	2.63	5.63	2.37	6.50	2.00	5.90 ± 0.40	2.30 ± 0.30
0.15 J/cm^2	4.75	3.50	4.12	3.88	5.25	3.25	4.70 ± 0.50	3.50 ± 0.30
0.2 J/cm^2	3.63	4.63	1.88	6.12	3.75	4.75	3.10 ± 0.90	5.20 ± 0.70
Hold	7.63	0.63	7.50	0.50	8.50	0.00	7.90 ± 0.40	0.40 ± 0.30

Note: The three replicates refer to three different bags of platelets.

associated WNV infections and 3 asymptomatic USUV cases were reported [24]. However, case numbers in the years 2020 and 2021 were influenced by the travel restrictions due to the SARS-CoV-2 pandemic and may rise now after travel restrictions have been lifted. Accordingly, from 2020, the Paul Ehrlich Institute, which is the national regulatory authority in Germany, has ordered testing for WNV RNA for blood donors who visited a WNV endemic area between 1 June and 30 November. Furthermore, WNV infections are subject to reporting following German regulations [36].

However, with the wider distribution of WNV and USUV in Germany and Europe, testing all blood donations with NAT methods will be costly. Thus, the demand for more cost-effective strategies to secure blood product safety is high. WNV transfusion transmission was first reported in 2002 during the WNV epidemic in the United States. Twenty-three patients had acquired WNV through transfused leukoreduced and non-leukoreduced red cells, PLTs or fresh frozen plasma [26]. These 23 TTI patients could be linked to 16 WNV-infected donors (confirmed retrospectively by NAT), of whom only 9 reported viral symptoms before or after donation. This underlines the importance of WNV detection or inactivation before donation, as almost half of the donors did not show any symptoms of an infection. Even with WNV testing implemented in blood donation services, nine infections occurred between 2003 and 2006 in South Dakota [28]. Furthermore, blood transfusion recipients are often immunocompromised, which makes them more prone to developing severe WNV infections. Indeed, of the 32 reported transfusion-associated WNV infections, more than half (19, 56%) developed WNND [26, 28].

Thus, an effective approach that ensures blood product safety against arboviruses is desirable. PI might be such a solution, as such systems are effective against a wide range of pathogens and may be economical [37]. The THERAFLEX UV-Platelets system by Macopharma has been shown to effectively inactivate a wide range of different pathogens including arboviruses such as DENV 1–4, chikungunya virus (CHIKV) and Ross river virus (RRV) [31]. We tested the THERAFLEX UV-Platelets system's capability to inactivate WNV and USUV.

The highest used UVC dose, which is also the standard dose, of 0.2 J/cm^2 resulted in a 4.40 ± 0.29 log reduction of WNV linage 2 stain (German isolate ED-I-33/18-UM, Table 2), a 3.59 ± 0.43 log reduction of a WNV linage 1 strain (NY99, Table 3) and a 5.20 ± 0.70 log reduction of the USUV strain HAN/SN/2018 titre (Table 4). This inactivation rate is similar to that found for other arboviruses such as DENV 1–4 (up to 4.43 log reduction), CHIKV (6.34 log reduction) and RRV (5.13 log reduction) [31]. Especially for WNV, the residual infectivity of the virus was close to or at the limit of quantification. Large-volume plating showed that WNV titres at the highest UVC dose were only 2.63 log_{10} $TCID_{50}$/mL. This remaining infecticity is due to the technical requirement to show a 4 log reduction of viral load for proper validation of an inactivation procedure, which made it necessary to use artificially high virus doses for initial spiking. Blood titres of 8 log_{10} TCID50/mL are not reached in humans naturally. The study reporting the first transfusion-associated WNV cases measured WNV titres in the donations ranging between 0.57 and 45.22 PFU/mL blood [26], while other studies reported titres between 0.06 and 0.6 PFU/mL blood [38–41]. Based on our inactivation efficiency of around 4 log_{10} at the highest UVC dose and the expected natural virus loads below 100 PFU/mL blood, it is rational to assume that there will be no residual infectivity in donor samples treated with the THERAFLEX UV-Platelets system.

We observed differences in log reduction following treatment between the two WNV strains and USUV (3.59 for NY99, 4.40 for ED-I-33/18-UM and 5.20 for USUV). However, this variation in log reduction after treatment is likely due to differences in input titres rather than to the biological features of the virus species and strains. The largest reduction was observed for USUV, which also had the highest input titre. Similarly, the smallest reduction was observed for WNV-NY99, which had the lowest input titre. If UVC illumination reduces the titers to comparable residual levels, different spiking levels could explain the observed variation in log reduction.

The influence of the THERAFLEX UV-Platelets system on the PLT characteristics was investigated in vitro as well as in vivo. UVC-treated PLTs are characterized by a slightly higher metabolic activity (glucose consumption and lactate accumulation) compared to

untreated controls. In addition, increased values for parameters such as CD62 expression, Annexin V binding and PAC-1 binding indicate a moderate activation of UVC-treated PLTs. [42–44]. However, preclinical and clinical studies showed good tolerability of repeated transfusions of PCs treated with the THERAFLEX UV-Platelets system [45, 46]. Recently, the efficacy and safety of UVC-treated, pathogen-reduced PLTs in patients were demonstrated in the multi-centre, randomized controlled CAPTURE Phase III trial [47].

The most prominent advantage of PI is its ability to inactivate many different pathogens. With its broad inactivation capacity, PI is especially useful as a proactive measure against unknown and known emerging pathogens threatening the blood supply. The log reduction observed for THERAFLEX UV-Platelets system in this study suggests that the UVC-based PI technology may effectively reduce the potential risk of TTI with the arboviruses WNV, USUV, DENV, RRV and CHIKV during PLT transfusion.

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U.G., T.J.S., A.S., S.B. and I.S. conceptualized the study; U.G., M. B., S.B. and I.S. contributed to the methodology; U.G., M.B., L.K. L.S., M.M. and I.S. to the investigation; U.G. and M.B. contributed to data curation; U.Z. and M.H.G. provided the resources; S.B. and I. S. supervised the study, helped acquire the funds and wrote the original draft. All authors contributed to reviewing and editing of the draft.

CONFLICT OF INTEREST STATEMENT

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DATA AVAILABILITY STATEMENT

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

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ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Characterization of transfusion-relevant bacteria reference strains in a lyophilized format

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Abstract

Background and Objectives: Blood safety measures used by blood establishments to increase blood component safety can be validated using Transfusion-Relevant Bacterial Reference Strains (TRBRS). Ultra-cold storage conditions and manual preparation of the current TRBRS may restrict their practical use. To address this issue, the ISBT Transfusion-Transmitted Infectious Diseases Working Party's Bacterial Subgroup organized an international study to validate TRBRS in a user-friendly, lyophilised format.

Materials and Methods: Two bacterial strains *Klebsiella pneumoniae* PEI-B-P-08 and *Staphylococcus aureus* PEI-B-P-63 were manufactured as lyophilised material. The lyophilised bacteria were distributed to 11 different labs worldwide to assess the robustness for enumeration, identification and determination of growth kinetics in platelet concentrates (PCs).

Results: Production of lyophilised TRBRS had no impact on the growth properties compared with the traditional format. The new format allows a direct low-quantity spiking of approximately 30 bacteria in PCs for transfusion-relevant experiments. In addition, the lyophilised bacteria exhibit long-term stability across a broad temperature range and can even be directly rehydrated in PCs without losing viability. Interlaboratory comparative study demonstrated the robustness of the new format as 100% of spiked PC exhibited growth.

Conclusion: Lyophilised TRBRS provide a user-friendly material for transfusion-related studies. TRBRS in the new format have improved features that may lead to a more frequent use in the quality control of transfusion-related safety measures in the future.

Keywords

bacteria, bacterial screening, blood safety, contamination, platelets, validation

Highlights

- Lyophilized transfusion-relevant bacterial reference strains offer a consistent low-count bacterial concentration important for transfusion safety studies.
- The new format is more stable at a wide temperature range, facilitating transport to and storage in blood establishments.
- The 100% growth success in all contaminated platelet concentrates demonstrates their reliable growth properties.

INTRODUCTION

Reference materials and standards have been used for a long time in the transfusion field, such as for blood group typing, antibody screening or viral testing [1–3]. The development of bacterial reference material became relevant when first test methods were routinely used for screening blood components for bacterial contamination. Due to several initiatives of members of the ISBT Working Party on Transfusion-Transmitted Infectious Diseases (TTID) subgroup on bacteria, transfusion-relevant bacteria reference strains (TRBRS) have been established for both platelets concentrates (PCs) and red blood cell components. Different bacterial isolates were evaluated about their dependable growth in blood components during the course of three international collaborative studies [4–6].

The currently available TRBRS are offered as frozen suspensions with a defined quantity of live cells. Typically, the strains have an approximate concentration of 1E6–1E7 Colony-Forming Units (CFU)/mL. To facilitate long-term storage, the cell suspension is mixed with a cryoprotectant agent and frozen at −80°C. In order to use the strains in a typical transfusion-relevant setup, a low-count inoculum is usually needed as the initial bacterial count of a contaminated blood component is generally considered to be below 100 bacteria per unit [7, 8]. In order to meet the low-count spiking requirement, a serial dilution of the bacteria stock is therefore required as a preliminary step. However, this step requires experience with handling bacteria, introduces dilution variability and, depending on local or national requirements and regulations, suitable equipment and facilities. Furthermore, the frozen bacteria have to be shipped at ultra-low temperature thereby excluding destinations for which the cold chain cannot be guaranteed by the shipper.

Consequently, the TTID subgroup bacteria decided to have TRBRS manufactured as BIOBALLs®, an alternative format that rectifies these deficiencies and provides a direct low-level CFU quantity and less demanding storage and shipping conditions. Most importantly, the growth properties in respective blood components must remain unchanged. For this purpose, two official reference strains *Klebsiella pneumoniae* PEI-B-P-08 and *Staphylococcus aureus* PEI-B-P-63 were chosen as model organisms for a first trial of a new TRBRS format. In this study, we present the characterization of TRBRS produced as lyophilised low-count material. The traditional and lyophilised formats were evaluated for growth in PC, long-term stability and accuracy of low CFU spikes. In the end, the new format was challenged in an international study to demonstrate robustness under various growth conditions.

MATERIALS AND METHODS

Collaborative international study

The study was coordinated by the Paul-Ehrlich-Institut (PEI) in collaboration with 11 centres in 8 different countries under the auspices of the ISBT TTID WP Bacterial Subgroup (Table S1). The lyophilised bacteria were sent to the study participants directly after production by

BTF (bioMérieux company, Sydney, Australia). In the course of the study, the participants provided the following information: (a) the number of viable cells of three lyophilised samples of each bacterial strain, (b) the number of CFUs over time for each inoculated PC, (c) identification of bacteria from each PC at the end of the study and (d) completed questionnaire with additional information including details of PC manufacturing and composition.

Study protocol

Each participating lab was required to determine the number of viable cells in the lyophilised material and spike PC to analyse the growth characteristics. To verify the low CFU count of the lyophilised TRBRS, three samples (stored between −18°C and −33°C) from each strain were re-hydrated individually with sterile saline and plated on agar plates. After incubation, colonies were counted the following day.

For growth analysis, the baseline sterility of each unit was tested according to the routine standard operating procedures used in each participating laboratory prior to PC spiking. Three lyophilised samples of each strain were rehydrated in 1 mL of sterile saline. PCs within the expiry period of the site were inoculated with 1 mL of the suspension. After spiking, the PC units were stored under local routine standard conditions. Aseptic sampling was performed on days 1, 2, 3, 4 and 7 from all PC units. A dilution series was performed from each sample and 100 µL of each dilution was plated in triplicate onto agar plates. The number of bacterial cells was determined by colony counting after an overnight incubation at 37°C. At day seven, species identity was confirmed by the standard identification methods routinely used in each participant laboratory. This included classical biochemical reactions (five labs), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (four labs), 16S rRNA gene sequencing (two labs) and fatty acid methyl ester analysis (1 lab).

In addition, a questionnaire on the production process and composition of the PC was sent to the participants to collect the following data: type of PC (apheresis/pool), bag system, additive solution/anticoagulant, total PC volume, platelet count and residual leukocytes per bag.

Platelet components

All sites used PC units in accordance with local practice. In total, four of the participants used apheresis and seven pool platelet units. Detailed information on the type and composition of the PC are listed in Table S2. Ethical Committee approval for the research use of clinical PC units was obtained at those centres where approval was required.

Bacterial strains

The present TRBRS panel is composed of 15 different species [4–6]. Within the scope of this feasibility study, 2 of the 15 strains were produced in a lyophilised format. *K. pneumoniae* PEI-B-P-08 and *S. aureus* PEI-B-P-63 were chosen as model organisms to cover different important bacterial characteristics. The Gram-negative *K. pneumoniae* strain exhibited the most rapid growth in PC stored under routine conditions in previous studies [5, 9], whereas the Gram-positive strain *S. aureus* is of special interest as isolates of this species frequently pose a challenge for culture-based screening methods [10–13].

The production of lyophilised bacteria was carried out by BTF (bioMérieux, Sydney, Australia). The In-house Isolate Service was used to produce SingleShot BIOBALLs® with a CFU specification of approximately 30 ± 3 CFU/BIOBALL® of *K. pneumoniae* PEI-B-P-08 and *S. aureus* PEI-B-P-63.

Accuracy of inoculum

In addition to the enumeration of the lyophilised samples performed by the study participants, an equivalent inoculum (30 CFU/mL) of the conventional TRBRS was prepared manually by five different operators. The relevant information required was taken from the TRBRS instruction leaflet [14] including the appropriate dilution scheme and the product insert providing the current live bacterial count of the two strains. Each operator prepared a low-count inoculum from three vials of *K. pneumoniae* and *S. aureus* respectively. The final inoculum was plated in triplicate on standard agar and the colonies were counted the following day after incubation at 37°C.

Long-term stability

To determine the impact of different storage temperatures on the viability of cells, aliquots of both formats were stored at −80°C, −20°C and, in the case of the lyophilised bacteria, +4°C and +20°C. The CFU of the lyophilised samples was determined after 1 and 2 years by rehydration with sterile saline and plating on agar plates, respectively. For the traditional format, the number of viable cells per sample was performed as previously described [6].

Growth comparison of traditional versus lyophilised format

To reveal a potential effect of the different manufacturing processes on the growth behaviour, PCs were spiked with both the traditional and the lyophilised TRBRS, respectively. TRBRS in the traditional format were serially diluted with sterile saline to create an identical low-count inoculum as the lyophilised format. For growth analysis, a two-arm pool-and-split study was conducted. Two ABO-matched platelets were pooled together and split into equal parts to produce two identical platelet concentrates (PCs). PCs were spiked with approximately 30 CFU per bag and stored under routine storage conditions. One millilitre of PCs was taken on days 1, 2, 3, 4 and 7. After serial dilution of the samples, 100 µL of each dilution was plated in

triplicates on agar plates. The seeded plates were incubated overnight at 37°C and the colonies were counted the following day.

A more detailed analysis of both TRBRS formats was performed with a focus on the different growth phases. For this purpose, the PC volume was reduced to allow the detection of bacterial growth with a low start inoculum of approximately 30 bacteria. One millilitre of PC was inoculated with 30 CFU of traditional and lyophilised TRBRS, respectively, and incubated at 22°C under agitation. For CFU determination, 2 × 50 µL samples were taken on a 2-h interval in case of *K. pneumoniae* starting at $t = 5$ h post-spiking. For *S. aureus*, sampling started 16 h post-spiking due to a prolonged lag-phase. Samples were automatically dispensed onto CASO plates (Roth, Karlsruhe, Germany) in a logarithmic dilution using spiral plater (Eddy Jet 2; IUL Instruments, Königswinter, Germany). Colonies were counted after overnight incubation at 37°C. The lower limit of quantification was 100 CFU/mL. The growth kinetic experiments were performed three times. An identical experimental setup was used to investigate the possibility of directly dissolving the lyophilised bacteria in PC serving as a rehydration fluid.

Statistical methods

Statistical analysis was performed on the data submitted by the participants transformed to $\log_{10}$ CFU/mL; zero CFU/mL was set to 1 before log transformation. Growth data were analysed per strain on each sampling day. For the long-term stability analysis, CFU values determined at time 0 were set to 100%.

RESULTS

Improved CFU accuracy

The strains *K. pneumoniae* PEI-B-P-08 and *S. aureus* PEI-B-P-63 were produced as lyophilised material with a target value of approximately 30 CFU per lyophilised ball. The bacterial concentration was verified by the participant laboratories reporting CFU values for both strains close to 30 CFU. The median value for *K. pneumoniae* was 29 CFU (CFU range, 15–36), whereas *S. aureus* showed slightly lower values with a median of 25 CFU (CFU range, 12–33) per lyophilised material (Figure 1). For precision comparison, an equivalent target inoculum of 30 CFU was exemplarily prepared by five operators using the original TRBRS material. Compared with the lyophilised format, the mean CFU value was similar, although the operator-dependent variation was higher. The median CFU value was 28 for both strains, and the range for *K. pneumoniae* was 13–51 CFU and 12–77 CFU for *S. aureus*.

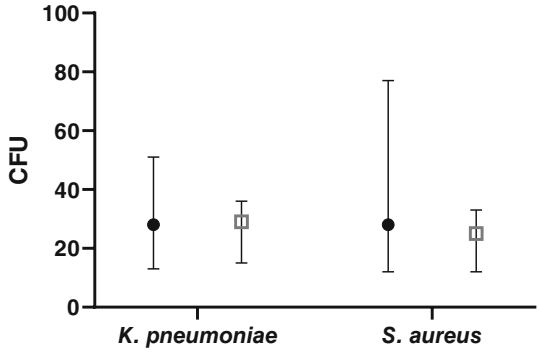

FIGURE 1 Inoculum precision. CFU (median and range; frozen $n = 43$, lyophilized $n = 33$) of both TRBRS formats (● = traditional; □ = lyophilised) was determined with a targeted inoculum of 30 CFU.

Improved stability

Shipment of the current TRBRS requires compliance with cold transport conditions of −80°C or below. Therefore, the temperature stability of both TRBRS formats was investigated to probably ease the transport conditions (Figure 2). Bacteria in both formats were highly stable at −80°C. Interestingly, freezing at −20°C was not sufficient to yield stable CFU results for the traditional format. *K. pneumoniae* cells were no longer cultivable after 1-year storage at −20°C. For *S. aureus*, less than 15% of cells were able to survive these warmer storage conditions. In contrast, the lyophilised bacteria were stable over a wide temperature range with more than 50% viable cells even after one-year storage at +4°C. However, long-term storage at temperatures above 4°C also resulted in a complete loss of viability. Only minor changes in stability were observed after a further year of storage. The number of viable *S. aureus* cells further decreased to approximately 6% for the traditional format at −20°C. For the new format, 50% of cells were still viable at +4°C. At colder temperatures, there was no change in the loss of quality compared with the 1-year storage results with a recovery of around 100%.

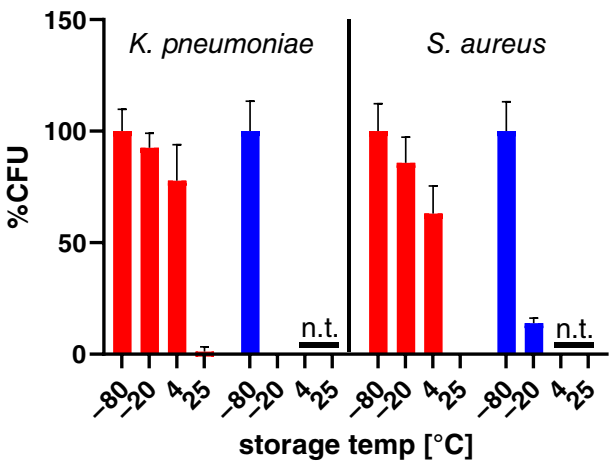

FIGURE 2 Long-term stability. TRBRS ($n = 3$) in both formats (lyophilized = red bars, frozen = blue bars) were stored at different temperatures for 1 year. The number of viable cells is normalized to the initial CFU values (n.t. = not tested).

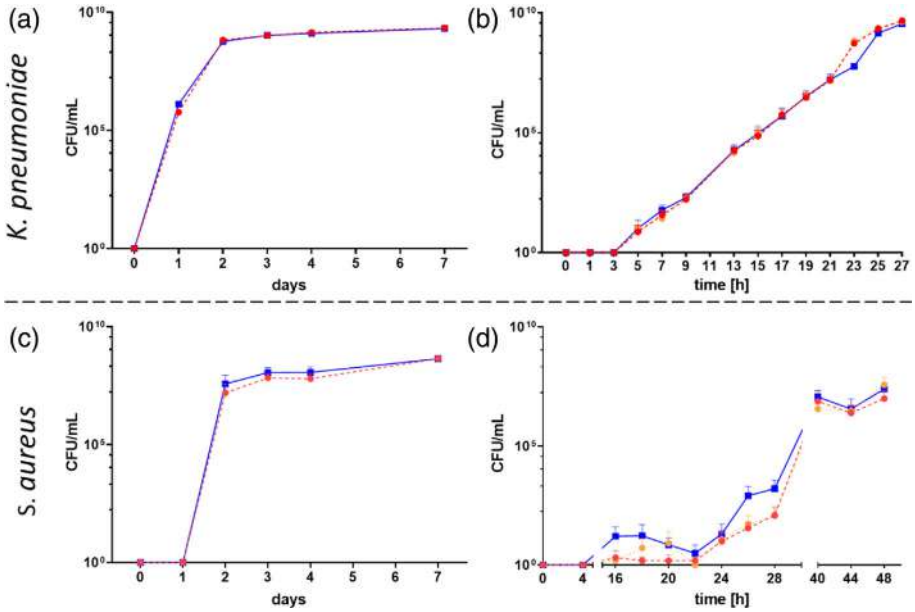

FIGURE 3 Growth comparison of traditional versus lyophilised format. Low-count inoculum of PC with traditional (blue square) or lyophilised (red dot) followed by CFU determination over time for 7 days (a, c). A detailed analysis of the lag phase (b, d) was performed within the first 27 and 48 h, respectively, including PC as rehydration fluid (orange dot).

Bacterial growth in PC

The production of the new format requires cultivation and passaging of the strain prior lyophilisation. The cultivation step can be critical as passage-dependent accumulation of mutations can occur [15]. Moreover, the freezing and freeze-drying step can be critical as it negatively affects both the viability and physiological state of the bacteria [16]. To investigate the potential impact of the manufacturing process on the growth characteristics, a comparative study with both classical and new formats was conducted (Figure 3). The analysis revealed no differences as the growth phenotype of both strains, independent of the format, was almost identical. Due to the fast replication of both strains between the sampling times, a proper analysis of potential differences in the lag-phase was impossible. Therefore, a more detailed growth analysis within the first 24 h for *K. pneumoniae* and 48 h for *S. aureus* was conducted. As shown in the previous growth experiments, *K. pneumoniae* reached the stationary phase at just over 24 h, regardless of the format (Figure 3a, b). For *S. aureus*, growth started with some delay due to a prolonged lag phase of about 28 h before entering the exponential phase (Figure 3c, d). The stationary phase was reached after approximately 40 h of growth. Interestingly, replacing saline with PC as rehydration fluid did not result in any different growth behaviour, indicating that TRBRS in the new format can be incubated directly in the PC matrix (Figure 3b, d).

To further challenge the new format in terms of robustness, an international study was organized. As each participating site used their own manufactured PC, different conditions were included such as apheresis and pool PC, different types of additive solutions and PC compositions (Table S2). A graphical analysis of the respective growth curves is summarized in Figure 4. *K. pneumoniae* exhibited a reproducible and homogenous growth behaviour independent of PC type reaching stationary phase after 2 days of incubation (Figure 4a). The average CFU on the first day post-inoculation was 3.0e5 CFU/mL indicating a rapid proliferation starting from an average initial bacterial load of 0.1 CFU/mL. In contrast, *S. aureus* showed a non-uniform growth behaviour based on different lengths of the lag-phase (Figure 4b). In 2 of 11 labs, no growth was detected in PC up to 48 h post-inoculation. One laboratory did not detect any viable cells in the first 4 days but reported more than 1E8 CFU/mL on day 7. The stationary phase was reached after 3–4 days with a maximum of 3E8 CFU/mL. The identification at the end of the growth revealed no deviation; each lab reported the correct species in all inoculated PC. In summary, both strains demonstrated 100% growth in a total of 33 spiked PCs.

DISCUSSION

Bacterial reference material should ideally meet defined criteria and be carefully selected based on specific requirements. With regard to transfusion safety-relevant purposes, the strains must be able to tolerate the challenging conditions prevalent in blood and its components, such as complement, phagocytes and nutrient or metabolite shortage [17–20]. Moreover, standards must meet low-count CFU criteria, be easy to use and have a good long-term stability.

Only some of the aforementioned requirements are fully met by TRBRS in its current frozen format. On the one hand, a highly concentrated stock solution must be prepared manually to achieve the low-count inoculum necessary for transfusion-related studies. On the other hand, transport and storage are restricted to ultra-low

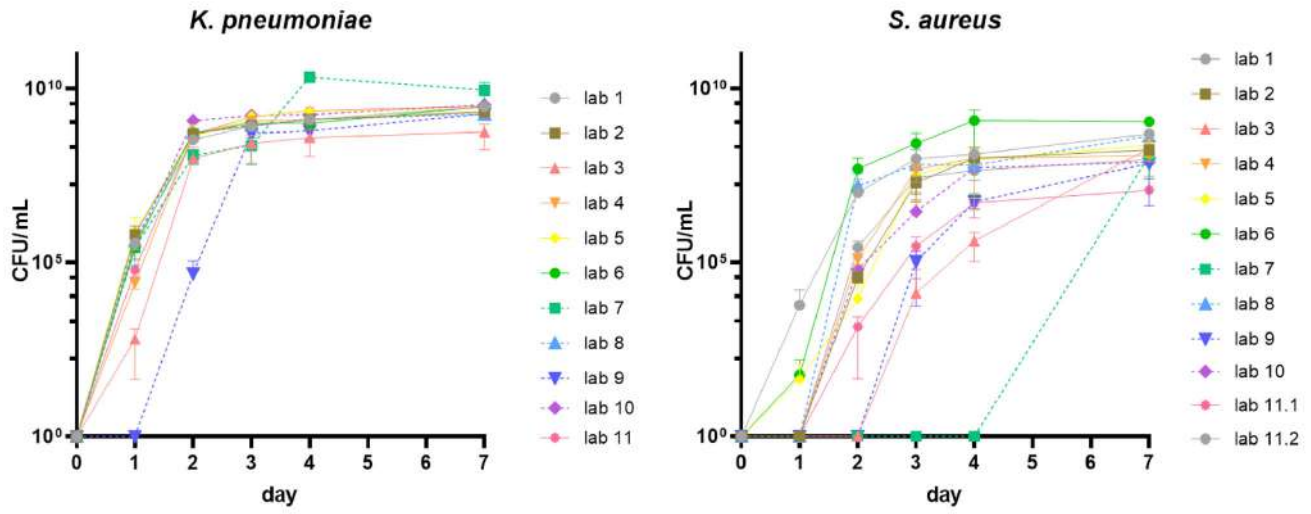

FIGURE 4 Growth curves of lyophilised *K. pneumoniae* and *S. aureus* in PC reported by each lab.

temperature conditions, which might limit its use due to difficulties in good distribution practices. The aim of the study was to develop the TRBRS into a more practical and user-friendly format. For this purpose, *K. pneumoniae* PEI-B-P-08 and *S. aureus* PEI-B-P-63 were manufactured as lyophilised material. Due to manufacturing procedure including several cultivation passages, a thorough and comprehensive quality control of the strains is required to ensure consistent growth properties.

Confirmation of the ability of the new strains to grow in PCs is the essential quality criterion. The possible effect of the preservation process on bacteria can be extremely variable and depends on both the species and the strain [21, 22]. Similarly, the processes of rewarming and rehydration can be critical and may lead to changes in the bacterial geno- and phenotype. Additionally, the growth passages that occur during cultivation prior to the freeze-drying step bear the risk of introducing random mutations into the genome [23]. Results from the study partners demonstrate a satisfactory growth behaviour in the inoculated PC. The physiological characteristics of the two bacterial strains, particularly serum resistance, are not adversely affected by the lyophilisation process. As a result, survival in PC is not different from that in the conventional format. Of note, based on our results, we cannot exclude the occurrence of genomic mutations that are irrelevant to PC survival and growth. Whole genome sequence alignment would allow a more comprehensive analysis. However, the analysis of single nucleotide substitutions in genomes based solely on NGS data can be bioinformatically challenging and may be difficult to interpret [24]. Therefore, in this study, we focused on characterizing the phenotypes of the two strains.

Another important quality criterion is the low-count quantity of the material. Due to the implementation of several mitigation strategies, the primary source of contaminated PC is presumably inadequate disinfection of the venipuncture site or donors with asymptomatic bacteraemia caused by small wounds or even teeth brushing [25]. The new format provides an inoculum that is well within the desired range enabling comparable starting conditions during spiking experiments. The deviation of the manually prepared inocula to achieve 30 CFU can be considered as non-critical, highlighting the robustness of the established format. It must be noted that most operators that took part were rather well-trained in handling bacteria. However, based on the results of previous international studies it is known that the desired target inocula can deviate strongly from the target value. For instance, the inocula of red blood cell reference strains were in a range of 1–215 CFU/unit [6]. Attaining an accurate initial inoculum is particularly important for culture-based screening approaches as initial bacterial concentrations post-blood collection are estimated to be below 1 CFU/mL PC and pose a sampling error challenge. The preparation of a low-count inoculum using the conventional TRBRS format is more error-prone depending on staff training and laboratory equipment. Therefore, the ready-to-use inoculum helps to meet this quantitative need.

Interestingly, inter-laboratory data for *S. aureus* demonstrate a very heterogeneous growth behaviour despite a defined and precise inoculum. The observed heterogeneity could not be related to either the platelet type or other PC-related parameters, such as the type of additive solution used, the bag material, or the amount of residual plasma and leukocytes. Another reason for this growth phenomenon could be the donor blood variance including nutrient composition, complement or species-specific antibodies. Particularly at low initial concentrations, bacteria might be hypersensitive to these conditions resulting in an erratic duration of the lag phase. Moreover, some factors might also contribute to an increased aggregation leading to an inhomogeneous distribution and consequent sampling error. Several reports describing misses or near-misses of *S. aureus*-contaminated PC by routine screening programs support this hypothesis [26–29]. Considering this inherent variability, it is even more crucial to attain the highest possible level of standardization for a valid assessment. In this context, it should be emphasized that both strains in the new format demonstrated 100% growth in each inoculated PC. This is in line with results from the traditional TRBRS in previous studies [4, 5] and again highlights its reliable and consistent performance.

Other key features of the newly produced TRBRS strains include long-term stability under different temperature conditions. Due to the less demanding storage requirements, the strains are easier to ship. With the traditional format, greater temperature fluctuations during transport would likely result in at least a partial loss of viability. Elevated temperatures for short periods should not result in a significant loss of CFU in lyophilised material.

In summary, the results of the study clearly show that the new TRBRS format offers a number of improved qualities. This may help to increase the frequency of its use in transfusion-related applications like method or assay validations, operator training or general performance evaluations in the future. A permanent availability of the two strains in the new format is not currently planned, nor is a general format change of all TRBRS. This will largely depend on future demand for the strains.

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S.R.-A. and C.P.M. conceived the study. M.P. designed the study protocol, and led the planning and execution of the study with international partners, collected and analysed the data. M.P. and O. K. wrote the manuscript which was reviewed by S.R.-A. and C.P.M. All co-authors performed testing enumeration, identification and growth measurement of the bacterial strains and contributed to the final approval of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

E-learning in transfusion medicine: An exploratory qualitative assessment

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International Society of Blood Transfusion

Abstract

Background and Objectives: E-learning programmes are increasingly offered in transfusion medicine (TM) education. The aim of this study was to explore facilitators and barriers to TM e-learning programmes, including assessment of learning outcomes and measures of effectiveness.

Materials and Methods: Participants selected from a prior survey and representing a diverse number of international e-learning programmes were invited to participate. A mixed methodology was employed, combining a survey and individual semi-structured one-on-one interviews. Interview data were analysed inductively to explore programme development, evaluation, and facilitators and barriers to implementation.

Results: Fourteen participants representing 13 institutions participated in the survey and 10 were interviewed. The e-learning programmes have been in use for a variable duration between 5 and 16 years. Funding sources varied, including government and institutional support. Learner assessment methods varied and encompassed multiple-choice-questions ($n = 12$), direct observation ($n = 4$) and competency assessment ($n = 4$). Most regional and national blood collection agencies rely on user feedback and short-term learning assessments to evaluate their programmes. Only one respondent indicated an attempt to correlate e-learning with clinical practices. Factors that facilitated programme implementation included support from management and external audits to ensure compliance with regulatory educational and training requirements. Barriers to programme implementation included the allocation of staff

time for in-house development, enforcing compliance, keeping educational content up-to-date and gaining access to outcome data for educational providers.

Conclusion: There is evidence of considerable diversity in the evaluation of e-learning programmes. Further work is needed to understand the ultimate impact of TM e-learning on transfusion practices and patient outcomes.

Keywords

E-learning, online education, transfusion

Highlights

- Facilitators for implementing e-learning programmes include funding allocation and leadership support, while barriers include staff time allocation, compliance enforcement and content updates.
- Evaluation of transfusion medicine e-learning ranges from learning assessment and user feedback/surveys to regulatory compliance assessment.
- Behavioural and outcomes-based assessments of e-learning programmes are limited.

INTRODUCTION

The adoption of e-learning has gained increasing attention in the field of transfusion medicine (TM) [1]. Our earlier international survey of 177 respondents revealed a widespread adoption of e-learning programmes in TM, with more than half being developed in-house, and targeting a range of audiences and topics [2]. A scoping review on e-learning in TM revealed limited published data on designs and behavioural impacts, although e-learning programmes often included learner knowledge assessment [3]. As part of an on-going programme of work in this area, we planned the present qualitative study to explore selected aspects of the development and design of existing TM e-learning programmes, with a focus on the facilitators and barriers to implementation, how assessment of learning is performed and how the effectiveness of the programme is evaluated.

We based our interview structure on the Kirkpatrick framework, which offers a structured four-level assessment for learning, each corresponding to different organizational aspects. Assessment refers to the assessment of the learner's knowledge, skills and abilities and can occur at multiple levels. Level 1, Reaction, measures learners' perceptions of training, regardless of its impact on knowledge or practice; Level 2, Learning, concentrates solely on knowledge acquisition; Level 3, Behaviour, scrutinizes changes in behaviour or practice resulting from learning and Level 4, Results, assesses if the training has achieved organizational goals and made a meaningful difference on the primary goals of the organization [4, 5], for example, a change in patient or patient blood management (PBM)-specific clinical outcomes due to the new learning.

MATERIALS AND METHODS

The study was conducted in two phases: a survey followed by qualitative interviews. Participants were selected from 177 respondents from a prior international survey among members of the International Society of Blood Transfusion to assess the utilization of e-learning in their institutions [2]. The participants were selected based on involvement in the development or implementation of e-learning programmes at their institutions, and who had indicated their willingness to be contacted for future research.

The survey and the interview questionnaire were designed by experts in TM education, e-learning and qualitative research. The initial survey was reviewed by the working group members for face and content validity to ensure it would capture the information needed. The survey was programmed electronically by a research team member (K.J). After multiple rounds of testing and editing, the electronic survey was piloted among the research team members ($n = 5$).

The survey comprised 44 questions that collected information on the participant's contact information, institutional details, description of the TM e-learning programme, topics and intended audience(s), development and implementation, and how learner assessment is performed (Supplementary Material A). The survey also aimed to gather information to guide the subsequent interview phase of the project.

The interviews were conducted from January to March 2023. The interview questions covered in-depth details of the e-learning programme, facilitators and barriers to implementation and how programme effectiveness is evaluated (Supplementary Material B). All interviews were recorded and detailed notes were compiled. We performed an expedited descriptive analysis, identifying commonalities and themes. We also included verbatim quotes from the recordings to provide supporting evidence.

This study obtained ethics approval from the Australian Red Cross Lifeblood Ethics Committee.

RESULTS

Nineteen participants were invited to participate in the survey and 16 participants agreed. Two responses were excluded due to lack of

sufficient data. Fourteen participants with complete responses represented 13 different institutions from 12 countries (Table 1). Nine institutions were university-affiliated (70%), 10 were involved in undergraduate education and 12 in postgraduate education and professional development (Supplementary Material C).

Ten survey participants from nine countries agreed to participate in the interview portion of the study. The results of the surveys and interviews are summarized herein.

Development of e-learning programmes

The majority of the programmes ($n = 10$, 77%) were developed in-house, in consultation with various stakeholders such as TM physicians, nurses and blood bank staff (Supplementary Material D). In many cases, these institutions contributed the TM content for the e-learning programmes but relied on a third-party technical provider to develop the digital learning experience.

The development of e-learning modules depended on the type and the function of the institution. For example, the development and delivery of TM e-learning was the impetus for the formation of the educational providers and is their sole function. Programmes at hospital-based institutions were often reported as resulting from the dedication of the institutions' TM specialists. Some specialists were tasked with creating e-learning materials as part of their regular duties, whereas in other cases, specialists personally initiated the development and implementation of TM e-learning.

Only one of the interviewees represented an institution that had embraced nationally accessible 'off-the-shelf' e-learning programmes. The blood administration and transfusion reactions training programme for nurses in one country was a national e-learning programme available to all hospitals.

> [It is] mandatory to study transfusion …in the nursing school. …[they are taught] blood component administration RBC (red blood cell) bags and after the session all participants …are evaluated by senior nurses every three months up to one and a half year.

E-learning topics and learners

The e-learning programmes were developed for different purposes and scopes including being a standard part of competency assessment ($n = 7$), professional development ($n = 6$) and certification ($n = 4$) (Supplementary Material E). The most common target learners are nurses/midwives and physicians. In one case, the training is mandatory for laboratory scientists every 3 years as part of their professional development. The programmes offered by educational providers and national blood services have a broad audience, reflecting the diverse roles these institutions play as blood collectors, education and training providers, and national TM authorities.

The e-learning programmes cover a variety of topics related to the operational needs of the institution, while educational providers and national blood centres appear to offer the most diverse set of topics (Table 1). Blood administration and transfusion reactions are by far the most common topics covered with every programme covering at least one of these topics and often both. Nine programmes include interactive features such as case studies ($n = 7$), interactive animation/video ($n = 5$) and problem-based learning ($n = 5$) (Supplementary Material F).

Interviewees mentioned that topics are often tailored to the specific roles of the learners. For example, one e-learning programme offers more comprehensive training to medical professionals, limited and role-specific training to phlebotomists and specimen collectors, and a short blood administration module for porters and couriers. Similarly, a programme used in a regional blood service/centre offers e-learning education on transfusion reactions and blood administration to nursing students, nurses and physicians, but exclusively offers education in PBM and haemovigilance to physicians.

There are also programmes that are available to learners from outside the institutions. Many institutions surveyed offer their e-learning materials to other institutions as part of their mandate as education providers. The programme developed in one hospital has been adopted by other hospitals that do not have resources to develop their own programmes. While national blood services/centres provide role-specific training to their internal staff, they also extend training to external healthcare providers.

Cost to the user

None of these programmes imposed any cost on the participants. Among the hospital institutions represented in the interviews, training is free to users and the institutions absorb the cost of time and wages lost when participants are temporarily unavailable for their regular duties. Notably, some larger hospitals provide their educational resources to other institutions at no cost, aiming to enhance TM training.

Funding of e-learning education

Funding sources for the development of the e-learning programmes varied, including institutional and governmental or provincial funds (Supplementary Material D). Interviewees generally observed that obtaining the required funding was not a significant obstacle for the development or maintenance of e-learning programmes.

> We have the budget or we are able to have the budget for it.

Interviewees based at hospitals discussed how e-learning programmes are supported by administrators in order to demonstrate

TABLE 1 Overview of e-learning programmes ($n = 13$).

Country	Institution type	Source	Topics covered						Learner assessment	Programme evaluation[a]
			Blood group systems	Blood administration	National Guidelines	Transfusion reactions	Apheresis	PBM		
Australia	Education Provider	In-house		✓	✓	✓		✓	MCQ	Level 1 Level 2 Level 3[b]
South Africa	Education Provider	In-house with external technical development				✓		✓	MCQ Open-ended questions Competency assessment	NA
Canada	Education Provider	In-house	✓	✓	✓	✓		✓	MCQ Competency assessment Practical exam	Level 1 Level 2[c] Level 3[b]
France	National Blood Service/ Centre	In-house with external technical development		✓	✓	✓			MCQ Competency assessment	Level 1 Level 2
England	National Blood Service/ Centre	In-house with external technical development	✓	✓	✓	✓		✓	MCQ Scenario-based assessment	Level 1 Level 2
Denmark[d]	Regional Blood Service/ Centre	In-house		✓	✓	✓		✓	MCQ	Level 1[c] Level 3[b]
India	Regional Blood Service/ Centre	A third-party developer	✓	✓	✓	✓	✓	✓	Nil	Level 1 Level 2
Pakistan	Regional Blood Service/ Centre	In-house	✓	✓	✓		✓		Open-ended questions Direct observation	NA
Norway	Hospital-based Blood Services	In-house with external technical development	✓	✓		✓			MCQ Direct observation	Level 1 Level 2[b] Level 3[b] Level 4 Other
Netherlands	Hospital-based Blood Transfusion Service	In-house	✓						MCQ Competency assessment	Level 1
Canada	Hospital-based Blood Transfusion Service	Externally by the Provincial Health Authority	✓	✓	✓	✓			Direct observation	Level 3[b] Other

(Continues)

TABLE 1 (Continued)

Country	Institution type	Source	Topics covered						Learner assessment	Programme evaluation[a]
			Blood group systems	Blood administration	National Guidelines	Transfusion reactions	Apheresis	PBM		
Japan	Hospital-based Blood Transfusion Service	Externally and used nationally		✓		✓			Direct observation	Other
USA	Hospital-based Blood Transfusion Service	In-house with external technical development		✓		✓		✓	Nil	Other

Abbreviations: MCQ, multiple choice question; N/A, not available; Other, compliance with regulatory obligations; PBM, patient blood management.
[a]Based on Kirkpatrick evaluation.
[b]Audit(s) conducted by an external agency.
[c]A discontinued type of evaluation.
[d]Physicians cover all topics while nurses only cover administration and reactions.

compliance with training regulations, and how funds for development and implementation were simply made available due to necessity.

> It probably costs something but I think the hospital wants to have this so I don't think it's a problem, if we talk about money.

> I think the funding and resources are pretty much okay for our institute… … it's an institute of national importance… But yeah, we are given priority as compared to the other institutes…

Delivery of e-learning education

The survey revealed that by far the most common format for e-learning programmes among the examined institutions was online, self-directed lessons. Only one programme used videos instead. The user experience varied among the programmes, including click-through presentations, a recorded lecture or podcast, or an interactive module with mixed media.

Notably hospital-based transfusion services often integrate the TM module into their existing e-learning infrastructure. Interviewees from these institutions explained that their organizations already had e-learning systems in place for various topics like fire safety training. This approach had clear advantages, such as reducing costs and implementation time. However, it also came with a drawback of the technological limitations of the existing systems. For instance, one centre's TM e-learning module could not track user data, including who had completed the modules, because this functionality was not originally built into the underlying system.

The duration of each institution's programme and modules significantly varies based on the type of institution and its specific requirements. Interviewees discussed that hospitals typically opted for relatively short and focused e-learning modules.

Assessment of learning and evaluation of effectiveness

Learning assessment was performed by multiple-choice questions (MCQs) ($n = 8$) open-ended questions ($n = 2$), direct observations ($n = 4$) and competency assessments to assess employee knowledge and areas of improvement ($n = 4$). Assessment records differ significantly among institutions, particularly between those whose core function is to provide education and those whose primary function is healthcare provision. National blood services and educational providers have the most comprehensive assessment records, including pass/fail grades and certificates of completion. In contrast, the hospital institutions surveyed typically rely on a single method of recording learner assessments, such as pass/fail grades, CME/CPD points or certificates of completion. One hospital does not appear to record assessments beyond fulfilling its compliance obligations.

Level 1 and level 2 evaluations

Six institutions (including two educational providers, two national blood services/centres, one regional blood service/centre and one hospital) conducted Kirkpatrick level 1 and level 2 programme evaluations (Table 1).

For Kirkpatrick level 1 evaluation, all six institutions use post-module surveys to assess user feedback and satisfaction. While the response rate is generally low, a substantial amount of feedback has been received. Such surveys were discontinued at two centres due to low response rate. As for knowledge gain by learners (Kirkpatrick Level 2), all institutions, except the hospital in Norway, include learning evaluation/quizzes in their modules and require learners to achieve a passing grade. It is important to note that users can retake these quizzes as many times as needed. One educational provider initially used pre- and post-testing to ensure the programme was resulting in improved learning outcomes, as the pre-test data revealed there was a clear need for the training among transfusion practitioners in the region, while the post-test data demonstrated knowledge improvement. However, this testing has been discontinued, due to concerns about time and usability. The revised programme will include MCQs as learning checks after each section. This programme was developed for all facilities in the province as a professional practice competency requirement (physicians, nurses, midwives, trainees and others), with input provided by the provincial TM service.

Level 3 and level 4 evaluations

Only one of the respondents indicated an audit linked to e-learning that may have demonstrated improvement in the hospital's transfusion practices. This hospital implemented its e-learning programme in response to a regulatory compliance audit by the Health Ministry in 2014. These audits involved staff interviews to evaluate whether they had been trained and if the education provided was of an adequate quality. The audit results pointed out areas where the hospital could improve in demonstrating that their staffs were well-prepared in TM practices. Subsequent external audits in 2019 and 2021 demonstrated improved staff recollection of TM practices (Level 2) and positive changes in TM practices due to the training (Level 3). The hospital also conducts internal audits of blood product utilization and demonstrated improved blood administration practices after introducing the e-learning programme (Level 4). These improvements, along with high training completion rates, were seen as indicators of the e-learning programme's effectiveness.

One of the education providers also employs surveys to measure perceived learning and assess whether users believe they have acquired new knowledge, which will lead to behavioural changes (Level 3). It also has attempted a Level 4 evaluation but faced challenges in obtaining the required governmental and organizational support, as this type of evaluation would need data from institutions using its modules. Instead, this education provider has passed external audits by regulatory bodies assessing whether the programme delivers high-quality TM education (a valid Level 4 results-based evaluation for an education provider), but it is worth noting that the scope of these audits was limited.

Similarly, another educational provider is interested in evaluating its programme's effectiveness in changing practices and improving patient outcomes at users' hospitals. However, it has limited visibility into patient outcomes, despite conducting audits on product usage and relevant indicators at some hospitals. These audits are slow, resource-intensive, and reportedly of limited value by the time the results are available. Additionally, the programme underwent review by independent external researchers who used the programme at their institution and demonstrated a modest positive effect on product usage and patient outcomes (Level 4).

Staff at one hospital expressed a desire for a higher-level evaluation of their e-learning programme. However, due to the COVID-19 pandemic, it has proven impossible to isolate the effects of the TM e-learning programme on blood administration practices.

> What we can really see now, last year and this year, is our blood—how much blood we are using—is going down all the time. But we thought it was a bit different because 2020 came—COVID-19. Naturally it gave a decrease in the spending of blood at the departments, but it has not gone up after the COVID-19 pandemic is over. Actually, we are seeing it's decreasing all the time so fast that we cannot hardly handle it in our blood banks… We have just this year minimised our—how many units we have in common, in-house. But I am not sure it is due to our e-learning program. I do not know.

> We had made an audit at the small hospital … and it actually revealed some points where they could definitely have got a lot of help… if they just had taken the e-learning program…

Other challenges at the hospital level were also described. One hospital-based blood transfusion service is facing limitations in conducting a patient outcomes-based evaluation of its e-learning program due to the implementation of a computer administration system. This implementation has temporarily affected data quality, making it challenging to undertake evaluations.

Facilitators for development and implementation

The implementation of TM e-learning was relatively easy in larger institutions such as national blood services/centres and educational providers. The interviewees from these institutions mentioned the feasibility of in-house development thanks to their access to qualified TM experts capable of providing high-quality educational content, whether to an in-house development team or to an external developer.

All hospital institutions highlighted that regulatory compliance was a core reason for implementing e-learning programmes and the interviewees from these institutions indicated being adherent to these (Table 1). Often, governments or regulating bodies mandate TM education for healthcare institutions under their jurisdictions, and these hospital institutions must provide adequate training to their staff and be able to demonstrate that adequate training has been provided.

> The audits from the health authorities helped and I also think this competence portal where you can put all that you need to go through to be certified and all the things you should do has made it much easier for the leaders to follow up and it's much easier to see when it's not followed up…

> We're only, I think, pulling [use data] to demonstrate compliance with regulations for PBM recertification.

Barriers to development and implementation

The primary challenge during the development phase usually revolved around time management, securing staff time for the development process and the maintenance of up-to-date educational content. For instance, one programme faced a significant barrier due to the need to switch digital platforms and revise and update e-learning courses.

For hospital-based institutions, institutional inertia also emerged as the most common obstacle to implementing a TM e-learning programme. A noticeable disconnect seems to exist between governing/regulatory bodies, executive management, lower-level management and the institution's TM practitioners. When a governing body mandates TM education after, for example, an unfavourable audit, upper management supports the development of TM e-learning by the institution's TM experts. However, lower-level managers, responsible for departments or clinical teams, are tasked with implementing the training but face competing demands for their and their physicians' and nurses' limited time.

Interviewees discussed how successful implementation of TM e-learning in a hospital-based institution was dependent on having upper management vocally support the new initiative while also supporting structural and procedural changes to allow space for the new training to occur. Institutional leaders that understand the nature of the problem that the new programme can help to solve—and who, crucially, can exert influence elsewhere in the organization to promote its implementation and uptake are the ideal start for any new initiative.

> The first years we had this e-learning program it was a challenge—the program was there but it was, I think, the leaders of the clinical departments, they were responsible for the education of their personnel but …. they were not very much aware the transfusion and e-learning programs so not very many had gone through this program [2] the first years and that I think it had to do with the consciousness of the leaders at the top of the system.

DISCUSSION

This study provides an exploratory evaluation of different elements of the development and use of TM e-learning in professional education and development. Interviews revealed facilitators for implementation like funding, upper management support and stakeholder management to ensure compliance remains a priority, especially within hospital settings. However, barriers and challenges remained, including in assessing the impact of learning on patient outcomes and organization objectives, due to assessment limitations and inability to access data on behavioural and institutional outcomes. Funding sources for the development of the e-learning programmes varied by institution type. Hospitals typically cover expenses from their budget for in-house initiatives, I and for other larger institutions funding is often allocated by governments for the organization to develop and provide training to smaller institutions or individuals. Institutions prioritize education differently, often providing free TM education to learners to cultivate a trained workforce. Most educational institutions and national providers conducted user feedback and knowledge assessments by learners.

Although only a small sample was interviewed, the content of e-learning programmes varied based on learner type and the learning objectives, with variable interactivity levels, potentially reflecting the differences in available resources for their development. Simple learning materials such as PowerPoint presentations and videos offer passive reading and can be quickly developed. However, interactive web-based training requires web developers and media editors [6]. Interactive, individualized and contextual learning, and integrating theory into practice are key enablers affecting e-learning in health science education [1].

A key finding was very limited data on whether e-learning had effects on transfusion practices (Level 3) or patient outcomes (Level 4). This finding is consistent with our scoping review, which identified very few publications describing the impact of e-learning on patient or PBM-specific outcomes [3, 7–10]. Examples of such evaluations of the impact of e-learning interventions on behaviour and patient outcomes include improvement in perinatal outcomes and maternal intensive care unit admissions related to postpartum haemorrhage [7], improvement in blood component ordering, usage and traceability [8] and institutional rates of red blood cell transfusions [9].

Hospital-based institutions primarily focus on regulatory compliance for mandatory training and use surrogate evaluations through performance in audits by external agencies, which does not translate necessarily into better 'down-stream' practices. Even if these audits suggest improved patient outcomes, they cannot definitively attribute these improvements solely to training. Individual and contextual factors such as organizational learning culture, workplace support, staffing and individual's motivation may also play a role [11, 12].

Studies specifically for transfusion have demonstrated that there are many factors in place that influence transfusion behaviour beyond knowledge, which include beliefs about capabilities (confidence in not transfusing a stable patient), beliefs about consequences (reducing infections, saving resources), social influences (from team members or patient family members), motivation and goals (opposing beliefs of the importance of restrictive transfusion and other goals) [13, 14].

In our study, while programme coordinators and institutional administrators expressed the desire to conduct higher Kirkpatrick level evaluations, significant barriers exist for educational providers such as limited access to patient and organizational outcomes from institutions that implemented their programmes, which may not be readily available or routinely collected. Performing these evaluations would require substantial collaboration, additional workload, and changes in data collection practices, which may not be feasible or seen as a top priority for many hospitals participating in these e-learning programmes.

This study offers a description of e-learning programmes in TM, building on our earlier international survey [2] and review [3]. However, it has limitations. The findings are based on data from a small sample of institutions who agreed to participate and cannot be broadly generalized to all institutions running e-learning programmes. Participants' ability to report on certain aspects may have been limited by their roles, tenure, and expertise. The information provided relied on participants' recollection and understanding. For instance, some interviewees lacked insight into programme implementation and integration with other learning forms, particularly when training materials were provided to external organizations. Finally, estimating programme duration was challenging during the interviews, since most interviewees had not personally undergone the training from the perspective of a learner.

In conclusion, our interview data extend the findings of our survey and review, identifying considerable variability in the development, content and evaluation of e-learning programmes. Further research is needed to assess the impact of TM e-learning on transfusion practices and organizational outcomes, including Kirkpatrick Level 3 and 4 assessments, as well as cost-effectiveness compared with other learning methods. This is especially the case with the lack of evidence to assert that e-learning is more cost-effective than face-to-face instruction in health professions education [15]. Moreover, e-learning is a time-, cost- and labour-intensive approach to education [11], and this can limit its application in less resourced countries [16]. Exploring new evaluation methods for learner behaviour and patient outcomes is also needed.

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A.Z.R. initiated the project idea, prepared the list of the participants, reviewed the data, and drafted the manuscript. K.J. wrote the research protocol, performed the interviews, designed the electronic survey, analysed its results and drafted the report of the findings. A.Z.R., C.S.O., B.S. and Y.L. reviewed the research protocol and finalized the interview questions. S.D. participated in drafting the introduction section of the manuscript. All authors participated in drafting the questions for the survey & interviews, and reviewed the manuscript.

CONFLICT OF INTEREST STATEMENT

Yulia Lin has research funding from Canadian Blood Services and Octapharma. She serves as a consultant for Choosing Wisely Canada. The rest of authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Is it time for the death knell of single-unit plasma?

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Abstract

Background and Objectives: A plasma transfusion dose should be weight-based (10–20 mL/kg), which equates to three to four units in an average-sized adult; therefore, the transfusion of single units under most circumstances is sub-therapeutic.

Materials and Methods: This retrospective observational study examined the prevalence of single-unit plasma transfusion in adults within a 12-hospital system from 1 January 2018, to 31 December 2019.

Results: During the study period, 5791 patients received plasma transfusions. The overall prevalence of single-unit plasma was 17.1% for 988 patients. The majority, 3047 (52.6%), occurred at one hospital, 2132 (36.9%) among five hospitals and 612 (10.7%) at the remaining six hospitals. Cardiac and gastrointestinal (GI)/transplant transfused 2707 (46.8%), combined respiratory, neurological, orthopaedic and congenital/dermatology/other comprised 2133 (36.9%) of the six hospitals that transfused less than 200 patients, four (66.7%) transfused single units above the overall prevalence.

Conclusion: In this hospital system, more than one in six patients received a transfusion of a single plasma unit. Six of the 12 hospitals had 89.5% of the patients who were transfused plasma. Six service lines transfused 83.7% of all patients receiving plasma. Hospitals that infrequently transfused plasma were more likely to under-dose.

Keywords

blood components, blood safety, patient blood management, transfusion strategy

Highlights

- A plasma transfusion dose should be weight-based (10–20 mL/kg), which equates to three to four units in an average-sized adult; therefore, the transfusion of single units under most circumstances is sub-therapeutic.
- This article will serve to educate medical students, advanced practice providers, physicians in training and those in practice.
- At this hospital system, more than one in six patients received a transfusion of a single unit of plasma. Hospitals that infrequently transfused plasma were more likely to under-dose.

INTRODUCTION

Patient blood management (PBM) programmes have increased the appropriateness of transfusion therapy of red blood cells (RBCs) and platelets. Multiple-unit transfusions given to haemodynamically stable, hospitalized adult patients have now been reduced, in many instances, to single-unit administration, with a post-transfusion laboratory check to determine whether additional units are needed.

An unintended consequence, either through lack of knowledge and education or due to misinterpretation of the single-unit RBC and platelet transfusion philosophy, is the regular practice of single-unit plasma transfusions. An appropriate plasma dose is 10–20 mL/kg, which equates to three to four units in an average-sized adult [1–3]. Doses below this threshold, except in specific clinical situations such as to treat hereditary angioedema and neonates, are considered sub-therapeutic. Supporting proper dosing of plasma, the magnitude of the correction of the international normalized ratio (INR) has been shown to increase as the plasma dose changed from three to four units ($p < 0.001$) [2].

As reports from three hospitals showed that at least one third of plasma transfusions were single units, it was decided to perform a study to examine the prevalence of this practice within the entire hospital system. This study has three aims. The first is to determine the prevalence of single-unit plasma dosing in adults at each hospital and then to compare the prevalence among all hospitals. The second is to determine the outcomes of patients who received single units compared with those who received the appropriate dose. The third aim is to identify the proportion of patients receiving sub-therapeutic dosing of plasma. It was also hypothesized that hospitals infrequently transfusing plasma would be more likely to under-dose.

MATERIALS AND METHODS

A retrospective observational study was conducted after two institutional review board (IRB) approvals (one in Ohio and one in Florida). The IRBs granted an exemption, as the information about biospecimens is recorded by the investigator in such a manner that the identity of the human subjects cannot readily be ascertained directly or through identifiers linked to the subjects, the investigator does not contact the subjects, and the investigator will not re-identify subjects.

Inclusion criteria were all patients ≥18 years old who received a plasma transfusion during an inpatient hospitalization at any of the 12 hospitals from 1 January 2018, to 31 December 2019. The hospitals ranged in size from 24 to 1299 beds, and were located in Ohio and Florida in the United States. All but one had an emergency department, and four were trauma centres. Data were sourced through EPIC (Epic Systems, Verona, WI), SunQuest (Tucson, AZ) and QlikSense® (King of Prussia, PA). The dataset contained gender, ethnicity, age, primary diagnosis, weight, length of stay (LOS), total number of plasma units transfused, highest INR, final INR, discharge status and hospital. A primary diagnosis of hereditary angioedema, lack of available data or transfusions not performed in the inpatient setting was excluded from the data set. Weight was obtained upon admission. Appropriate dosing was calculated on the number of 250 mL units (standard unit volume) needed to achieve what was defined in this study as a minimum therapeutic dose of 10 mL/kg. A subgroup of 100 patients had their charts reviewed for the clinical reasoning for their plasma orders.

Data were organized by regrouping discharge status and primary diagnosis. Discharge status was coded as expired, home, hospice, rehab/skilled nursing or transfer. The diagnosis was coded as autoimmune/infection, cancer/haematology and oncology, cardiac, congenital/dermatology/other, endocrine/metabolic, gastrointestinal (GI)/transplant, gynaecology and genitourinary/renal, neurology/psychology, orthopaedics or respiratory. Sub-therapeutic treatments were marked where the number of total plasma units was less than the minimum dose weight. The data were excluded if total plasma units or minimum dose weight were missing. INR difference was calculated as the highest INR minus the last INR. The values with a > for the highest or last INR were used as the number for difference calculations (i.e., a value of '>16.5' for the highest INR was treated as '16.5' to calculate 'INR difference').

Statistical analysis

Categorical variables were described using frequencies and percentages, and comparisons used Pearson's Chi-square tests. Continuous variables were described using means, standard deviations or medians with quartiles or minimum and maximum values. Analyses comparing groups on LOS used Wilcoxon rank-sum tests. Pairwise comparisons were run using a Bonferroni adjustment when comparisons involving more than two groups were significant. Analyses were performed using SAS® Software (version 9.4; Cary, NC).

RESULTS

There were 5813 total patients. However, 22 records that did not have a response for the highest INR were removed, leaving 5791 records in the analysis cohort. There were 90 'unavailable' responses coded as missing values for ethnicity. A total of 988 transfused patients (17.1%) received a single unit. Cardiac and GI/transplant transfused 2707 patients (46.8%), and combined respiratory, neurological, orthopaedic and congenital/dermatology/other comprised 2133 (36.9%). The median (minimum, maximum) total number of transfused plasma units was 2.0 (1.0, 283.0). Per weight, the median (minimum, maximum) units that should have been transfused was 3.3 (0.98, 10.9), and by the calculated actual dose, the median (minimum, maximum) units that should have been transfused was 7.4 (1.08, 1013.1) (Table 1).

An overall summary of the data demonstrates that 3047 patients (52.6%) received plasma transfusions at one hospital (Main), 2132 patients (36.9%) among five hospitals (Weston, Tradition, North, Hillcrest, Fairview) and 612 patients (10.7%) at the remaining six hospitals (Southpoint, South, Medina, Marymount, Euclid and Avon). The comparison of single doses by location among the 12 hospitals was significant ($p < 0.001$). Of the six hospitals that transfused less than 200 patients, four (66.7%) transfused single units above the overall prevalence of 17.1%. One hospital administered 64 patients in single units; 41.3% of all plasma was transfused to adults, well above the other hospitals. Interestingly, six hospitals transfused a single dose to more than 20% of patients (Table 2).

TABLE 1 Plasma units transfused at each hospital.

	Total patients ($N = 5791$)	
Factor	***N***	**Statistics number (percent)**
Hospital name, *n* (%)	5791	
Avon, OH		102 (1.8)
Main, OH		3047 (52.6)
Euclid, OH		71 (1.2)
Fairview, OH		388 (6.7)
Hillcrest, OH		452 (7.8)
Marymount, OH		86 (1.5)
Medina, OH		61 (1.1)
North Hospital, FL		481 (8.3)
South Hospital, FL		137 (2.4)
South Pointe, OH		155 (2.7)
Tradition Hospital, FL		281 (4.9)
Weston, FL		530 (9.2)
Age (years) discharge, mean ± SD	5791	65.4 ± 15.2
Classification (modified), *n* (%)	5789	
Autoimmune/infection		242 (4.2)
Cancer/haematology and oncology		290 (5.0)
Cardiac		1603 (27.7)
Congenital/dermatology/other		469 (8.1)
Endocrine/metabolic		157 (2.7)
Gastrointestinal/transplant		1104 (19.1)
Gynaecology and genitourinary/renal		260 (4.5)
Neurology/psychology		623 (10.8)
Orthopaedics		566 (9.8)
Respiratory		475 (8.2)
LOS (days), mean ± SD	5774	16.7 ± 20.2
Weight kg, median (min, max)	5669	83.1 (24.4, 272.3)
Total plasma units, median (min, max)	5791	2.0 (1.00, 283.0)
Calculated actual dose, median (min, max)	5669	7.4 (1.08, 1013.1)
Minimum dose per weight, median (min, max)	5669	3.3 (0.98, 10.9)
Discharge status (modified), *n* (%)	5791	
Expired		704 (12.2)
Home		3011 (52.0)
Hospice		279 (4.8)
Rehab/skilled nursing		1348 (23.3)
Transfer		449 (7.8)
Single dose, *n* (%)	5791	988 (17.1)
Sub-therapeutic treatment, *n* (%)	5669	3625 (63.9)
Expired, *n* (%)	5791	704 (12.2)
Highest INR, *n* (%)	5791	
1.8+		3662 (63.2)
<1.8		2129 (36.8)
INR difference, median (min, max)	5791	0.50 (0.00, 17.1)

Note: Statistics are presented as mean ± standard deviation (SD), median (min, max), *N* (column %).
Abbreviations: FL, Florida; INR, international normalized ratio; kg, kilograms; LOS, length of stay; OH, Ohio.

TABLE 2 Comparison of single dose by location (overall $p < 0.001$[a]).

Hospital location	No single dose number, (percent)	Single dose number, (percent)
Overall ($N = 5791$)	4803 (82.9)	988 (17.1)
Avon ($N = 102$)	80 (78.4)	22 (21.6)
Main ($N = 3047$)	2607 (85.6)[b,c,d,e]	440 (14.4)
Euclid ($N = 71$)	61 (85.9)[d]	10 (14.1)
Fairview ($N = 388$)	304 (78.4)[d,f,g]	84 (21.6)
Hillcrest ($N = 452$)	384 (85.0)[d]	68 (15.0)
Marymount ($N = 86$)	65 (75.6)	21 (24.4)
Medina ($N = 61$)	51 (83.6)[d]	10 (16.4)
North Hospital ($N = 481$)	386 (80.2)[d]	95 (19.8)
South Hospital ($N = 137$)	101 (73.7)[f,g]	36 (26.3)
South Pointe ($N = 155$)	97 (58.7)[b,e,f,g,h,i,j,k]	64 (41.3)
Tradition Hospital ($N = 281$)	213 (75.8)[d,f,g]	68 (24.2)
Weston ($N = 530$)	460 (86.8)[b,c,d,e]	70 (13.2)

Note: Statistics are presented as *N* (column %). Post-hoc pairwise comparisons were done using Bonferroni adjustment.
[a]Pearson's chi-square test (*p*-Values).
[b]Significantly different from Fairview.
[c]Significantly different from South Hospital.
[d]Significantly different from South Pointe.
[e]Significantly different from Tradition Hospital.
[f]Significantly different from Cleveland Clinic.
[g]Significantly different from Weston.
[h]Significantly different from Euclid.
[i]Significantly different from Hillcrest.
[j]Significantly different from Medina.
[k]Significantly different from North Hospital.

Table 3 shows a statistically significant difference of single dose versus multiple doses of plasma when evaluating using service lines ($p = 0.004$), discharge status ($p < 0.001$), LOS ($p < 0.001$) and INR difference ($p < 0.001$), but not by ethnicity ($p = 0.61$). Cardiac (24.8%) and GI/transplant (23.0%) were transfused in single doses well above other service lines. Patients receiving multiple doses of plasma compared with a single dose had a higher percentage of death (13.4% vs. 6.0%), longer LOS (11.0 days [6.0, 21.0] vs. 9.0 days [5.0, 15.0]) and a more significant INR difference (0.50 [0.10, 1.4] vs. 0.30 [0.00, 1.1]). Table 4 shows that from the 5813 patients whose weight was available, almost two thirds of 3642 (62.6%) received a sub-therapeutic dose of plasma.

The subgroup analysis of 100 patients found that 57% had no clinical reasoning for plasma transfusion documented, 13% were for coagulopathy, 10% were for warfarin reversal, 10% were for bleeding prophylaxis, 9% for acute blood loss and 1% for volume expander (Figure 1).

DISCUSSION

Single-unit RBCs and platelets are appropriate for adult hospitalized non-bleeding stable patients, and in recent years, much work has been done to educate providers on this practice [4–6]. The emphasis on single-unit transfusions, however, may have unintended consequences for determining plasma dosing for adult patients. This study found that

TABLE 3 Comparison of plasma dosing for all patients—Number (percent).

	Overall ($N = 5791$)		Multiple doses ($N = 4803$)		Single dose ($N = 988$)		
Factor	*N*	Statistics	*N*	Statistics	*N*	Statistics	*p*
Classification, *n* (%)	5789		4802		987		0.004[a]
Autoimmune/infection		242 (4.2)		195 (4.1)		47 (4.8)	
Cancer/haematology and oncology		290 (5.0)		229 (4.8)		61 (6.2)	
Cardiac		1603 (27.7)		1358 (28.3)		245 (24.8)	
Congenital/dermatology/other		469 (8.1)		385 (8.0)		84 (8.5)	
Endocrine/metabolic		157 (2.7)		135 (2.8)		22 (2.2)	
Gastrointestinal/transplant		1104 (19.1)		877 (18.3)		227 (23.0)	
Gynaecology and genitourinary/renal		260 (4.5)		215 (4.5)		45 (4.6)	
Neurology/psychology		623 (10.8)		530 (11.0)		93 (9.4)	
Orthopaedic		566 (9.8)		469 (9.8)		97 (9.8)	
Respiratory		475 (8.2)		409 (8.5)		66 (6.7)	
Discharge status, *n* (%)	5791		4803		988		<0.001[a]
Expired		704 (12.2)		645 (13.4)		59 (6.0)	
Home		3011 (52.0)		2450 (51.0)		561 (56.8)	
Hospice		279 (4.8)		217 (4.5)		62 (6.3)	
Rehab/skilled nursing		1348 (23.3)		1110 (23.1)		238 (24.1)	
Transfer		449 (7.8)		381 (7.9)		68 (6.9)	
LOS days, median (Q1, Q3)	5774	11.0 (6.0, 20.0)	4791	11.0 (6.0, 21.0)	983	9.0 (5.0, 15.0)	<0.001[b]
INR difference, median (Q1, Q3)	5791	0.50 (0.10, 1.4)	4803	0.50 (0.10, 1.4)	988	0.30 (0.00, 1.1)	<0.001[b]

Note: Statistics presented as median (P25, P75), *N* (column %).
Abbreviations: INR, international normalized ratio; LOS, length of stay.
[a]Pearson's chi-square test (*p*-values).
[b]Wilcoxon rank-sum test (*p*-values).

more than one in six adult patients (17.1%) being transfused for diagnoses other than hereditary angioedema at a large hospital system received a single-unit plasma transfusion. Of the six hospitals that transfused less plasma (<200 units) during the study period, patients were more likely to receive single units, with four (66.7%) transfusing above the mean healthcare system rate of 17.1%. It is believed that the clearest cause for this is the lack of experience and familiarity with the indications for plasma, as well as with the volume-based dosing.

Patients treated in all cardiac and GI/transplant services received single doses far more often than those who presented to other services. It needed to be clarified why the ordering practices differed from other locations. Did the inter-hospital differences in RBC transfusion practice result from variations in practice by individual surgeons? When this was tested at five university teaching hospitals

TABLE 4 Dose of plasma administered ($N = 5813$).

Actual plasma dose (mL/kg)	Number of patients	Percentage of total with weights (%)
Less than 10 mL/kg	3642	62.6
10–20 mL/kg	1401	24.1
Greater than 20 mL/kg	770	13.2

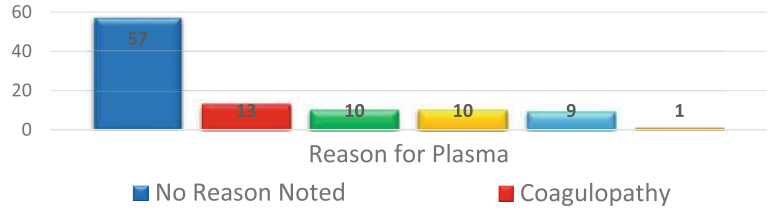

FIGURE 1 Documented clinical reasons for plasma orders.

TABLE 5 Comparison by international normalized ratio (INR).

	Overall ($N = 5791$)		1.8 + ($N = 3662$)		<1.8 ($N = 2129$)		
Factor	***N***	**Statistics**	***N***	**Statistics**	***N***	**Statistics**	***p*-Value**
Classification, *n* (%)	5789		3661		2128		*<0.001*[a]
Autoimmune/infection		242 (4.2)		199 (5.4)		43 (2.0)	
Cancer/haematology and oncology		290 (5.0)		187 (5.1)		103 (4.8)	
Cardiac		1603 (27.7)		855 (23.4)		748 (35.2)	
Congenital/dermatology/other		469 (8.1)		281 (7.7)		188 (8.8)	
Endocrine/metabolic		157 (2.7)		108 (3.0)		49 (2.3)	
Gastrointestinal/transplant		1104 (19.1)		807 (22.0)		297 (14.0)	
Gynaecology and genitourinary/Renal		260 (4.5)		169 (4.6)		91 (4.3)	
Neurology/psychology		623 (10.8)		317 (8.7)		306 (14.4)	
Orthopaedic		566 (9.8)		407 (11.1)		159 (7.5)	
Respiratory		475 (8.2)		331 (9.0)		144 (6.8)	
Discharge status, *n* (%)	5791		3662		2129		*<0.001*[a]
Expired		704 (12.2)		579 (15.8)		125 (5.9)	
Home		3011 (52.0)		1667 (45.5)		1344 (63.1)	
Hospice		279 (4.8)		221 (6.0)		58 (2.7)	
Rehab/skilled nursing		1348 (23.3)		908 (24.8)		440 (20.7)	
Transfer		449 (7.8)		287 (7.8)		162 (7.6)	
LOS, median (Q1, Q3)	5774	11.0 (6.0, 20.0)	3653	11.0 (6.0, 21.0)	2121	10.0 (6.0, 18.0)	0.13[b]
INR difference, median (Q1, Q3)	5791	0.50 (0.10, 1.4)	3662	1.00 (0.40, 2.2)	2129	0.20 (0.00, 0.30)	*<0.001*[b]
Ethnicity, *n* (%)	5701		3606		2095		0.83[a]
Hispanic		281 (4.9)		176 (4.9)		105 (5.0)	
Non-Hispanic		5420 (95.1)		3430 (95.1)		1990 (95.0)	

Note: Statistics are presented as median (P25, P75), *N* (column %). $p \le 0.05$ considered statistically significant (in italics).
Abbreviations: INR, international normalized ratio; LOS, length of stay.
[a]Pearson's chi-square test (*p*-values).
[b]Wilcoxon rank-sum test (*p*-values).

concerning the percentage of patients receiving transfusion(s) and the number of units transfused, the surgeons in each hospital saw a surprising uniformity of practice. In only one instance, in one hospital, did a surgeon's aggregate RBC transfusion practice differ significantly from that of another surgeon in the same hospital [7]? Based on this study, it is possible that in this healthcare system, the uniformity of practice by these service lines led to a pattern of higher than the overall prevalence of single-unit plasma usage.

Significant differences were seen in those receiving single units versus multiple units in discharge status, LOS and INR status categories. Individuals who received a single dose of plasma were more likely to survive and be discharged to home, while rates of discharge to hospice, rehab or skilled nursing and a transfer were similar between the two groups. Patients who received single dose also had shorter LOS (mean 9.0 days) than those who received multiple doses (mean 11.0 days). The reasons for the differences between the two groups cannot be easily explained from the findings of this study. One possibility shown elsewhere is that blood transfusions can result in transfusion-related immune modulation (TRIM). However, this was not evaluated as part of this study [8, 9].

Almost two thirds of patients received a sub-therapeutic dose of <10 mL/kg. This is higher than the 28.6% of plasma donations deemed inappropriate in a study conducted in Ontario [10]. It should be noted that the last plasma guidelines were published in *Transfusion* in 2010 and did not include any recommendations regarding weight-based dosing nor recommendations on what is considered an appropriate INR upon which to transfuse. One recommends that plasma transfusion for invasive procedures should occur when the prothrombin time (PT) and activated partial thromboplastin time (aPTT) indicate factor levels ≤30% or when the INR is ≥2.0 [11]. Based upon clinical consensus, the hospital system involved in this study now uses an INR of 1.8 systemwide for prophylactic transfusions. This threshold was set by the hospital system several years ago, and none of the authors were involved in this decision. It was noted that while the majority of patients, 3662 (63.2%), received plasma with an INR of 1.8 or greater, a significant minority, 2129 (36.8%), did not ($p < 0.001$) (Table 5), representing a significant opportunity for education regarding hospital policy and plasma transfusion thresholds.

While this study showed that under-transfusion of plasma occurred in this hospital system, plasma should be used judiciously. It should be noted that specialty-specific guidelines speak to a consensus on the lack of any proven benefit of treating elevated INRs in isolation prior to procedures [12, 13].

The limitations of this study include that it was conducted at one hospital system, and the results may not be more generalizable to all hospital systems and patients. Another limitation was that the study only reviewed a single-patient admission and the number of plasma transfusion doses that occurred. Still, it did not cover additional scenarios, such as transfusions in the outpatient setting. Finally, the differences in LOS, discharge status and changes in INR were based on data available, but they may have also been influenced by the patient's underlying medical conditions. It is possible that the data were confounded by indication, meaning that the patients who

Plasma Dosage [1]		
Recommended adult therapeutic dose of plasma is 10–20 mL/kg and guided by clinical situation and coagulation results.† Not for Massive Transfusions.		
Calculations of one Adult Therapeutic dose of plasma (in units)		
10 mL/kg	Patient Weight (kg)	20 mL/kg
	less than 50 kg	
2 units Plasma	50 kg	4 units Plasma
	55 kg	
3 units Plasma	60 kg	5 units Plasma
	65 kg	
	70 kg	6 units Plasma
	75 kg	
	80 kg	
4 units Plasma	85 kg	7 units Plasma
	90 kg	
	95 kg	8 units Plasma
	100 kg	
10 mL/kg	greater than 100 kg	20 mL/kg
†Volume of Plasma in a unit is variable, calculations based on mean volume ~250 mL		
This table is intended as a guide to the correct adult dose of Plasma, it is not a directive		
Use caution in using this table for greater than 100kg patients as the volume suggested		
[1]Accessed at: AABB Technical Manual. 19th ed. AABB Press. 2017.		

FIGURE 2 Plasma dosing chart that providers can see when ordering in electronic health record.

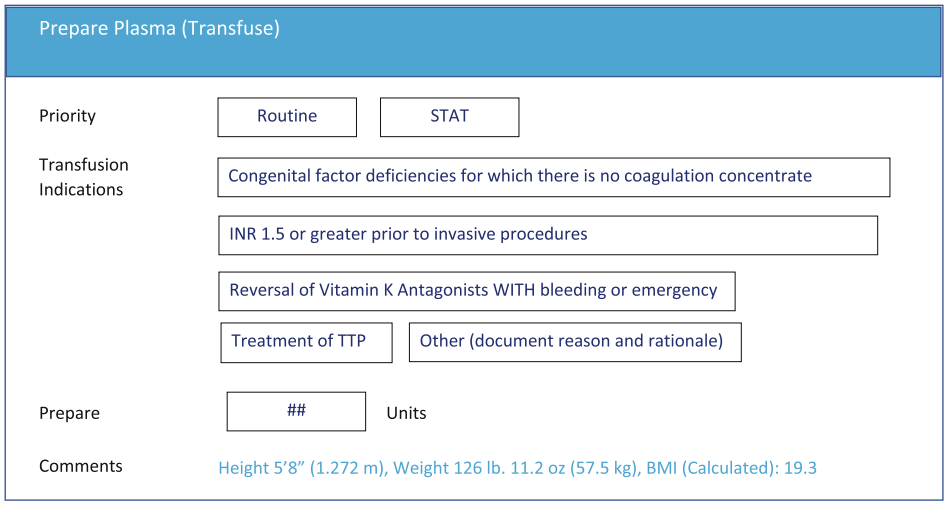

FIGURE 3 Patient weight displays when ordering plasma. BMI, body mass index.

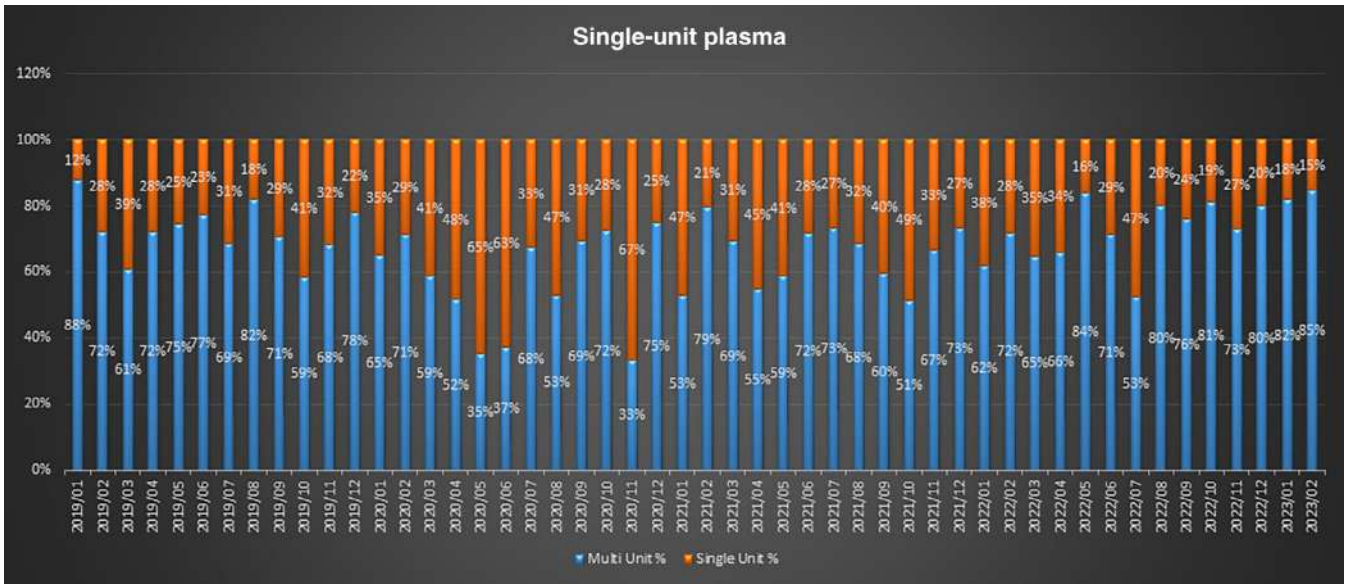

FIGURE 4 Historical data (January 2019–December 2022), after which the multiple plasma unit threshold of 65% was implemented in January 2023.

received multiple plasma units were, in the aggregate, sicker than those who received just one unit, as, on average, patients who received more blood products tend to be sicker than those who did not [14].

This hospital system has active PBM programmes at both its Ohio and Florida locations, and based on the findings of this study, an active campaign to decrease single-unit plasma use has been undertaken. In Florida, plasma transfusion threshold procedures have been modified from 1.5 to 1.8, consistent with Ohio procedures. A weight-based dose chart has been added to the electronic health record (EHR), and the patient's weight is now readily available to the ordering physician as a tool to encourage appropriate ordering practices. (Figures 2 and 3) After a physician performs a chart review, letters are sent to providers who order single units of plasma that are not considered justified, asking for a response and serving as an educational tool. To increase awareness, quarterly reports are sent to department leaders. Appropriate plasma dosing has been added to annual physician and nursing education. In January 2023, a target goal of a minimum of 65% of plasma transfusions to be multiple units was established based on historical data (Figure 4). Finally, a grand rounds session on appropriate plasma dosing was conducted to educate providers.

In Ohio, multiple measures have been taken, including educational efforts and recurrent presentations of data performance, to leadership. Educational efforts include presentations at the Quality Education Forum, which encompasses all quality leaders from all institutes

and departments; the Taussig Cancer Center staff meeting, presenting to all the Haematology/Oncology clinicians; the Grand Rounds for Hospital Medicine, as well as an iterative presentation to the Internal Medicine Residency Program; and it is part of the annual onboarding for all surgical trainees. A monthly report of the prevalence of single-unit transfusion is presented to the hospital leadership.

At this hospital system, single-unit plasma transfusions were not likely to have improved patient-centred outcomes and appeared to be a sign of clinician uncertainty and unindicated, potentially harmful transfusion practices. In conclusion, the goal with access to robust data and active PBM programmes is to promote appropriate evidence and weight-based plasma transfusions to optimize efficacy. While PBM does not prohibit transfusions, it does promote appropriate transfusions.

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R.R.G., M.A., D.T. and M.D. contributed equally to the writing of this manuscript. R.R.G. contributed to manuscript editing and reviewing. James Bena and Shannon Morrison contributed to statistical analysis. Aaron Frisbie and Matt Jones created reports through business intelligence.

CONFLICT OF INTEREST STATEMENT

Richard Gammon is a member of the CSL Behring, Patient Blood Management Advisory Board. All other authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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ORIGINAL ARTICLE

Vox Sanguinis ISBT International Society of Blood Transfusion

Disease severity in subsequent pregnancies with RhD immunization: A nationwide cohort

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Abstract

Background and Objectives: To evaluate the severity of haemolytic disease of the foetus and newborn (HDFN) in subsequent pregnancies with RhD immunization and to identify predictive factors for severe disease.

Materials and Methods: Nationwide prospective cohort study, including all pregnant women with RhD antibodies. All women with at least two pregnancies with RhD antibodies and RhD-positive foetuses were selected. The main outcome measure was the severity of HDFN in the first and subsequent pregnancy at risk. A subgroup analysis was performed for the group of women where RhD antibodies developed after giving birth to an RhD-positive child and thus after receiving anti-D at least twice (group A) or during the first pregnancy at risk for immunization (group B).

Results: Sixty-two RhD immunized women with a total of 150 RhD-positive children were included. The severity of HDFN increased for the whole group significantly in the subsequent pregnancy ($p < 0.001$), although it remained equal or even decreased in 44% of women. When antibodies were already detected at first trimester screening in the first immunized pregnancy, after giving birth to an RhD-positive child (group A), severe HDFN in the next pregnancy was uncommon (22%). Especially when no therapy or only non-intensive phototherapy was indicated during the first immunized pregnancy (6%) or if the antibody-dependent cell-mediated cytotoxicity result remained <10%. Contrarily, women with a negative first trimester screening and RhD antibodies detected later during the first pregnancy of an RhD-positive

child (group B), often before they had ever received anti-D prophylaxis, were most prone for severe disease in a subsequent pregnancy (48%).

Conclusion: RhD-mediated HDFN in a subsequent pregnancy is generally more severe than in the first pregnancy at risk and can be estimated using moment of antibody detection and severity in the first immunized pregnancy. Women developing antibodies in their first pregnancy of an RhD-positive child are at highest risk of severe disease in the next pregnancy.

Keywords

alloimmunization in pregnancy, foetal anaemia, foetal hydrops, haemolytic disease of the foetus and newborn, intra-uterine transfusion, natural course of disease, pregnancy complications, red cell immunization in pregnancy

Highlights

- The severity of haemolytic disease of the foetus and newborn (HDFN) increased significantly in the subsequent pregnancy compared with the first immunized pregnancy.
- Women with antibodies detected late (27th week or later) during the first immunized pregnancy, even before they had ever given birth to an RhD-positive child, or most often before they had ever received anti-D prophylaxis, were most prone to severe disease in a subsequent pregnancy.
- The risk of severe HDFN in a subsequent pregnancy can be estimated using the moment of antibody detection, antibody characteristics as reflected by antibody-dependent cellular cytotoxicity test results and the severity of HDFN in the first immunized pregnancy.

INTRODUCTION

Haemolytic disease of the foetus and newborn (HDFN) is a serious, and nowadays rare, condition caused by maternal alloantibodies against foetal red cells. The subsequent haemolysis may result in neonatal anaemia and hyperbilirubinemia, requiring phototherapy, red cell transfusions or exchange transfusions. In severe cases, anaemia occurs prenatally and intervention with intra-uterine transfusion(s) (IUT) is needed. Although the introduction of anti-D prophylaxis has greatly reduced the RhD immunization-rate, it still has remained the major cause of severe HDFN cases [1].

As blood transfusions are ABO- and RhD-matched, RhD alloimmunization is mostly the result of maternal exposure to foetal red cell antigens, inherited from the father [2]. The risk of alloimmunization depends on the duration and amount of foetomaternal haemorrhage, characteristics of the maternal immune system and of the nature of the red blood cell antigens [3].

A generally accepted idea is that the severity of HDFN increases in every subsequent pregnancy, as a rise in the amount of stillbirths in every following pregnancy affected with HDFN was already reported in 1957, before the introduction of anti-D prophylaxis [4, 5]. It has been observed that administration of anti-D prophylaxis seems to be associated with a lower immune response [6, 7].

The aim of this study is to assess the severity of HDFN in consecutive pregnancies with RhD immunization and RhD-positive foetuses, in the presence of routine antenatal and postnatal anti-D prophylaxis, to properly counsel and manage women after a first RhD immunized pregnancy. Furthermore, we evaluated which factors from the first immunized pregnancy are associated with severe disease in a subsequent pregnancy at risk.

MATERIALS AND METHODS

Setting

To prevent RhD immunization induced by pregnancy, RhD-negative mothers carrying RhD-positive foetuses receive both antenatal (around 30 weeks gestation) and postnatal anti-D prophylaxis in the Netherlands.

All pregnant women are screened for the presence of alloantibodies in the first trimester of pregnancy. Furthermore, RhD-negative and c-negative women are additionally screened in week 27. The coverage of this screening program is almost 100% [8]. All maternal blood samples with a positive screening result, identified at routine screening or at any other moment in pregnancy, are sent to one of the two national referral laboratories (Sanquin Diagnostic Services and Special Institute for Blood group Investigations [BIBO]). Here, the clinical relevance of the antibody is evaluated by, among other things, assessing whether the foetus is antigen-positive, with serological typing of the father as the first step in most of the pregnancies in this study. If the foetus is RhD-positive, the risk on foetal haemolysis is assessed by serially determining the antibody titre and antibody-dependent cell-mediated cytotoxicity (ADCC; performed only at Sanquin Diagnostic Services), a monocyte-based assessment of the destructive capacity of the antibodies [9, 10].

Study design and population

This study was part of the OPZI 2.0 study, a nationwide cohort study on RhD immunization in pregnancy. All pregnant women with a positive screening for RhD antibodies at any moment in pregnancy, identified at Sanquin Diagnostic Services during our study period, were eligible for inclusion. Positive screenings as a result of an anti-D prophylaxis administration were not included. Women were identified from two time periods (for practical reasons): from 1 July 2014 to 31 March 2015 and from 1 August 2015 to 28 February 2017. Women were excluded if the mother additionally had another antibody with a titre higher than that of RhD (and an antigen-positive child). In addition, women who did not receive regular antenatal and postnatal anti-D prophylaxis were excluded from this cohort. Risk factors for RhD alloimmunization in this cohort have been reported previously [11].

The local care provider of eligible pregnant women was contacted to obtain patient's informed consent. Subsequently, clinical data were collected from the care provider in a detailed questionnaire. If outcome data were incomplete, the researchers made at least three attempts to contact care providers or study participants directly to complete the questionnaire. Missing data on receiving anti-D prophylaxis in a previous pregnancy was obtained from the Department for Vaccine Supply and Prevention Programs (RIVM-DVP).

To test the hypothesis that HDFN is more severe in the subsequent pregnancy with RhD immunization than in the first immunized pregnancy, we selected all women with at least two pregnancies with RhD antibodies and RhD-positive foetuses from the OPZI 2.0 cohort. To assess the risk of selection and non-response bias, characteristics of included and non-included cases were compared (Supporting Information).

Sample size calculation

Based on the literature [6, 12] and an interim analysis of our data, we expected approximately 20% of cases to be treated with IUT, exchange transfusion or ending in foetal or neonatal death in the first immunized pregnancy and 45% in the second pregnancy. With a significance of 0.05 and a power of 0.8, a total of 56 women with two immunized pregnancies of RhD-positive foetuses would be required.

Data collection and outcome definitions

Relevant clinical data from all previous non-immunized and immunized pregnancies were collected in the OPZI 2.0 database. Furthermore, we obtained treatment details to assess the severity of HDFN of all pregnancies with RhD antibodies and RhD-positive foetuses. From Sanquin Diagnostic Services, laboratory data were retrieved (including antibody titres, ADCC results and the presence of additional antibodies). A list of collected data is available in Table S1. Data on previous drug use as a risk factor for immunization is missing.

In the current study, 'first immunized pregnancy' is defined as the first pregnancy with RhD antibodies and an RhD-positive child. 'Subsequent pregnancy' is defined as the second pregnancy with RhD antibodies and an RhD-positive child.

Our main outcome was disease severity, which was categorized as follows:

1. No HDFN: no antenatal or postnatal treatment.
2. Mild HDFN: non-intensive phototherapy (≤2 lamps), or only 1 day intensive phototherapy (>2 lamps), with or without a red blood cell transfusion during the first month after birth.
3. Moderate HDFN: intensive phototherapy (>2 lamps) for more than 1 day or neonatal exchange transfusion (in the Netherlands neonatal exchange transfusion has been gradually replaced by intensive phototherapy).
4. Severe HDFN: intra-uterine transfusion or HDFN-related death.

In case of missing data on disease severity, patients were assigned to a disease category based on the other, non-missing disease parameters (laboratory results, phototherapy duration and intensity, etc.). In twin pregnancy, disease severity was categorized according to the most severely affected child.

Ethical considerations

The medical ethics committee of the Leiden University Medical Center approved the protocol (P15.101/NV/nv). Written informed consent was obtained from all mothers included in this study.

Statistical analysis

All outcomes were analysed according to a predefined analysis strategy that was conducted in collaboration with our clinical epidemiologist (J.G.B.).

For our main outcome, sensitivity and subgroup analyses on the difference in severity of HDFN between two subsequent pregnancies, a Wilcoxon Signed-Rank test was used. With this test, the number of positive differences in severity (+1 to +3 disease categories), negative differences (−1 to −3) and ties are ranked.

Differences in severity of HDFN between two non-paired groups were analysed with a multinomial logistic regression. In other, non-paired analyses, the Pearson's Chi-square test or logistic regression (or Fisher's exact test if appropriate) was used for the comparison of proportions. Comparisons of non-parametric outcomes were analysed with the Mann–Whitney *U* test. A sensitivity analysis was performed among patients in whom all the information on disease outcome was available and disease severity was thus not imputed. As the mechanism and thus severity of HDFN might be different if RhD antibodies are developed after giving birth to an RhD-positive child and thus after receiving anti-D at least twice (group A), or in the first pregnancy at risk for immunization (group B), a subgroup analysis was performed between these groups.

To identify factors possibly predicting severe HDFN (IUT or death) in a subsequent pregnancy for counselling purposes, a

prediction model was constructed including variables known or thought to be associated with HDFN severity from the literature, the potential predictors. All potential predictors with a p value <0.25 in univariate analysis were included in a multivariate logistic regression model. The prediction model was further improved by applying manual backward selection, excluding the variable with the highest p value at every step. Eventually, all variables with a p value <0.1 remained in the final prediction model.

RESULTS

Selection and characteristics of study population

Three hundred eleven pregnant women with RhD immunization were found eligible for inclusion in the OPZI 2.0 study. Figure 1 shows how the study population for the present analysis on HDFN severity in subsequent pregnancies was selected. In total, 62 women were included, with 155 pregnancies complicated by RhD antibodies and with an RhD-positive child (38 women with two, 19 women with three, 4 women with four and 1 woman with six pregnancies of RhD-positive children after her RhD antibodies were detected). Including two twins makes a total of 157 RhD-positive children. Table 1 shows the characteristics of included women and their children. To assess the risk of selection bias by selecting women with two or more subsequent pregnancies only, disease severity in the first immunized pregnancy of patients with and without a subsequent pregnancy was compared and showed a similar distribution (Table S2).

Severity of HDFN in the first immunized and the subsequent pregnancy

In this cohort of 157 RhD-positive children out of pregnancies complicated by RhD antibodies, no children died as a result of HDFN. One foetal death occurred due to a cause other than HDFN (severe growth restriction and placental infarction by pathological examination). As the severity of HDFN of this deceased child cannot be categorized nor compared with the subsequent children (four RhD-positive children since detection of antibodies), it is not reported in outcome tables and figures.

In two sets of twins, RhD antibodies were detected in the first immunized pregnancy; all children were typed postnatally as RhD-positive and showed mild disease.

Table 2 demonstrates that the severity of HDFN was significantly higher in the subsequent pregnancies, compared with the first immunized pregnancy ($p < 0.001$). HDFN was more severe in the subsequent pregnancy in 34/61 women (56%, maximum of three categories more), equally severe in 19/61 (31%) and less severe in 8 women compared with the first immunized pregnancy (13%, maximum of one HDFN category less). For two patients, intensity of phototherapy was missing, and HDFN severity was thus imputed. As their charts reported 'short phototherapy' and '2 days of phototherapy,' disease severity was imputed as mild disease. The sensitivity analysis without these patients showed a similar result ($p < 0.001$). Figure 2a demonstrates the severity of HDFN in subsequent pregnancies in relation to the severity in the first immunized pregnancy. HDFN severity in third and later pregnancies is available in Table S3 and the accompanying text.

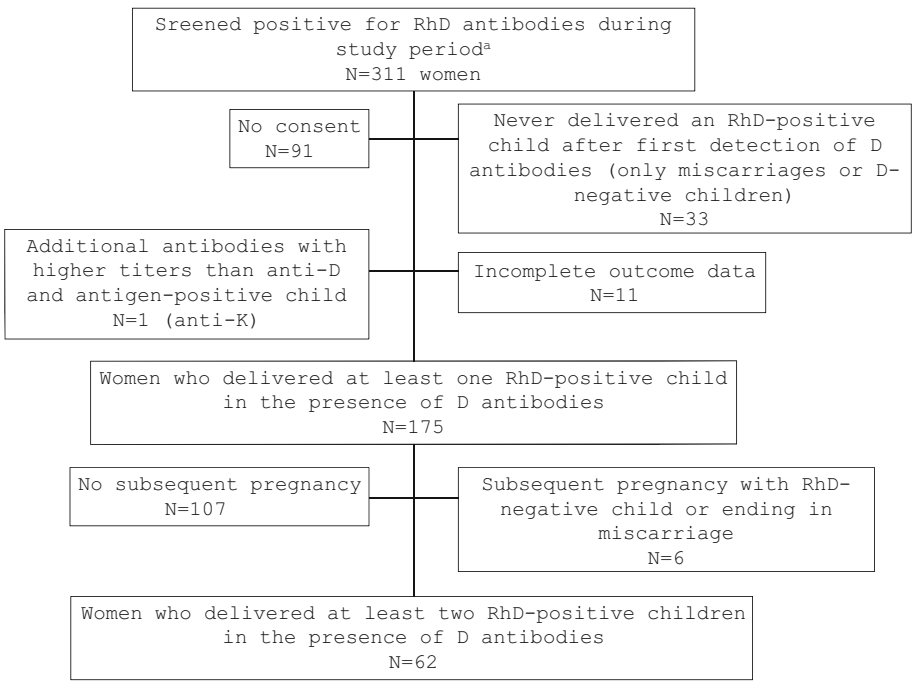

FIGURE 1 Composition of the study population. Red boxes reflect exclusions and unselected cases. Blue boxes show the number of women before and after exclusions, and the green box reflects the final population for this study. [a]Newly immunized women or a new pregnancy after previous immunization.

TABLE 1 Baseline characteristics of 62 women with two or more pregnancies with RhD antibodies and an RhD-positive child.

Characteristics	First immunized pregnancy (*N* = 62 women)
	All women
White ethnicity	44 (73)
Maternal age at first positive screening	27 [19–35]
Number of (spontaneous) abortions before immunization	0 [0–3]
Group A[a]	0 [0–3]
Group B[a]	0 [0–1]
Number of births before immunization[b]	1 [0–3]
First positive screening during pregnancy	
Early first trimester screening	21 (34)
Delayed 'first trimester' screening, ≥20 weeks	3 (5)
27th week screening	34 (55)
Between 27th week screening and birth	4 (7)

Characteristics	Events occurring between the first and subsequent pregnancy with an RhD-positive child		
	Group A[a] (*N* = 27 women)	Group B[a] (*N* = 25 women)	All women (*N* = 62 women)[c]
Time, years[d]	3 [1–10]	2 [1–15]	2 [1–15]
Number of (spontaneous) abortions	0 [0–1]	0 [0–5]	0 [0–5]
Number of births of RhD-negative foetuses			
0	25 (100)	22 (88)	58 (94)
1		2 (8)	3 (5)
2		1 (4)	1 (2)

Characteristic	First immunized pregnancy (*N* = 64 children, two sets of twins)	Subsequent pregnancy (*N* = 62 children)
Sex of child		
Male	41 (64)	32 (53)
Female	23 (36)	29 (48)

Note: Data presented in *N* (%) or median [range]. The first immunized pregnancy is the first pregnancy with RhD antibodies and an RhD-positive child, and the subsequent pregnancy is the second pregnancy with RhD antibodies and an RhD-positive child.
[a]Group A: RhD antibodies developed after giving birth to an RhD-positive child and thus after receiving anti-D at least twice, or during the first ongoing pregnancy at risk for immunization (group B).
[b](RhD-positive and RhD-negative for group A, RhD-negative only for group B).
[c]Twenty-seven women in group A, 25 in group B, 9 women with unclear immunizing moment not analysed in subgroups (positive first trimester screening without giving birth to an RhD-positive child).
[d]Years between due dates of first and subsequent immunized pregnancy.

TABLE 2 Severity of HDFN in the first immunized and the subsequent pregnancy with an RhD-positive child.

HDFN severity	First immunized pregnancies *N* = 61	Subsequent pregnancies *N* = 61	*p* value
No HDFN	19 (31)	11 (18)	<0.001
Mild HDFN	20 (33)[a]	14 (23)[b]	
Moderate HDFN	20 (33)	17 (28)	
Severe HDFN	2 (3)	19 (31)	

Note: Data shown in *n* (%). Wilcoxon Signed-Rank test performed to compare HDFN severity in first and subsequent immunized pregnancy.
[a]Intensity of phototherapy missing in one child (2 days of phototherapy), interpreted as mild.
[b]Intensity of phototherapy missing in one child ('short phototherapy'), interpreted as mild.

Table S4 presents the raw data on indicators of HDFN and treatment details in first immunized pregnancies and in subsequent pregnancy with an RhD-positive child. Most of these disease parameters indicated more severe disease in the second immunized compared with the first immunized pregnancy (upon simple inspection).

Severity of HDFN according to the time of antibody detection

Figure 2a,b illustrate severity of HDFN in subsequent pregnancies in relation to the severity in the first immunized pregnancy for the subgroups with RhD antibodies detected after giving birth to an RhD-positive child (group A) or during the first pregnancy at risk for immunization (group B). The median HDFN severity was mild in the first immunized pregnancy in both subgroups and did not differ significantly between group A and B ($p = 0.794$). In both subgroups, disease severity increased significantly in the subsequent pregnancy as compared with the first pregnancy (more severe in 14/27 [52%] women in group A, equal in 10 [37%], equal and less severe in 3 [11%], $p = 0.007$ and 18/25 [72%] more severe, 4 [16%] equal and 3 [12%] less severe in group B, $p = 0.001$). The change in HDFN severity in subsequent pregnancies did not differ between these subgroups (both median + 1 HDFN categories change, range: −1 to +3, $p = 0.053$).

In group A, 6/27 (22%) of women experienced severe HDFN in the second pregnancy, as opposed to 12/25 (48%) of women who developed antibodies in the first pregnancy at risk (group B, odds ratio [OR]: 3.22, 95% confidence interval [CI]: 1.0–10.8, $p = 0.051$).

Predicting severe disease in the second pregnancy with RhD antibodies

The association between severity in the first and the second pregnancy with RhD antibodies was assessed in both subgroups. In

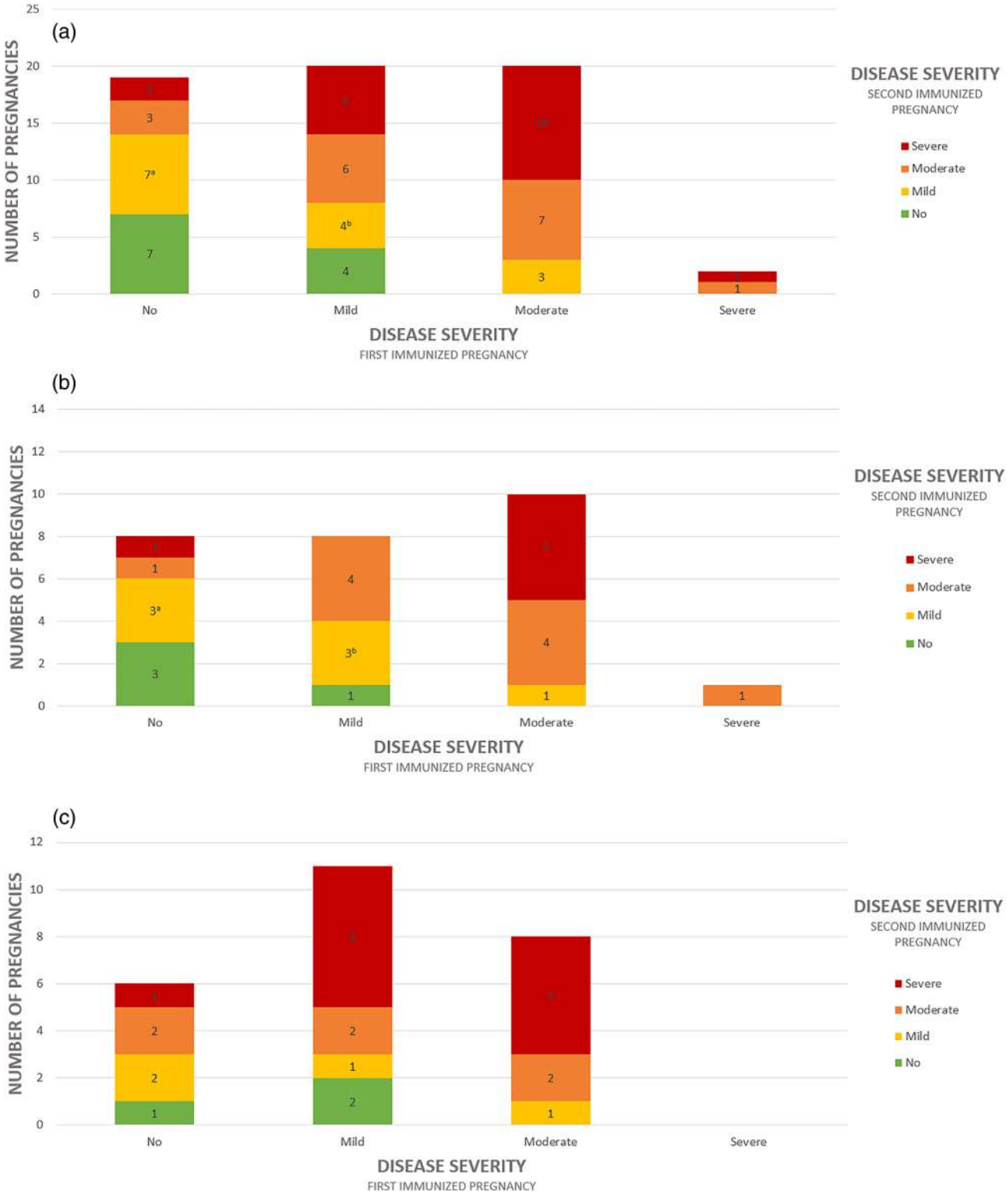

FIGURE 2 Severity of haemolytic disease of the foetus and newborn (HDFN) in first and subsequent immunized pregnancies with RhD-positive children. (a) Subgroup of 27 women who gave birth to at least one RhD-positive child before antibodies were detected (group A). (b) Subgroup of 25 women who developed antibodies in their first pregnancy at risk for immunization (no previous RhD-positive child, group B). (c) All 61 women. Outcome of woman with foetal death to a cause other than HDFN not shown. [a]Intensity of phototherapy missing in one child ('short phototherapy'), interpreted as mild. [b]Intensity of phototherapy missing in one child (2 days of phototherapy), interpreted as mild.

group A, 5/11 (45%) women with moderate to severe disease in the first pregnancy with RhD immunization developed severe disease in the second pregnancy as compared with 1/16 women with previous no or mild disease (6%, OR: 12.5, 95% CI: 1.2–130.6, $p = 0.027$). In group B, this was 5/8 (63%) as compared with 7/17 women with previous no or mild disease (41%, OR: 2.4, 95% CI: 0.4–13.4, $p = 0.411$).

Factors from the first immunized pregnancy possibly predicting severe disease in the subsequent pregnancy with an RhD-positive foetus were assessed in a multivariate prediction model per subgroup (Table S5). In group A, the highest ADCC result in the first immunized pregnancy remained as the only factor associated with severe disease in the subsequent pregnancy. The predictive value of this test is summarized in Table S6. If the ADCC test did not exceed 10% in the first pregnancy, 89% (95% CI: 55%–98%) of subsequent RhD-positive children will not be treated with intra-uterine transfusion(s).

In group B, no predictive factors were found in this multivariate analysis.

DISCUSSION

In this unselected national cohort of 157 RhD-positive children of 62 women with RhD antibodies, HDFN severity in the first pregnancy with anti-RhD antibodies with an RhD-positive child and subsequent pregnancies at risk was evaluated. The severity of HDFN increased significantly in 56% of women. Women who developed RhD antibodies in the first pregnancy at risk for immunization seemed more prone for severe disease in the subsequent pregnancy.

In this study, severe HDFN occurred more often in subsequent (31%) compared with first immunized (3%) pregnancies, in line with findings of others. For example, Tiblad et al. found 1.7% (5/288) severe HDFN in first immunized pregnancies, according to our definitions, and 19% in the second pregnancy at risk [12]. Similar to our findings, mothers who were already immunized during their first ongoing pregnancy (before giving birth to an RhD-positive child) received more treatment for HDFN, although not significantly. Other authors observed 0% severe disease in first immunized pregnancies and 19% in 'reactivation' of RhD immunization [13]. Our study is, however, the first directly comparing the first and subsequent immunized pregnancy of the same woman, which demonstrated that the severity of HDFN did not increase in 44% of the cohort. This challenges the general accepted concept that every next child at risk for HDFN will be more severely affected.

We found that the proportion of severe disease in a subsequent pregnancy of women who developed antibodies during their first pregnancy of an RhD-positive child, before anti-D prophylaxis could even be administered (group B), was as high as 48%. This is twice as much as compared with the subgroup that developed antibodies after giving birth to an RhD-positive child, despite receiving full prophylaxis (group A, 22%), a finding that approached statistical significance (Figure 2b,c). Several mechanisms might contribute to this difference in course of disease.

First tempting hypothesis is that women already developing antibodies during their first ongoing pregnancy with an RhD-positive child (group B) may be classified as 'high responders'. Recent publications revealed associations between the intensity of an antibody response and a combination of genetic risk factors such as carrying human leucocyte antigen-DRB1*1501 and Fc gamma Receptor IIc ORF alleles [14–16]. If in the future 'high responders' could be identified early, additional anti-D prophylaxis before the conventional antenatal administration might prevent immunization during the first pregnancy at risk. In this study, no association was found in this subgroup between clinical or biochemical (ADCC/titre) disease severity and severe disease in the subsequent pregnancy. Therefore, all women who develop RhD antibodies in their first pregnancy at risk for immunization are to be monitored closely.

Second hypothesis is that in line with the observations that administration of anti-D prophylaxis is associated with a lower immune response [6, 7, 12, 17], in the current study, we observed that a stronger antibody response occurred in women who have never received anti-D prophylaxis (group B), as opposed to women who received prophylaxis antenatally and postnatally (group A).

A third hypothesis is that women in group A and B have different immunoglobulin G (IgG)-Fc-glycosylation profile of their anti-D antibodies, which correlates with clinical and biochemical (ADCC) HDFN severity, but this needs to be studied in more detail [18, 19]. Interestingly, we have previously shown that the IgG-Fc glycosylation profile is sustained in subsequent pregnancies [19]. Already before anti-D prophylaxis was available, disease severity in subsequent pregnancies seemed to be interrelated [4]. This correlates with our finding of a persistent tendency to milder disease in the subsequent pregnancy in group A: only one of 16 women with no or mild disease in her first immunized pregnancy developed severe disease, and a low ADCC result in the first immunized pregnancy was the best predicting factor for no severe HDFN in the next pregnancy. These associations were not found in group B, possible reflecting a different IgG-Fc-glycosylation profile.

Lastly, an additional factor influencing the relation between severity in the first and subsequent immunized pregnancies might be the inherited foetal Fc-receptor profiles, as we have previously shown that this profile influences the risk of severe HDFN [16].

The major strength of our study is the unselected study population: as coverage of the national screening program is near 100% in the Netherlands [8] and serological assessment (titres and ADCC tests) for the risk on HDFN is performed at Sanquin Diagnostic services only, all women with D antibodies in the Netherlands who were pregnant during our study period were identified.

Another strong point of this study was our response rate of 73%. Furthermore, no selection bias seems to be induced by selecting women with two or more subsequent pregnancies only (Supporting Information text and Table S2).

A limitation of this study is, however, that cut-offs for the disease categories are somewhat arbitrary, as the clinical rationale for treatment decisions is not always clear in retrospect and might vary over time. Our main finding that disease severity increases in the majority of subsequent pregnancies at risk is, however, supported by the increase in almost all raw disease characteristics in Table S4. It may be that ABO incompatibility or compatibility between the mother and the foetus is a modifier of the immune response, but we could not study this in detail because registration of ABO types of neonates was incomplete.

In conclusion, the severity of anti-RhD-mediated HDFN increases in the majority of subsequent pregnancies with RhD-positive foetuses. The risk of severe HDFN in a subsequent pregnancy can be

estimated using the moment of antibody detection, antibody characteristics as reflected by ADCC test results and the severity of HDFN in the first immunized pregnancy. Mothers with antibodies occurring during their first pregnancy of an RhD-positive child and before antenatal administration of anti-D prophylaxis, are more at risk for developing severe disease in a subsequent pregnancy. Further research should focus on identifying this group of 'high responders' to establish whether an additional, early administration of anti-D prophylaxis could be beneficial. Furthermore, the development of more effective non-invasive treatment options for foetuses affected by HDFN could possibly ameliorate outcome.

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C.Z. and Y.M.S. initiated and planned the study and were responsible for the data collection, analysing and writing the manuscript; J.M.K., I.L.v.K., E.L., D.O. and M.d.H. were closely involved in initiation and planning and reviewing the results and manuscript; P.C.L. was responsible for organizing data collection and analysing the results; J.G.v.d.B. reviewed and supervised the study methods and analysis plan and reviewed the manuscript; C.E.v.d.S. was involved in initiation of the studies and reviewed the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data is available from corresponding author.

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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

Criteria for storage of cord blood units at Japan's largest cord blood bank

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Abstract

Background and Objectives: In Japan, cord blood transplantations exceed those done with adult-sourced unrelated stem cells. This study analyses cord blood (CB) storage criteria to maintain high-quality CB units.

Materials and Methods: The Kanto-Koshinetsu Cord Blood Bank received 29,795 units from 2014 to 2021, mostly >60 mL, and 5486 (18.4%) were stored as transplantable units. We investigated the mother's gestational period, CB volume, total nucleated cells (TNCs), CD34+ cells, total colony-forming units (CFUs), time from collection to reception and cryopreservation, cell viability, and the reasons for not storing a unit.

Results: The average time from collection to reception of 29,795 units was 18.0 h. The most common reason for not storing a CB unit was low cell numbers (pre-processing TNC count <1.2 billion), accounting for 67.9% of the units received. There was no correlation between the CB volume and the CD34+ cell count. The shorter the gestational period, the lower the TNC count, but the higher the CD34+ cell count. There was no correlation between the time from collection to cryopreservation, within a 36-h time limit, and the CD34+ cell recovery rate.

Conclusion: We could accept units with a TNC count <1.2 billion and a CB volume <60 mL from a gestational period of 38 weeks or less if we did a pre-processing CD34+ cell count. This would secure more units rich in CD34+ cells.

Keywords

CD34+ cells, cord blood, quality

Highlights

- There was no correlation between the cord blood volume and CD34+ cell number, but there was a correlation between the number of CD34+ cells and colony-forming unit counts at both pre-freezing and after thawing.
- The shorter the gestational period, the lower the pre-processing total nucleated cells, but the higher the post-processing CD34+ cell count.
- There was no correlation between the time from collection to cryopreservation, with a 36-h time limit, and the CD34+ cell recovery rate.

INTRODUCTION

The first cord blood transplantation (CBT) from a related donor took place in 1988 [1, 2] and the first from an unrelated donor in 1993 [3]. In Japan, the first related CBT was performed in 1994 [4] and the first unrelated in 1997 [5]. The Japanese Red Cross (JRC) cord blood banks (CBBs) formally became part of the blood service in 2011, and in 2012, the national government passed the first law to regulate cord blood (CB) banking. Currently, there are six public CBBs in Japan, four of which are managed by the JRC. The annual number of unrelated CBTs has increased from 13 in 1997 to 1496 in 2020. From 1997 through July 2023, the total number of unrelated CBTs was 23,201. As of August 2023, there were 9608 CB units available for transplantation, of which 3348 units were managed by the JRC Kanto-Koshinetsu CBB.

CBBs have the responsibility to improve processing and storage criteria by analysing the data from before and after processing and after freezing and thawing. Recently, Morishima et al. reported that a higher CD34+ cell dose was associated not only with improved engraftment but also with better overall survival [6]. Therefore, the number and viability of CD34+ cells are as important as other quality measurements of the CB units. The report also showed the importance of the colony-forming units-granulocyte macrophage (CFU-GM) counts in the CB units.

The purpose of this study was to analyse the CB data from the Kanto-Koshinetsu CBB, one of the four CBBs managed by the JRC and the largest in Japan, to improve CB processing and cryopreservation criteria for the effective storage of CB units of the highest quality.

MATERIALS AND METHODS

Cord blood

We received 29,795 units from CB collection centres from April 2014 through December 2021. Those centres had been instructed to send the CB unit only when they collect over 60 mL, but units with a smaller volume were also shipped to the processing site from time to time. TNC were counted even in such low-volume units. We obtained written informed consent from all donors before CB collection. The CB was collected in utero when the mother and baby did not have an obvious infection or inheritable disease risk and generally after a gestational period of 37–41 weeks, or earlier depending on the obstetrician's decision. Gestational period information was not required for CB processing and cryopreservation. After the collection, a sample of the mother's blood (10 mL) was also collected. The CB was transported at temperatures ranging from 4 to 25°C. When received, it was kept at 10°C after October 2021, but at 15°C before then.

After arriving from the collection centre, the CB was weighed and 600 μL was taken as a test sample. Reception had to be within 33 h after collection. Processing began if there was no blood clot and documentation was adequate (including a consent form, questionnaire, family history and delivery record), and the TNC and/or CD34+ cell counts were sufficient. Freezing of the processed CB was started within 36 h of collection.

We investigated the gestational period, sex and volume of the CB, the numbers of nucleated red blood cells, total nucleated cells (TNCs), CD34+ cells, and total CFU (the sum of CFU-GM, burst forming units-erythroid [BFU-E] and CFU-mix), the time from collection to cryopreservation, time from reception to cryopreservation, cell viability (CD45+ cells and CD34+ cells), and the cryopreservation period. Post-thaw tests using an attached segment were conducted 2–3 weeks before supplying the CB unit.

This study was approved by the ethics committee of the JRC (No. 2022-029-1) and adhered to the principles laid out in the Declaration of Helsinki.

Processing, cryopreservation and testing

CB arriving from the collection centre was weighed and the TNC were counted. Through 2020, the CB was processed if the TNC count was 1.2 billion or over. Beginning in 2021, if the TNC count was between 1.2 and 1.4 billion, the CD34+ cells were counted, and if they totalled 2.5 million or more, then the CB was processed. Units with over 1.4 billion TNC were processed without a CD34+ cell count. After processing, all units were measured for the TNC, CD34+ cells and CFU counts before freezing.

Processing was performed manually in a clean room. The CB in the collection bag, which contained 28 mL of CPDA (KBS-200CA8L (C), Kawasumi, Kawasaki, Japan), was mixed with a 1/5 volume of 6% HES (HES40, Nipro, Osaka, Japan), to a final concentration of 1% HES, centrifuged at 50*g* for 5 min, and then placed to stand for 20 min to 1 h. The supernatant, containing plasma and white blood cells, was squeezed out manually into a blood separation bag (KBP-200C, Kawasumi), connected using a sterile tubing welder (TSCD-II, Terumo, Lakewood, CO, USA), leaving the sedimented red blood cell fraction. The supernatant fraction was centrifuged at 400*g* for 10 min, and then the plasma was partially removed by squeezing it out manually to another blood separation bag (KBP-200C), connected with the tubing welder, to concentrate the nucleated cell fraction in a volume of 23.4 mL. After collecting a test sample of 0.2 mL for TNC and HLA typing, the nucleated cell fraction was mixed with 4.4 mL of cryoprotectant, consisting of 55% (w/v) DMSO and 5% (w/v) dextran 40 (CryoSure-DEX40, USP grade, WAK-Chemie Medical GmbH, Steinbach, Germany) to a final DMSO concentration of 8%. The total volume of 27.6 mL was transferred to a freezing bag (F-025B, Nipro). Samples for the sterility test were aliquoted from the freezing bag, and then the connecting tube was sealed to make three segments with a total volume of about 1.0 mL. Approximately 25 mL of the processed CB was frozen by a programmed freezer and kept at −180°C in a vapour phase of liquid nitrogen, pending negative test results for infectious markers and cultures; it was then moved to liquid nitrogen for storage. The average TNC recovery rate after processing was 82.1%.

The tests for infectious markers, using the mother's serum, included a nucleic amplification test (NAT) for HBV, HCV and HIV, (and HEV since 2020), serological testing for anti-HBs, -HBc, -HCV, -HIV-1/2, -HTLV-1, (and -HTLV-2 since 2017), parvovirus B19-Ag, HBs-Ag, and syphilis, and a biochemical test (ALT). These screening tests for CB are the same as those for blood donations at the JRC [7]. For cytomegalovirus, we conducted an antibody test using the mother's serum and a NAT was carried out using pre-processed CB. Bacterial–fungal culture tests, using 10 mL of a sedimented RBC fraction in both aerobic and anaerobic culture bottles, were carried out using Bact/Alert (bioMérieux, Marcy-l'Étoile, France). In addition, 0.5 mL of processed CB from the cryopreservation bag was cultured in the Oxoid Signal blood culture system (Thermo Fisher Scientific, Waltham, MA, USA), which detects both aerobic and anaerobic bacteria.

If the CB was not stored for transplantation, maternal blood tests were not performed. Personal information is kept for 10 years after CB collection and, if the CB was used for transplantation, for 30 years after the transplantation. After these data storage periods, the personal information is properly disposed of based on the Personal Information Protection Law. If a CB unit was not used for transplantation within 10 years, it was either used for research, without the personal information, or discarded.

The stability of the CB has been confirmed by a research project at our CBB, examining CB units after more than 10 years of storage.

TNC, CD34+ cells, viability, CFU assay and HLA typing

TNC were measured using an automated analyser (XN-1000, Sysmex, Kobe, Japan). CD34+ cells were measured by flow cytometry (Navios with Stem-Kit, Beckman Coulter, Brea, CA, USA), gating for lymphocyte-sized cells of CD45 weak positive, CD34 positive and 7-aminoactinomycin D (7-AAD) negative. The viability of CD45+ and CD34+ cells was measured by flow cytometry with 7-AAD staining (Beckman Coulter). For the viability test, flow cytometry with 7-AAD was introduced at the start of April 2014. Therefore, the data analysis period of this study was set from April 2014. For the CFU assay, 4.0×10^4 CB cells were plated in 1.1 mL of methylcellulose medium (Methocult H4034 Optimum, StemCell Technologies, Vancouver, BC, Canada) in three dishes and incubated at 37°C in a

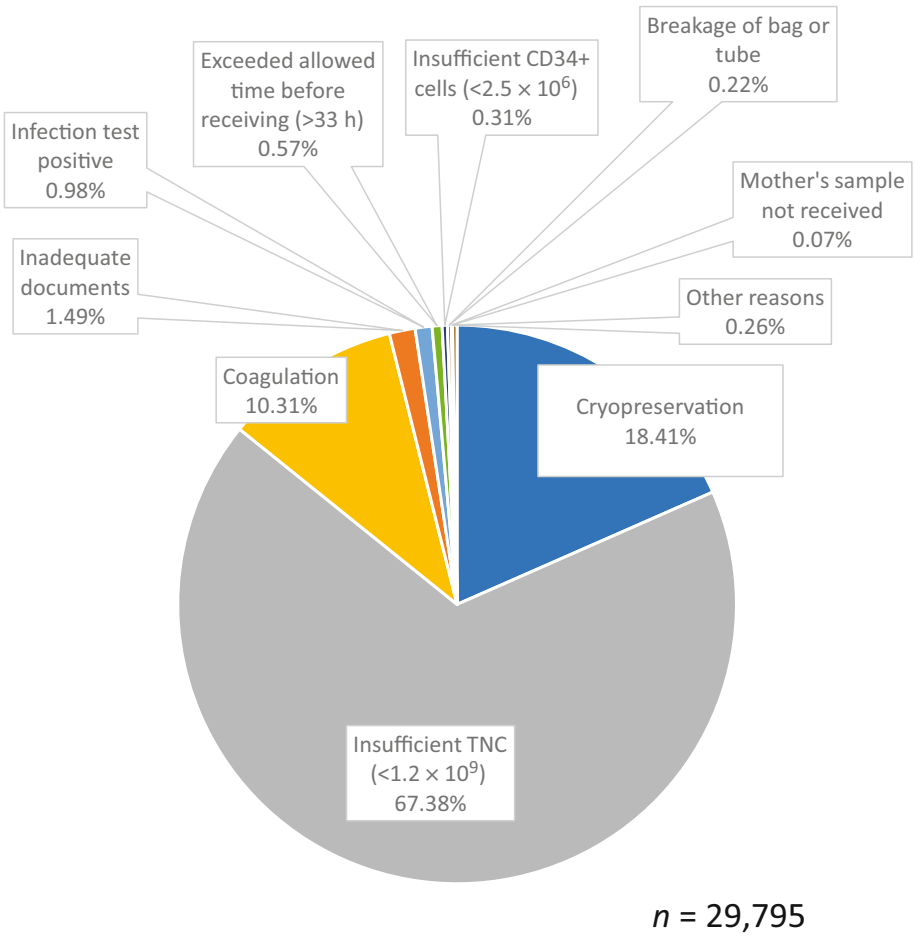

FIGURE 1 The reasons for not cryopreserving cord blood received by the Kanto-Koshinetsu cord blood bank from April 2014 to December 2021. TNC, total nucleated cell.

humidified 5% CO_2 incubator for 14 days. The colony numbers were counted by trained personnel using an inverted phase contrast microscope as CFU-GM, BFU-E or CFU-mix and the mean colony count of three plates for each CFU was recorded. To identify HLA types, genomic DNA was extracted from 200 μL of the whole blood samples that remained after the TNC count, using an automated DNA extractor (Biomek-NX, Beckman Coulter) with QuickGene-HT (Kurabo, Osaka, Japan). HLA types (HLA-A, -B, -C and -DRB1) were determined by polymerase chain reaction–reverse sequence-specific oligonucleotide (PCR-rSSO) using a WAK Flow HLA-DNA typing kit LABType

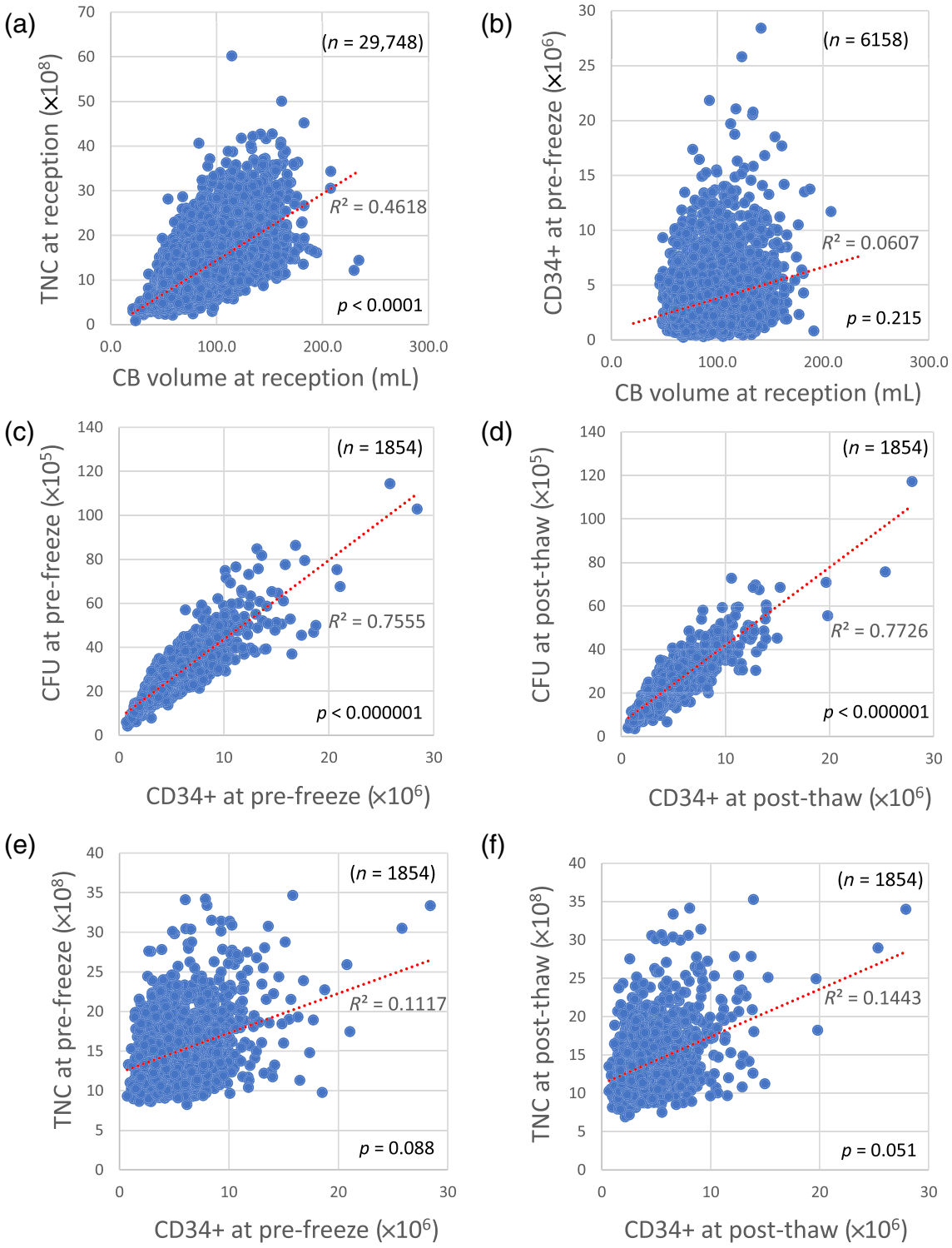

FIGURE 2 Correlations between (a) cord blood (CB) volume and total nucleated cell (TNC) count at reception, (b) CB volume at reception and CD34+ cell count at pre-freeze, (c) CD34+ cell count and colony-forming unit (CFU) at pre-freeze, (d) CD34+ cell count and CFU at post-thaw, (e) CD34+ cell count and TNC at pre-freeze, and (f) CD34+ cell count and TNC at post-thaw.

(Wakunaga Pharmaceutical, Tokyo, Japan). Each CB unit with all relevant data was uploaded to the donor search system after the minimum follow-up of 9 months, which was needed to confirm the baby's health information. These data included the counts of TNC, CD34+ cells and CFU-GM, along with the HLA type. When a CB unit was requested, we used a segment tube sample for HLA confirmation testing, and a second segment for TNC, CD34+ cell and CFU counting. For this study, statistical analyses were performed using SAS (Ver. 9.4, SAS Institute, Cary, NC, USA). The Tukey's HSD test was used for multiple comparisons of the CD34+ cell count, TNC count and CB volume with the gestational period (weeks). The significance level was set at $p < 0.05$.

RESULTS

17.5% of the CB collected was made available for transplantation

The Kanto-Koshinetsu CBB accepted 29,795 units from the CB collection centres from April 2014 through December 2021. Of the accepted CB, 5912 units (19.9%) were processed, 5486 units (18.4%) were stored as transplantable units and finally 5241 units (17.5%) were uploaded into the donor search system. From them, 1907 units (6.4%) were requested, and 1651 units (5.5%) were transplanted by the end of 2021.

The most common reason for not cryopreserving CB was insufficient cell number

We investigated the reasons for not cryopreserving CB (Figure 1). The most common reason was insufficient cell number (TNC < 1.2 billion), accounting for 67.38% of the total CB units received, followed by coagulation for 10.31%. Another 171 units (0.57%) were not processed because 33 h had expired between collection and reception. There were 291 units (0.98%) with positive bacterial/fungal cultures or other positive infectious marker screening tests. Some of the units that were not stored were used for research. Those who want to use CB for research must receive approval by the Institutional Review Board. During this study period, 6147 unprocessed units and 46 units for which the cryopreservation had exceeded 10 years were sent to researchers.

We have received about 4000 CB units annually. The days with the highest number of collections have been Tuesdays, Wednesdays and Thursdays, and processing was performed the most on Wednesdays, Thursdays and Fridays. To increase the number of CB units received, we began to accept CB on Sundays and holidays. The number of CB units received on Sundays remained small, but on holidays, the number received was similar to weekdays.

There was no correlation between the CB volume and CD34+ cell number

The average volume of the CB units received was 76.8 mL, due mainly to the collection centres being instructed to send the CB unit only when they collect over 60 mL. At reception, the average number of TNC for all units was 1.09 billion. The CB volume and the TNC count were correlated but not strongly (Figure 2a, $R^2 = 0.462$, $p < 0.0001$). Therefore, it is reasonable to measure the number of TNC regardless of what the volume is. Since 2021, if the TNC count was between 1.2 and 1.4 billion, the CD34+ cells were counted at reception. Of the 2335 units with 1.2–1.4 billion TNC pre-processing, 1123 units (48%) exceeded the CD34+ minimum criterion of 2.5 million cells. The CD34+ cell count at pre-processing (mean ± SD) was 3.61 ± 2.09 million, and post-processing was 3.53 ± 1.61 million, with a recovery rate of 78.54 ± 10.86%. The CB volume and post-processing/pre-freezing CD34+ cell count were not correlated (Figure 2b, $R^2 = 0.061$, $p = 0.215$).

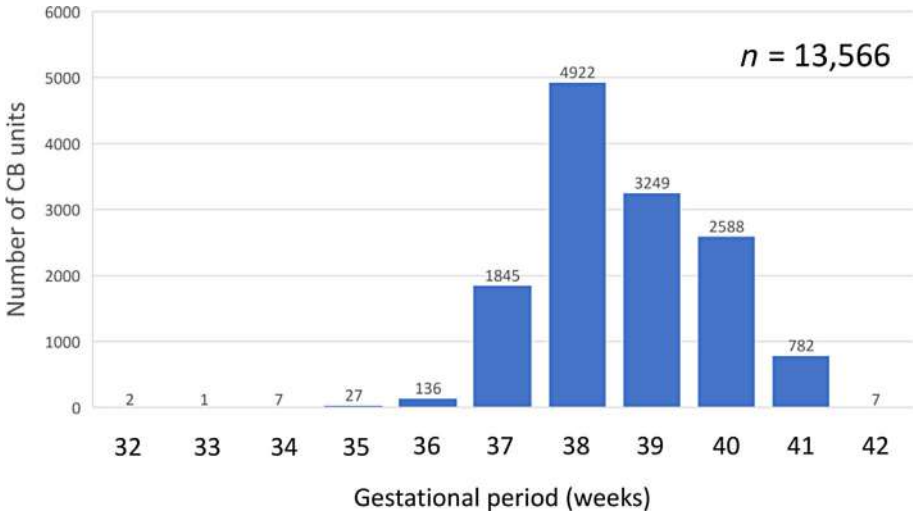

FIGURE 3 Histogram of the gestational period for cord blood (CB) units that were received with gestational information.

Importantly, there was a strong correlation between the number of CD34+ cells and CFU both before freezing and after thawing (Figure 2c,d, $R^2 = 0.756$ and 0.773, $p < 0.000001$). On the other hand, there was no correlation between TNC and CD34+ cell numbers (Figure 2e,f, $R^2 = 0.112$, $p = 0.088$ and $R^2 = 0.144$, $p = 0.051$).

The shorter the gestational period, the higher the number of CD34+ cells

Of all the CB received, 46% had their gestational period information. The range of the gestational periods was from 32 to 42 weeks (Figure 3). Collection at less than 37 weeks was performed at the discretion of the obstetrician. The average gestational period of the accepted and processed CB units was 38.8 weeks. Interestingly, the shorter the gestational period, the higher the number of CD34+ cells (Figure 4a). The number of CD34+ cells after processing was significantly higher for each of the gestational periods of 35, 36, 37 and 38 weeks compared with each or most of the longer gestational periods, as shown in Figure 4a. There were no significant differences between the counts at 39-week gestation compared with each of 40 or 41 weeks. In contrast, the shorter the gestational period, the lower the TNC count (Figure 4b). The gestational period and CB volume were not correlated (Figure 4c). Therefore, if the gestational period is 38 weeks or less, it is advisable to measure the CD34+ cell count even if the TNC count is low or the CB volume is lower than 60 mL.

There was no correlation between the time from collection to cryopreservation and the CD34+ cell recovery rate

We started processing CB units within 33 h from collection, and the average time from collection to reception was 17.83 h. The time was not correlated with the number of TNC and CD34+ cells after processing within the permitted 33 h (Figure 5a–c).

In units requested for transplantation, the average time from collection to reception was 17.25 h. The average time from collection to cryopreservation was 21.64 h, with a range of 4.57–35.32 h (Figure 6a). After processing, the average TNC was 1.49 billion, CD34+ cells were 5.17 million and CFU were 2.63 million. Histograms comparing the received and requested units are in Figure S1. The units requested for transplantation tended to be those with high cell numbers, given the relatively low importance of HLA matching in adult CB transplantations [8].

After freezing and thawing, the recovery rates (mean ± SD) were 93.4 ± 6.8% for TNC, 83.1 ± 13.0% for CD34+ and 83.1 ± 19.5% for CFU. There was no correlation between the time from collection to cryopreservation and either the CD34+ cell recovery rate (Figure 6b)

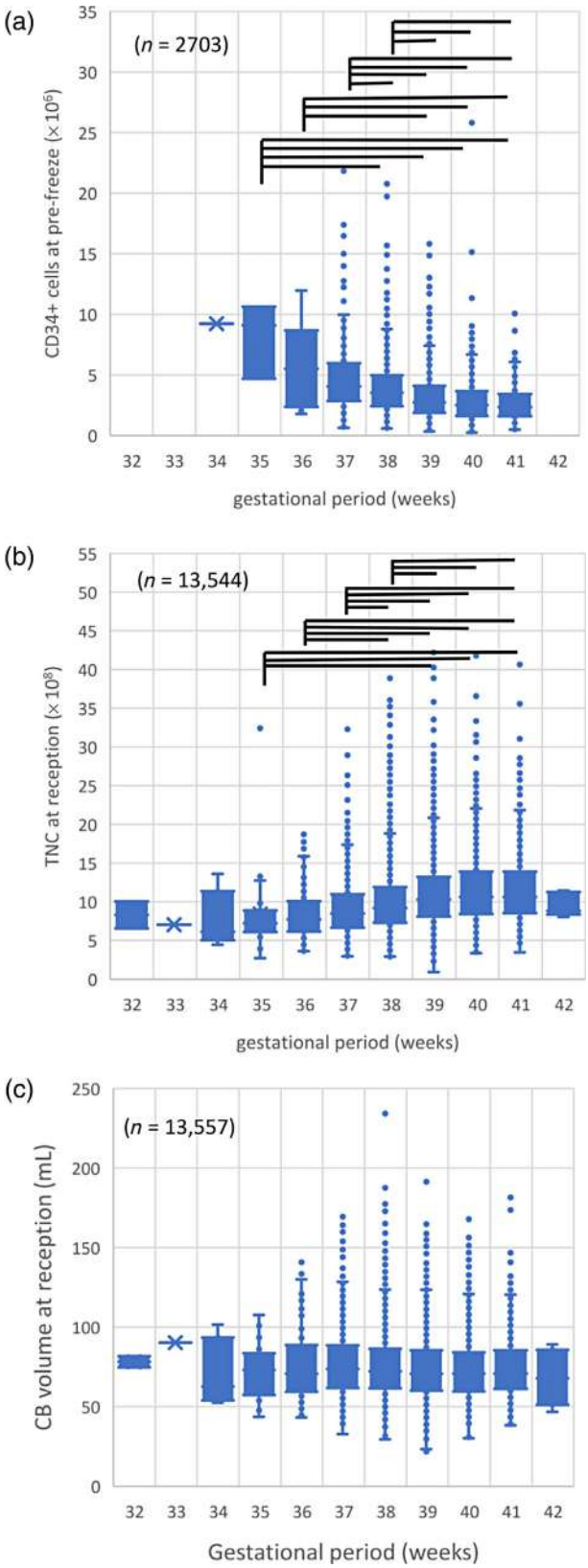

FIGURE 4 (a) CD34+ cell count by weeks of gestation. (b) Total nucleated cell (TNC) count by weeks of gestation. (c) Cord blood (CB) volume by weeks of gestation. Bars indicate significant differences ($p < 0.05$).

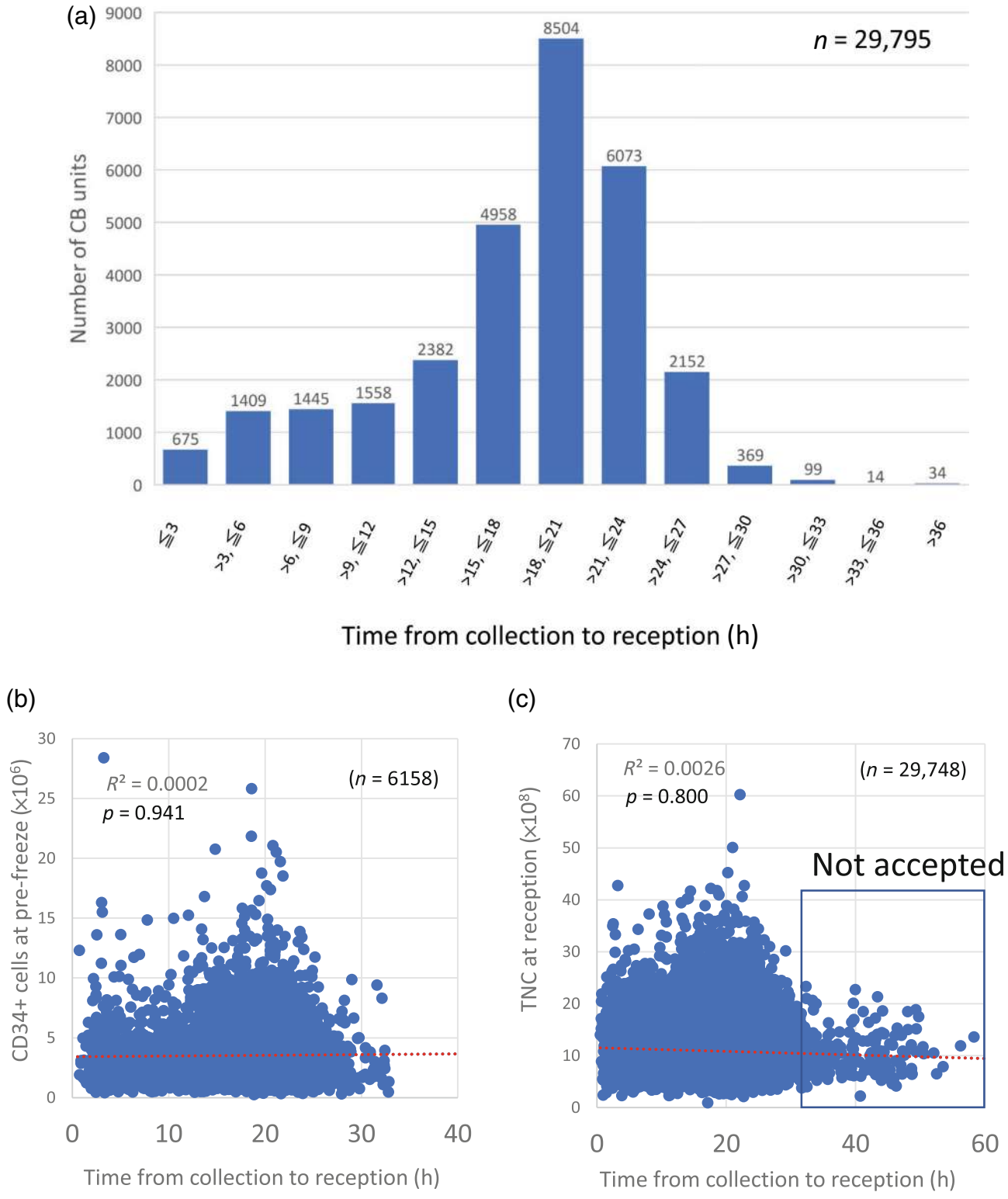

FIGURE 5 (a) Histogram of the time from collection to reception of all cord blood (CB) units. (b) Correlation between the time from collection to reception and the number of CD34+ cells after processing. (c) Correlation between the time from collection to reception and the number of total nucleated cell (TNC) before processing.

or the TNC recovery rate (Figure 6c). The average time in cryopreservation for units used for transplantation was 686 days and the maximum was 2738 days. There was no correlation between the storage period and either the CD34+ cell recovery rate (Figure 7a) or the CFU recovery rate (Figure 7b). For the transplanted units, 68.8% were used within 2 years after cryopreservation (Figure 7c).

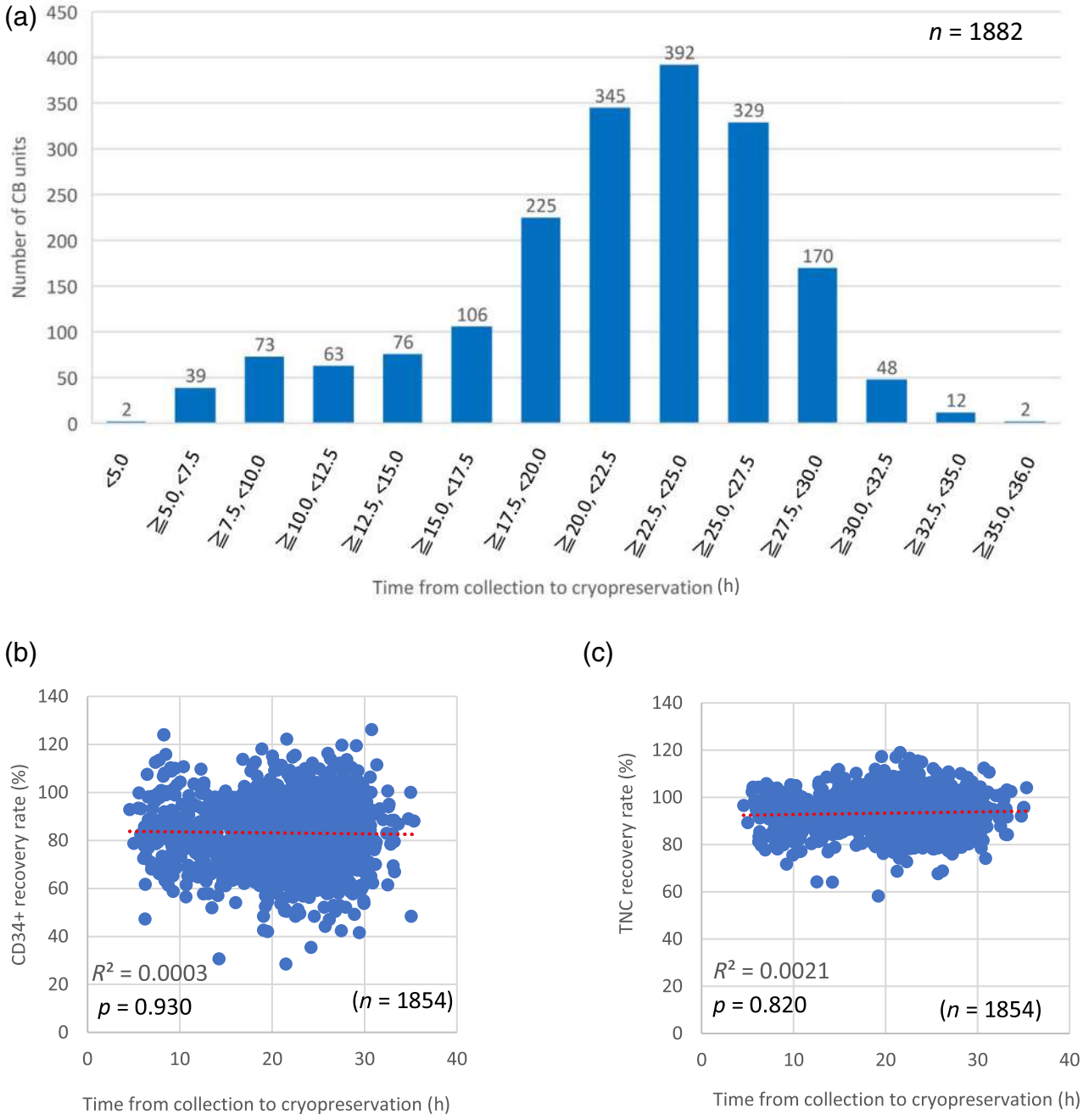

FIGURE 6 (a) Histogram of the time from collection to cryopreservation for the cord blood (CB) units requested for transplantation. (b, c) Correlation between the time from collection to cryopreservation and the CD34+ cell recovery rate and total nucleated cell (TNC) recovery rate from freeze and thaw.

DISCUSSION

The CB volume was not strongly correlated with the TNC count ($R^2 = 0.462$, $p < 0.0001$), and not at all with the CD34+ cell count ($R^2 = 0.061$, $p = 0.215$) in this study. Therefore, we recommend measuring the number of TNC and CD34+ cells regardless of the volume.

The shorter the gestational period, the higher the number of CD34+ cells. In contrast, the shorter the gestational period, the lower the TNC count. The gestational period and CB volume were not correlated. Thus, if the gestational period is 38 weeks or less, it is advisable to measure the CD34+ cell count even if the TNC count or the CB volume is low.

We have found some units with a high CD34+ count when the gestational period is 38 weeks or less. Also, the transplant centres now tend to choose CB by the CD34+ count rather than TNC. In response, our CBB introduced pre-processing CD34+ cell counting for units with TNC of 1.2–1.4 billion. Our decision to count CD34+ cells based on the TNC counts was practical, considering laboratory workload, testing, processing and storage costs, as well as CB banking efficiency.

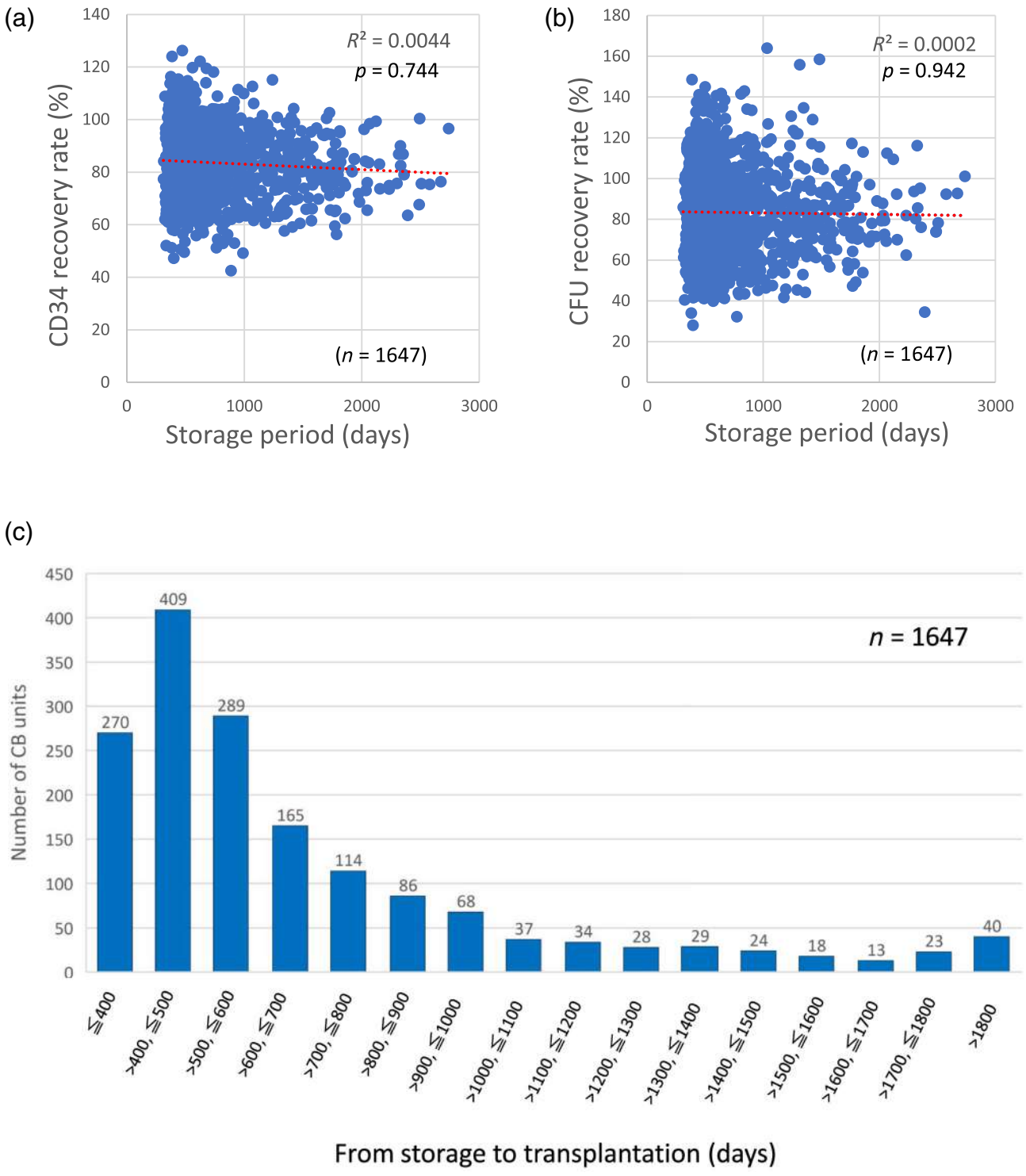

FIGURE 7 (a) Correlation between storage time and CD34+ recovery rate. (b) Correlation between storage time and colony-forming unit (CFU) recovery rate. (c) Histogram of the storage period of the cord blood (CB) units requested for transplantation.

We are now preparing to measure CD34+ cell counts for units of 1.1–1.4 billion TNC, thus lowering the minimum. Because of the higher CD34+ counts in shorter gestations, we should consider counting CD34+ cells regardless of TNC for units at less than 39 weeks, although we have to consider the laboratory workload. CB units with over 2.5 million CD34+ cells should be processed for storage. The CB volume limit could also be lowered.

We found a high incidence of clot formation. To try to reduce this, we started changing the size of the collection bag from 200 to 400 mL, which doubled the amount of CPDA used, after the period of this study. In addition, our processing of the CB units has been manual, and the size limit for a blood clot to be recorded is not defined in Japan. We record even very small clots. Overseas, the processing of CB is mechanized and it is passed through a filter first, which may

allow small clots to be ignored. After studying the effects of filters, we decided to filter blood clots from our CB units from April 2024.

The time between CB collection and its cryopreservation had no effect on post-thaw CD34+ cell recovery within the 36 h that we allow. Leukocyte viability decreases when that period is between 49 and 80 h [9], and internationally, some facilities allow up to 48 hours before beginning cryopreservation [10, 11]. The average time was 21.64 h in this study.

The recovery rate for CD34+ cells and CFU after thawing was not affected by the storage period. Therefore, with high-count CB units, we can thaw and test an attached segment before it is needed for transplantation. Then, on receiving a request, we could send such a unit immediately to the transplant centre. For units that have a high likelihood of being selected, each CBB can decide which ones to test post-thaw, with consideration for workload and testing cost.

Post-thaw data analysis, which is done at the CBBs and given to the transplantation centres, requires accurate testing of cell viability. There have been significant differences reported in CD34+ cell viability among CBBs around the world. Wagner et al. reported that only 43% of CBBs provided results of cell viability testing as of 2006 [12], and that cell viability reported by transplantation centres appeared to be 33% lower than what was reported by CBBs. More recently, the Biomedical Excellence for Safer Transfusion (BEST) Collaborative surveyed laboratory practices for viability testing and suggested testing by flow cytometry with a 7-AAD dye as a first step towards standardization [11]. The Foundation for the Accreditation of Cellular Therapy (FACT) standards for CB banking requires CBBs to examine the viability of TNC, CD34+ and CFU or use another validated potency assay after thawing a contiguous segment or a representative sample before releasing CB to a transplantation centre [10].

The FDA requires 'TNC count, viability and viable CD34+ cells' as minimum testing criteria [13]. Regrettably, none of these three items indicate engraftment ability. Even if the TNC is high, there may be no stem cells in the CB unit, and there is little reliable scientific information as to the suitability of the unit for transplantation [14]. TNC contains many kinds of cells and is inadequate for assessing the quality and potency of stem cells. The potential for engraftment cannot be accurately predicted by TNC. Although the CFU assay is difficult to standardize and has been shown to yield very high inter-laboratory coefficients of variation, CFUs are important for assessing the quality of the CB unit. No growth of CFU is an indicator of poor haematopoietic reconstitution potential [12]. Based on the importance of the CFU assay and the clear correlation in our laboratory between the CD34+ cell counts and CFU, we count the CD34+ cells if the TNC number is between 1.2 and 1.4 billion when we receive the CB, to decide whether to process it for cryopreservation. To use CD34+ as the testing criterion, it is important for each laboratory to confirm the correlation between the CD34+ cell counts and CFU.

A limitation of this study is that the data shown here are from only one of our CBBs. In the future, it would be desirable to analyse data from all Japanese CBBs. We believe that this report will contribute to improving the efficiency of CBBs and the results of CBTs.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST STATEMENT

There are 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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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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Anti-monkeypox virus-neutralizing activities of human immunoglobulin manufactured between 1999 and 2021 and derived from donors in the United States and Japan

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Abstract

Background and Objectives: In May 2022, the United Kingdom reported the first case of chained transmission of the monkeypox (mpox) virus without any known epidemiological links to west or central Africa. The monthly number of mpox patients currently has passed a peak and is declining globally, and infected patients include both non-vaccinated and vaccinated individuals. Herein, the virus-neutralizing (VN) activity against vaccinia viruses, which are considered to cross-react with the mpox virus, in the intravenous immunoglobulin (IVIG) lots derived from donors, including vaccinated Japanese populations, was evaluated to clarify the status of the Japanese blood donor population.

Materials and Methods: VN titres against vaccinia and human mpox viruses in IVIG lots derived from donors in Japan and the United States manufactured between 1999 and 2021 and 1995 and 2001, respectively, were evaluated by neutralization testing.

Results: VN titres of IVIG derived from donors in Japan and the United States against vaccinia and mpox viruses showed a slowly decreasing trend between 1999 and 2021.

Conclusion: VN titres are expected to decrease in the future since the percentage of vaccinated donors in the donor population seems to have decreased. Therefore, continuous monitoring of VN titres is required.

Keywords

IVIG, mpox, neutralization, vaccinia

Highlights

- The anti-monkeypox (mpox) virus-neutralizing (VN) activities of human immunoglobulin were investigated.
- The study revealed that the VN titres were stable or had slightly reduced over the last 20 years.
- These titres are expected to be reduced in the future because mpox-vaccinated donors will stop donating blood and plasma.

INTRODUCTION

Variola, also known as smallpox, and human monkeypox (mpox) are infectious diseases caused by variola and mpox viruses, respectively. These viruses belong to the genus *Orthopoxvirus*, and globally, smallpox vaccine strains are developed from the vaccinia virus, which belongs to the same genus. The latest case report of variola was published in 1977, and the World Health Organization (WHO) declared its eradication in 1980 [1]. In Japan, the vaccination programme was discontinued in 1976 [2]. Individuals born after 1976 in Japan have no antibodies against the virus since they had no opportunity of natural infection, and the population that received the smallpox vaccine did not receive additive immunization by natural infection after the programme's discontinuation. However, the immunoglobulin G (IgG) positivity against the vaccinia virus has persisted for at least 25 years after the discontinuation of the vaccine. According to a previous study, the seropositivity against the vaccinia virus in healthcare workers in Japan collected in 2002 by enzyme-linked immunosorbent assays (EIA) (OD405 ≥ 0.30) was 56.5% (495/876) and by virus-neutralizing (VN) assay (≥4) was 65.1% (99/152) [2]. In addition, the seropositivity of the donors born after 1975 by EIA was 0%. Another study by Takeuchi et al. using influenza vaccine-administered donors collected in 2002–2003 reported that seropositivity against vaccinia virus by the VN assay (≥4) was 55.8% (67/120) [3]. On the other hand, VN titres against the vaccinia virus ranged from 66.0 to 198.3 (mean titre = 121.0) in intravenous immunoglobulin (IVIG) products manufactured in 2003 in the United States and European countries [4].

The calculated equivalent VN titre range in the source plasma was 13.2–39.7 as the IVIG products used in the study contain 5% IgG (50 mg/mL), which is approximately five times concentrated from in plasma. The titres reflect the seropositivity of the donor population in the plasma collection region.

Mpox is a zoonosis that circulates in west or central Africa. The first chained transmission case without known epidemiological links to west or central Africa was reported in the United Kingdom in May 2022, and further cases were reported in European countries [5]. The current worldwide situation of mpox is that the number of infected patients passed a peak and went up and down at a lesser level each month [6]. The reported mpox patients include those who had smallpox vaccination [7]. The WHO has recommended that only some risk holders should receive the smallpox vaccine and that mass vaccination is not required [8]. The current seroprevalence of the population and the antibody titres of IVIG products against the mpox virus have not yet been clarified, as approximately 50 years have already passed since the discontinuation of vaccination. To clarify the situation in the Japanese population, normal IVIG lots derived from plasmas donated in Japan and the United States that were manufactured between 1999 and 2021 and 1995 and 2001, respectively, were evaluated for VN titres against vaccinia and mpox viruses.

MATERIALS AND METHODS

The vaccinia virus WR strain [9] and an mpox virus clinical isolate from a specimen of the patient in Osaka, Japan, in March 2023 (manuscript in preparation, Japan/Osaka/RIMD_OCGH-2/2023, GenBank Accession No. PP587855) used for the VN assay were propagated in African green monkey kidney cells CV-1 and Vero, respectively. A normal IVIG product, Venoglobulin-IH 5% (containing 50 mg/mL IgG, Japan Blood Products Organization, Tokyo, Japan), which is derived from plasma recovered from whole blood and apheresis plasmas donated in Japan (VGIH-JP) and manufactured in 1999, 2012, 2018 and 2021, and those derived from apheresis plasmas donated in the United States (VGIH-US) and manufactured in 1995, 1999 and 2001 (the VGIH-US was discontinued in 2001) were used.

The VN assay was designed to evaluate the infection and propagation steps, as diluted IVIG after reaction with the virus in the culture media was not removed. The CV-1 or Vero cells were seeded in 24-well plates and cultured in minimum essential medium supplemented with 10% foetal bovine serum (FBS) and then cultured till approximately 90% confluent. The IVIG sample was diluted four times with phosphate-buffered saline containing 1% FBS, and then serially diluted twice with a minimum essential medium containing 5% FBS. The diluted samples were mixed with each virus (approximately 50 plaque-forming units) and incubated for 60 min at room temperature. Reacted samples (100 μL) were then inoculated into CV-1 or Vero cells in a 24-well plate. Three days after inoculation, the cells were fixed with formalin in phosphate-buffered saline and stained with methylene blue. Plaques were observed, and VN titres were expressed as the highest dilution with more than 50% plaque reduction. Measurements were performed in duplicate or triplicate, and the mean titre was calculated from three tests. When calculating the mean titre, the VN titre was set to zero if the titre was <4. The difference in the VN titres of the VGIH-JP manufactured between 1999 and 2021 was evaluated by a standard *t*-test. The percentages of the population born before 1976 in each year of the total population and blood donor population in Japan were calculated using open-source data [10, 11]. Furthermore, the estimated percentages after 2023 are predicted based on an equal share of the data obtained in 2022. This study was approved by the Ethics Committee of the Japan Blood Products Organization (2021–003). Blood donors born before 1976 in Japan received the smallpox vaccine.

RESULTS

The VN titres of VGIH-JP and VGIH-US against vaccinia and mpox viruses are summarized in Table 1. The titres against the vaccinia virus in VGIH-JP lots manufactured in 1999, 2012, 2018 and 2021 were 21.3, 16.0, 13.3 and 13.3, respectively. The data indicate a slowly decreasing trend in titres, although not statistically significant, between 1999 and 2021. In contrast, the titres of VGIH-US manufactured in 1995, 1999 and 2001 were 64.0, 32.0 and 21.3, respectively. The data indicated that the titres decreased between 1995 and 2001. The titres of VGIH-US were higher than those of VGIH-JP. The titres against the mpox virus of VGIH-JP manufactured in 1999, 2012, 2018 and 2021 were weaker than those against the vaccinia virus, as the anti-mpox virus titres were 4.0, 5.3, 1.3 and <4.0, respectively. Similarly, the titres of VGIH-US manufactured in 1995, 1999 and

TABLE 1 Neutralizing titres and percentage reduction in plaques against vaccinia and mpox viruses.

		Derived from plasmas donated in Japan				Derived from plasmas donated in the United States		
Manufactured year		**1999**	**2012**	**2018**	**2021**	**1995**	**1999**	**2001**
VN titres Mean ± SE	Vaccinia	21.3 ± 4.4	16.0 ± 0.0	13.3 ± 2.2	13.3 ± 2.2	64.0 ± 0.0	32.0 ± 0.0	21.3 ± 4.4
	Mpox	4.0 ± 1.9	5.3 ± 2.2	1.3 ± 1.1	<4 ± 0.0	10.7 ± 2.2	13.3 ± 2.2	2.7 ± 1.1
Percent reduction at ×4 dilution Mean ± SE	Vaccinia	84.0 ± 3.3	76.4 ± 4.2	81.3 ± 3.9	81.4 ± 1.9	90.6 ± 1.7	89.8 ± 2.0	89.3 ± 1.7
	Mpox	50.1 ± 2.2	48.3 ± 3.2	46.0 ± 2.9	40.3 ± 0.5	62.2 ± 4.9	55.8 ± 3.8	51.9 ± 1.7

Abbreviations: mpox, monkeypox; SE, standard error; VN, virus-neutralizing.

2001 were 10.7, 13.3 and 2.7, respectively. These data indicate that the VN titres against vaccinia and mpox viruses of VGIH-JP and VGIH-US showed a slowly decreasing trend.

Our experiments indicated that the virus was not neutralized completely since viral plaques were detected at a minimum dilution. It has been reported that the vaccinia virus has three mechanisms of resistance against the immune system, one of which is resistance to antibody neutralization against the virus [12]. Therefore, the plaque reduction percentages were also evaluated (Table 1). The reduction percentages against the vaccinia virus of VGIH-JP manufactured in 1999, 2012, 2018 and 2021 were 84.0%, 76.4%, 81.3% and 81.4%, respectively. In contrast, the VGIH-US manufactured in 1995, 1999 and 2001 were 90.6%, 89.8% and 89.3%, respectively. The data indicate that the plaque reduction percentages were at the same level during all periods for the VGIH-JP and VGIH-US. The reduction percentages of the mpox virus in VGIH-JP manufactured in 1999, 2012, 2018 and 2021 are 50.1%, 48.3%, 46.0% and 40.3%, respectively. In addition, the plaque reduction percentages of VGIH-US manufactured in 1995, 1999 and 2001 were 62.2%, 55.8% and 51.9%, respectively. The data indicated that the plaque reduction percentages were at the same level during all periods for the VGIH-JP and VGIH-US, and both percentages may decrease slightly over time.

The VN titres against the vaccinia virus were combined with the percentages of the population born before 1976 in each year of the total population and blood donor population of Japan (Figure 1). The percentage of the population born before 1976 among the blood donor population in Japan is still reducing, and the VN titres seem to be slowly decreasing.

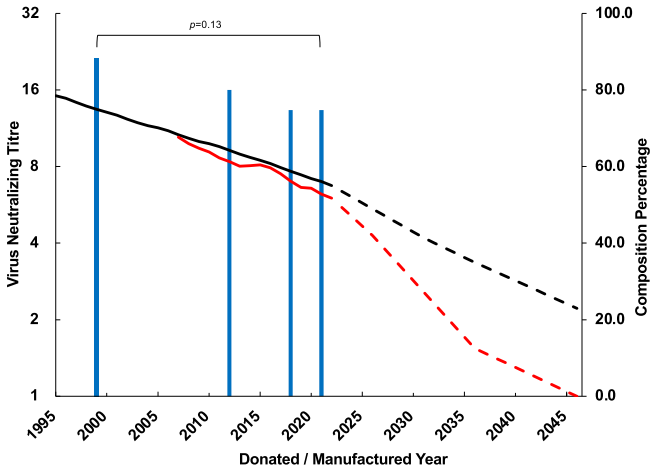

FIGURE 1 The transition of the composition of the population born before 1976 in the overall population in Japan or blood donor population from 1995 to 2045 and the virus-neutralizing (VN) titres against vaccinia virus from 1999 to 2023. The percentage of the population born before 1976 in the overall population between 1995 and 2022 is represented by a black solid line, and an estimated percentage between 2023 and 2045 is shown by a black broken line. The percentage of blood donor population born before 1976 in the donor population between 1995 and 2022 is shown by a red solid line, and an estimated percentage between 2023 and 2045 is represented by a red broken line. The VN titres against the vaccinia virus of Venoglobulin-IH 5% derived from plasma donated in Japan manufactured between 1999 and 2021 (shown as blue bars).

DISCUSSION

In 2022, a threat of mpox emerged approximately 50 years after the discontinuation of the smallpox vaccination. After a vaccine programme is discontinued, neither the general population that has received vaccinations nor the population that was born after the programme was discontinued have any opportunity of receiving additional immunization by natural infection or booster doses. Therefore, it is important to understand the current seropositivity level of the population to implement measures against the threat of mpox. The seropositivity status of the donated population, time period and region is reflected in the immunoglobulin products, which come from tens of thousands of donors. In the present study, to clarify the state of seropositivity in Japan, VN titres against the vaccinia virus and mpox virus clinical isolates of VGIH-JP manufactured between 1999 and 2021 were evaluated. In addition, VGIH-US manufactured between 1995 and 2001 were also evaluated.

The VN titres against the vaccinia virus of the VGIH-JP manufactured between 1999 and 2021 were 21.3–13.3. The equivalent titres of plasma level were calculated to be 4.3–2.7. The titres showed a slowly decreasing trend between 1999 and 2021. Thus, it seems that the seropositivity of the donor population is weak because non-immunized individuals are also included in the donor population. Although the titres against mpox were also very weak, the donor population may have cross-reactive antibodies against the vaccinia and

mpox viruses. We should pay attention to a lack of clinical evidence of the effectiveness of neutralization titres on actual viral infection. The VN titres against the vaccinia virus of normal IVIG products manufactured in the United States and European countries in 2003 were 66.0–198.3 (mean titre was 121.0) [4]. Takeuchi et al. reported that the estimated average VN titre across all age groups of plasma donated in 2002–2003 was 10.2 [3] and that the calculated equivalent VN titre of 5% IVIG was 51.0. Therefore, IVIG titres in the United States and EU seem to be higher than those in Japan. The reason for this difference remains unclear. Seropositivity and titre against the pathogen of IVIG are reflected in the donor's background, such as collected region, period, frequency and pooled numbers. The VGIH-JP lot was constituted of several tens of thousands of plasmas, and the maximum donation count of recovered or apheresis plasmas was 6 or 24 per year, respectively. Contrarily, the VGIH-US lot was constituted of a few tens of thousands of apheresis plasmas, and the maximum donation count of each donor was twice per 7 days. To clarify the reason for the difference, it is necessary to investigate the background of the donor population in the United States and Japan, as described above.

It is expected that in the future, the vaccinated population in Japan will decrease. This will be especially noticeable among blood donors, as the maximum retirement age for donors is 69 years. By the year 2045, there will likely be no vaccinated donors in Japan. In Japan, blood donation is allowed for people aged between 16 and 65 years, with some certified donors being able to donate until 69 years of age. Clearly, the seropositivity of the total population in Japan will continue to decrease in the future. The titres of IVIG against the vaccinia virus seemed to rapidly decrease in 2045, reflecting the seropositivity of the blood donor population. It is possible that measures against not only mpox but also other epidemic pox viruses, such as the development of new vaccines or medicines, would be required in the future.

Different products utilize different manufacturing process conditions. Different reactivity against pathogens of different products derived from the same collection region of the source plasma was reported [13]. Furthermore, it is considered that the difference in the collection region of the source plasma that is affected by the epidemiological situation more strongly affects reactivity against pathogens than manufacturing process conditions. Therefore, possible limitations of the present study may include difficulty in generalization of our findings to other products in the world.

Sarkar et al. evaluated the minimum protective neutralizing titres using volunteers among the contacts of smallpox cases [14]. Volunteers with vaccination scars were considered smallpox vaccinees, and they did not develop smallpox, even with a neutralization titre of <10. The results suggested that the vaccinees could have memorized cellular immunity and did not develop smallpox. Contrarily, the minimum protective neutralizing titre of the volunteers who did not receive the vaccination and may not have memorized immunity was 20. Currently, the threat of infectious diseases caused by *Orthopoxvirus* infections, such as mpox, in the population, especially without vaccination, is increasing. Therefore, a minimum protective titre of 20 is noteworthy, although it is difficult to directly compare their results with ours. To evaluate the mass immunity against the threat, further studies, such as continuous monitoring for the transition of seropositivity and cellular immunity of the population with and without the vaccination, are required.

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M.Y. contributed to study conception, design, VN assay, data management, data interpretation and writing of the original draft; R.K.-K. contributed to study design, VN assay and data interpretation; T.S. contributed to supervision, study design and data interpretation.

CONFLICT OF INTEREST STATEMENT

Mikihiro Yunoki is an employee of Japan Blood Products Organization. The other authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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SHORT REPORT

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A novel haemodilution chip mounted on the total thrombus-formation analysis system, a flow chamber system, enables stable analysis of platelet products under low platelet concentration/high shear conditions

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Abstract

Background and Objectives: The total thrombus-formation analysis system (T-TAS) can quantitatively analyse the contribution of platelets to haemostasis using reconstituted blood samples. However, it is unsuitable in cases with low platelet counts. We introduced a haemodilution (HD) chip with a shallow chamber depth, adapted to low platelet counts and high shear conditions (1500 s^{-1}).

Materials and Methods: Blood samples were prepared by mixing red blood cell products, standard human plasma and platelet products; the final platelet count was 50×10^3/μL. Aggregation tests were performed by using the aggregation inducers collagen, adenosine diphosphate (ADP) and ristocetin. Samples with 2-, 4- and 9-day-old platelet products ($N = 10$) were evaluated.

Results: The HD chip enabled the stable analysis of the haemostatic function of all samples at a platelet count of 50×10^3/μL. Haemostatic function was correlated with ADP aggregation (time to 10 kPa [T_{10}]: $r = -0.53$; area under the curve for 30 min: $r = 0.40$) and storage period (T_{10}: $r = 0.44$).

Conclusion: The HD chip-mounted T-TAS can stably analyse haemostatic function under low platelet counts and high shear conditions; this approach is expected to serve as a bridge to in vivo haemostatic tests with experimental animals.

Keywords

haemostasis, high shear stress, low platelet count, total thrombus-formation analysis system

Highlights

- A modified microchip-based flow chamber system was developed to analyse haemostatic function under low platelet counts/high shear conditions.
- The properties of red blood cells on day 3 after blood collection in reconstituted blood, even when the donors were different, had little effect on haemostatic function.
- The haemostatic function of platelet concentrates was correlated with platelet aggregation induced by adenosine diphosphate and their storage period.

INTRODUCTION

The haemostasis mechanism consists of platelet adhesion to the vascular injury site, platelet thrombi (primary haemostasis) in which adhered platelets form aggregates and fibrin thrombus (secondary haemostasis) in which fibrinogen incorporated into platelet thrombi accumulates as fibrin. In vitro, primary haemostasis has been analysed using an aggregation test with platelet-rich plasma and secondary haemostasis using a coagulation assay with platelet-poor plasma [1, 2]. However, accurately reflecting the mechanisms of in vivo haemostasis through these assays is challenging. This is because haemostasis progresses through platelet adhesion, as platelets are extruded by red blood cells (RBCs) into the vascular endothelium (margination) and through the interaction of activated platelets with coagulation factors [3]. To overcome these points, flow chamber systems, which allow for real-time observation and analysis of the thrombus formation process under flow conditions, have contributed greatly to the improved understanding of haemostatic mechanisms.

A simple thrombus-formation evaluation kit, called the total thrombus-formation analysis system (T-TAS; Fujimori Kogyo, Tokyo, Japan), has been developed to comprehensively evaluate primary and secondary haemostases in whole blood samples under flow conditions (Figure S1) [4]. The microchip used in the T-TAS is coated with type I collagen and tissue thromboplastin to monitor changes in flow pressure that reflect the growth of platelet- and fibrin-rich thrombi. In our previous study, we reported the usefulness of the T-TAS as a novel in vitro test to quantitatively analyse the contribution of platelet products to haemostasis in reconstituted blood samples prepared from RBCs derived from healthy individuals, standard human plasma (SHP; Siemens Healthcare Diagnostics GmbH, Forchheim, Germany) and various concentrations of platelet products [5]. This system may have the potential to more closely resemble the clinical situation of post-platelet transfusion than other conventional in vitro haemostatic tests and may be considered as platelet transfusion in a test tube; however, it has some limitations. First, low platelet concentration may not be measurable because the T-TAS quantifies changes in flow pressure with thrombus formation. Second, high shear conditions cannot be reproduced. Recently, to overcome these points, a modified chip (haemodilution [HD] chip; Fujimori Kogyo) with a shallower chamber depth has been developed, allowing higher sensitivity in detecting elevation of flow pressure caused by growth of an occlusive thrombus in the T-TAS analytical chamber for evaluations under low platelet concentration [6]. Compared with the conventional chip, the HD chip allows for the haemostatic function evaluation under high shear stress (1500 s^{-1}; conventional chips: 600 s^{-1}), allowing for a comprehensive haemostasis evaluation that is more dependent on platelet function than plasma factors. Samanbar et al. [7] have reported the usefulness of the HD chip-mounted T-TAS as a new tool for evaluation of haemostasis in thrombocytopenic patients after platelet transfusion.

In this study, we aimed to establish a sensitive and quantitative evaluation method to detect haemostatic function changes of platelet concentrates (PCs) in low platelet concentration/high shear conditions using an HD chip-mounted T-TAS. In addition, changes in haemostatic function with PC storage were measured using an HD chip-mounted T-TAS, compared with conventional platelet test results, and the physiological and functional significance of the novel T-TAS-based haemostatic test was discussed.

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).

Preparation of PCs

The PCs were collected using an apheresis system, Trima Accel (Terumo BCT, Tokyo, Japan) or Component Collection System (Haemonetics, Boston, MA, USA), from healthy volunteers, according to the donor selection guidelines of the Japanese Red Cross Society. The PCs were irradiated with 15 Gy of x-rays (MBR-1530A-TW; Hitachi Healthcare Systems, Tokyo, Japan) and were stored at 22°C with agitation at 60 cycles/min (NR-30; Panasonic, Osaka, Japan).

Novel haemostatic test and conventional aggregation tests

Platelet samples were collected on day 2 of PC storage (setting the day of platelet collection to day 1), the freshest platelets available for the study; day 4, the expiration date; and day 9, 1 week after the initial collection. The sampling was performed by aseptic collection of 3 mL each using an 80 mL bag (BB-T008FJ; Terumo BCT).

We introduced an HD chip-mounted T-TAS (Fujimori Kogyo) [4, 6]. An overview of the T-TAS instrument with the HD chip and analysis parameters is shown in Figure S1. Since the test samples were reconstituted blood mixed with citrate-treated standard RBCs, SHP and platelet samples, they were mixed with 20 μL of 0.3 M $CaCl_2$ containing 1.25 mg/mL of corn trypsin inhibitor immediately before analysis to restore the coagulation system, as described previously [5]. Briefly, the reconstituted blood was prepared from the RBC pellets obtained by adding an equal volume of phosphate-buffered saline to the RBC products stored at 4°C for 3 days and washing by centrifugation at 500*g* for 15 min and SHP not for transfusion with anticoagulant citric acid containing 0.86–1.06 IU/mL coagulation factors and 2.57 g/L fibrinogen and the platelet sample. The target values were a platelet count of 50 × 10^3/μL and haematocrit value of 40%. The haemostatic test was performed on 10 PCs from different donors. Standard RBCs were obtained from random donors for each day of the test, and the same lot products were used for SHP. The flow rate can be set to 4 or 10 μL/min. We selected 10 μL/min, which corresponds to an initial wall shear rate of approximately 1500 s^{-1} in the chamber because high shear stress allows for the analysis of haemostatic function caused by platelets rather than coagulation factors.

The maximum percentage of aggregation was determined using an aggregometer (PRP313M; Taiyo, Osaka, Japan). The samples were prepared by mixing the platelet sample and the autologous plasma obtained by centrifuging the platelet sample (1810*g*; 10 min). The target platelet count was set at $300 \times 10^3/\mu L$. Aggregation inducers, that is, collagen, adenosine diphosphate (ADP) and ristocetin, were prepared as previously reported [8]. The methods for determining the platelet count; mean platelet volume and expression levels of CD62P, procaspase activating compound-1 (PAC-1) and annexin V are shown in Supporting Information S1.

The effect of different donor-derived RBC products on the haemostatic function

Reconstituted blood with a platelet count of $50 \times 10^3/\mu L$ and haematocrit value of 40% was prepared using the same sample sources for plasma and platelets, but using different donor-derived sample sources for RBCs only, and haemostatic function was measured. SHP was used in the same lot containing 0.86–1.06 IU/mL coagulation factors and 2.57 g/L fibrinogen, and platelets were collected from one donor-derived product and used on day 2 of platelet storage. On the other hand, the RBCs were collected from five different donors and used day 3 of RBC storage.

Statistical analyses

Data analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Results are expressed as the mean ± standard deviation. All parameters on day 2 versus days 4 and 9 of platelet collection were compared using repeated measures analysis of variance, followed by Dunnett's multiple comparisons post hoc tests. Spearman's rank correlation coefficients were calculated

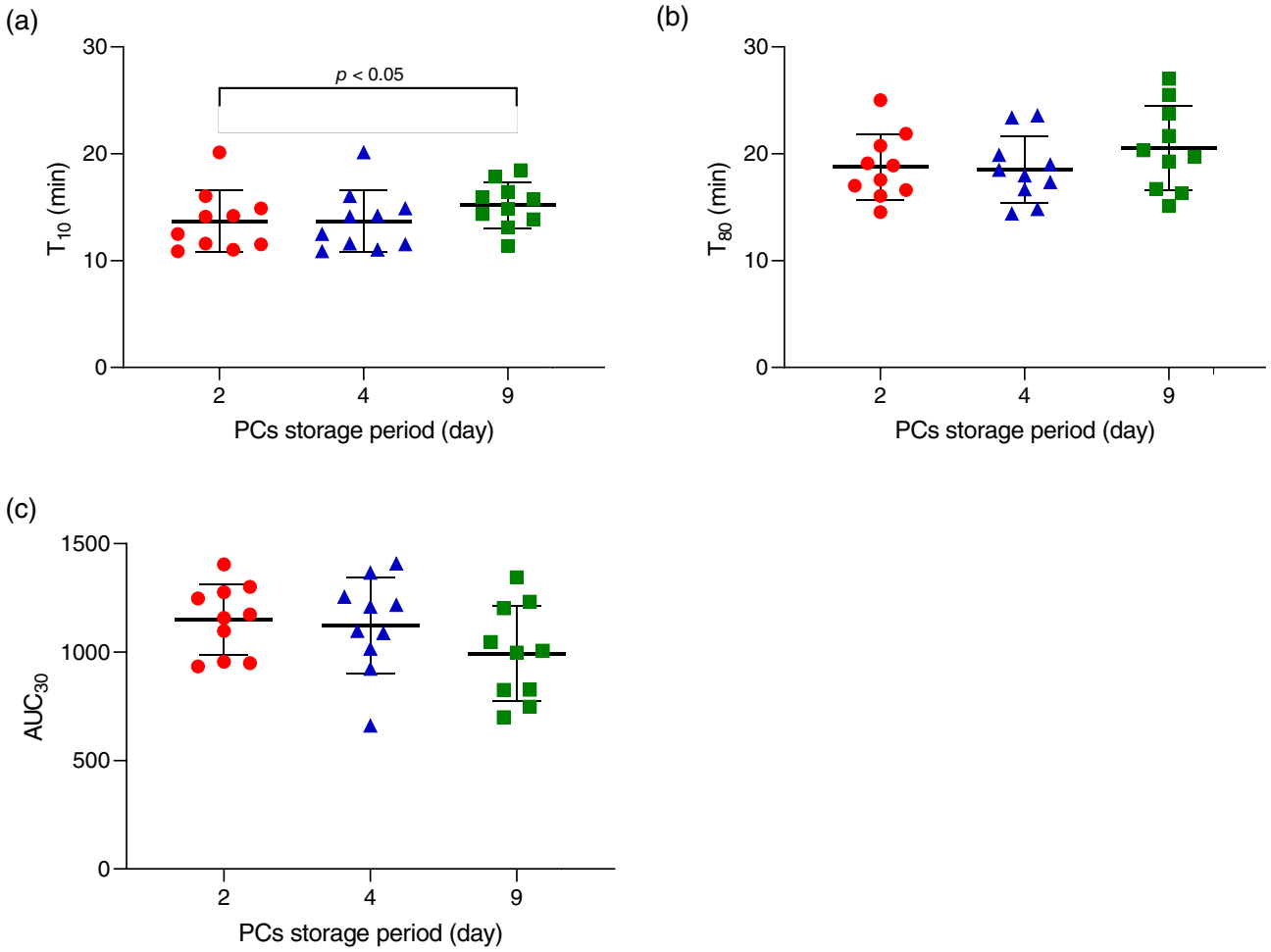

FIGURE 1 Haemostatic function of platelet concentrates (PCs) during storage with agitation at 22°C. (a) T_{10} (time in minutes for the flow pressure to reach 10 kPa), (b) T_{80} (time to 80 kPa: occlusion time in minutes) and (c) area under curve for 30 min (AUC_{30}) are indicated. We introduce a haemodilution chip-mounted total thrombus-formation analysis system (Fujimori Kogyo, Tokyo, Japan). The test samples are reconstituted blood (platelet count of $50 \pm 2 \times 10^3/\mu L$; haematocrit value of 37.3 ± 0.9%) prepared from platelet samples, standard red blood cells and standard human plasma. Data are expressed as the mean ± standard deviation, $N = 10$. T-TAS parameters on day 2 and on days 4 and 9 of platelet collection are compared by repeated measures analysis of variance followed by Dunnett's multiple comparisons post hoc tests. Statistical significance is set at $p < 0.05$.

between in vitro properties and the T-TAS parameters in the PCs. Statistical significance was set at $p < 0.05$.

RESULTS

Times to 10 kPa (T_{10}), a measure of initial thrombus formation, were 1.09-fold ($p = 0.108$) and 1.21-fold ($p < 0.05$) higher for platelets collected on days 4 and 9 than on day 2, respectively (Figure 1a). The times to 80 kPa, a measure of thrombus occlusion (enough to almost completely occlude the capillary) were 0.99-fold ($p = 0.954$) and 1.10-fold ($p = 0.225$) higher on days 4 and 9 than on day 2, respectively (Figure 1b). The areas under the curve for 30 min (AUC_{30}), a quantitative index of haemostatic function, were 0.98-fold ($p = 0.814$) and 0.86-fold ($p = 0.063$) higher on days 4 and 9 than on day 2, respectively (Figure 1c). The maximum percentage of aggregation decreased for collagen, ADP and their simultaneous aggregation on days 4 and 9 of platelet collection (Figure 2). Levels of the platelet activation marker CD62P and annexin V binding increased on days 4 and 9, and PAC-1 binding increased on day 9 (Table S1). Haemostatic function was correlated with the storage period (T_{10}: $r = 0.44$) and ADP aggregation (T_{10}: $r = -0.53$; AUC_{30}: $r = 0.40$) (Table 1).

Haemostatic function of reconstituted blood samples from five different RBC donors is presented in Table S2. The coefficients of variation for T_{10}, T_{80} and AUC_{30} were 5.1%, 4.9% and 5.8%, respectively. These results indicate that the use of different donor-derived RBCs on day 3 of storage had little effect on the T-TAS parameters.

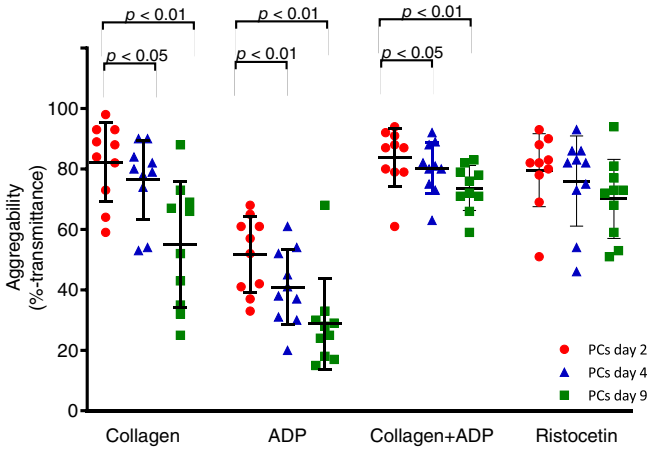

FIGURE 2 Aggregation abilities of platelet concentrates (PCs) during storage with agitation at 22°C. Aggregation abilities are measured using platelet-rich plasma (platelet counts of 295 ± 10 × 10^3/μL) prepared from platelet samples and autologous plasma using an aggregometer (PRP313M; Taiyo, Osaka, Japan). Data are expressed as the mean ± standard deviation, $N = 10$. Aggregation parameters on day 2 and on days 4 and 9 of platelet collection are compared by repeated measures analysis of variance followed by Dunnett's multiple comparisons post hoc tests. Statistical significance is set at $p < 0.05$.

TABLE 1 Spearman's rank correlation coefficients between in vitro properties and the total thrombus-formation analysis system parameters in platelet concentrates.

	T_{10}	T_{80}	AUC_{30}
Storage period	0.44*	0.21	−0.29
Collagen	−0.35	0.02	0.16
ADP	−0.53**	−0.27	0.40*
Collagen + ADP	−0.30	0.10	0.07
Ristocetin	−0.36	−0.15	0.31
Platelet count	−0.27	−0.06	0.12
Mean platelet volume	0.15	0.15	−0.12
CD62P	0.26	0.08	−0.13
PAC-1	0.00	−0.05	0.04
Annexin V	0.23	0.03	−0.09

Abbreviations: ADP, adenosine diphosphate; AUC_{30}, area under the curve for 30 min; PAC-1, procaspase activating compound-1; T_{10}, time to 10 kPa; T_{80}, time to 80 kPa.
*$p < 0.05$; **$p < 0.01$ ($N = 10$).

DISCUSSION

In this study, improvements were made to the T-TAS test in order to establish a more sensitive and quantitative evaluation method to detect haemostatic function changes associated with the preservation of PCs in low platelet concentration/high shear conditions compared with the current T-TAS test. In our previous study using the conventional chip, quantitative evaluation allowed for the stable analysis of the haemostatic function in only 4 out of 10 experiments with platelet counts of 50 × 10^3/μL; however the use of an HD chip with a shallower chamber depth enabled us to stably analyse the haemostatic function of all reconstituted blood samples with low platelet counts under high shear conditions [5].

The contributions of RBCs to haemostasis and thrombosis have become increasingly clear [3]. Marin et al. [9] reported that the percentage (mean ± standard deviation) of normal-type RBCs on day 3 of storage was 80.0 ± 3.9%. Even if RBCs are derived from different blood donors, fresh RBCs on day 3 after blood collection had a large number of discoid forms, and the difference between the donors was small. As a result, the influence of donor differences on the haemostatic function of RBCs was also small, and the coefficient of variation of the T-TAS parameters was estimated to be less than 10% (Table S2).

The novel T-TAS-based haemostatic test was capable of comprehensively quantifying haemostatic function under low platelet counts/high shear conditions; accordingly, we believe that the test could evaluate the haemostatic function of platelet products under more physiologically relevant conditions than the current in vitro platelet tests. This system may have the potential to more closely resemble the clinical situation of post-platelet transfusion and may be considered as platelet transfusion in a test tube regardless of the transfusion recipient. The novel T-TAS-based haemostatic test is expected to serve as a

bridge from the current in vitro platelet test to in vivo haemostatic tests with experimental animals.

This study had two limitations. First, blood components in the reconstituted blood might have been damaged to some extent during the preparation. Second, unlikely in vivo, reconstituted blood samples contain few leukocytes and do not reflect coagulation-promoting events, such as neutrophil extracellular traps [10]. Thus, our current method may not yet adequately reflect haemostatic potential in laboratory animals or humans. The novel T-TAS-based haemostatic test is, however, superior to the in-vivo haemostatic test in terms of the ethics, labour and reproducibility. Our assay has the potential to be an alternative to the in vivo haemostatic test as a method to evaluate the haemostatic function of platelet products under low platelet concentration/high shear conditions.

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A.F. performed the research and wrote the first draft of the manuscript. A.F., K.Y., T.H., M.T. and K.S. designed the research study. C.O., T.O.-W. and K.H. participated in T-TAS analysis. K.Y., F.H., T.K. 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 and haemodilution chip are products of Fujimori Kogyo Co., Ltd., where C.O., T.O.-W. and K.H. are employed. The remaining 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

Vox Sanguinis ISBT International Society of Blood Transfusion

Transfusion-associated acute lung injury following albumin treatment in liver disease

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Abstract

Background and Objectives: Transfusion-related acute lung injury is an infrequent adverse reaction observed in patients receiving blood products. The lung injury can range in severity and can be associated with both mortality and mortality. All blood products except albumin have been linked to cases of transfusion-related acute lung injury. In fact, albumin may be used as a salvage modality in severe transfusion-related acute lung injury. We report an alcoholic patient who developed lung injury following treatment with albumin in the setting of hypoalbuminaemia.

Materials and Methods: A 41-year-old male with alcoholic liver disease was admitted for severe ascites and alcoholic hepatitis. Chest x-ray showed small pleural effusions at the lung bases with no overt pulmonary oedema. He received high doses of furosemide for lower extremity oedema. The patient received a total of two albumin infusions to augment the diuresis effect.

Results: He subsequently developed acute hypoxic respiratory failure with imaging showing interstitial and airspace abnormalities concerning for pulmonary oedema. He showed no additional signs of volume overload and was treated supportively until the condition improved.

Conclusion: This is the first reported case of albumin-associated lung injury proximally related to albumin infusion. We aim to increase awareness of this possible sequelae among physicians.

Keywords

albumin treatment, circulatory overload, respiratory distress, TRALI, transfusion-associated acute lung injury

Highlights

- Albumin is a widely used treatment option for patients with liver disease.
- We report here, to our knowledge, the first case report describing the development of transfusion-associated acute lung injury (TRALI) associated with albumin infusion.
- The management of patients with albumin-associated TRALI involves supportive care and diuresis.

INTRODUCTION

Transfusion-associated acute lung injury (TRALI) manifests with respiratory insufficiency and hypoxemia occurring during or within 6 h of blood or blood product transfusion, often evidenced by bilateral fluffy infiltrates on imaging indicative of pulmonary oedema. A recent consensus has refined TRALI into two types: TRALI type I, lacking acute respiratory distress syndrome (ARDS) risk factors, and TRALI type II, associated with ARDS risk factors or mild pre-existing ARDS [1].

Many cirrhotic patients have derangements in fluid homeostasis, which results in large amounts of extracellular fluid causing ascites or other forms of interstitial oedema. This results from a disparity in the Starling equilibrium due to increased hydrostatic pressure from portal hypertension and decreased oncotic pressure due to a hypoalbuminaemia state. The result is an increased fluid movement from the intravascular space to the interstitial space.

Albumin is a low-molecular-weight protein used in various clinical settings to increase intravascular volume and oncotic pressure. Healthy individuals may tolerate this treatment with no complications. However, serum albumin transfusion may lead to complications encompassing circulatory overload similar to blood transfusion in critically ill patients. Non-cardiogenic pulmonary oedema may arise following treatment with albumin, especially in albumin-deficient patients. This may be due to the rapid expansion of the intravascular volume and associated increased oncotic pressure and subsequent hydrostatic pressure, leading to impaired driving pressure homeostasis and resultant fluid leakage into the alveolar spaces [2]. Interestingly, this is one theorized mechanism for the development of TRALI.

We describe a case of a 41-year-old male patient with alcoholic liver disease who presented to the hospital with alcoholic hepatitis, moderate ascites, lower extremity oedema and significantly decreased serum albumin. This patient developed acute hypoxic respiratory failure in the setting of TRALI following treatment with IV albumin.

CASE PRESENTATION

A 41-year-old male with a history of an alcohol use disorder, alcoholic hepatitis, cirrhosis and hepatic encephalopathy was admitted due to complaints of worsening abdominal distension and pain associated with bilateral lower extremities swelling for 3 weeks. A review of systems upon admission was negative for fever, chills, chest pain, haemoptysis, haematemesis, black stools and confusion. Of note, he had been admitted to the same hospital 1 month prior similar symptoms. He was treated at that time for acute alcoholic hepatitis and hepatic encephalopathy with spironolactone, furosemide, methylprednisolone and lactulose with rifaximin. He was asked to follow up in the outpatient setting without success.

During physical examination after admission, the patient was uncomfortable, appeared jaundiced and had scleral icterus. He had decreased breath sounds at the lung bases and no abnormal heart sounds, including S3. He had tense ascites and +4 bilateral lower extremity oedema to the knees with no other stigmata of chronic liver disease. Initial vital signs were significant for a temperature of 98.7 °F, heart rate of 101 beats/min, blood pressure of 164/84 mmHg, respiratory rate of 22 breaths/min and SpO_2 of 97% on room air.

Initial findings showed an alcohol level of 58 mg/dL, total bilirubin of 8.8 mg/dL, aspartate aminotransferase 75 U/L, alanine aminotransferase 41 U/L, albumin 1.9 g/dL serum ammonia 89 mcmol/L, international normalised ratio (INR); 2.1, haemoglobin 10.6 g/dL with Mean corpuscular volume 104 fL, platelet count 31 B/L, white blood cell count 5.5 B/L and NT-pro B-type natriuretic peptide (NT-proBNP) 92 ph/mL (reference range: ≤125 pg/mL) (Table 1). Chest x-ray followed by computerized tomography (CT) chest showed possible small pleural effusions at the lung bases (Figure 1).

IN-HOSPITAL LABORATORY RESULTS

The patient was admitted to the hospital and continued on steroid taper (due to active drinking and lack of benefit for his alcoholic hepatitis per the Lille score). He was treated with increasing doses of furosemide with minimal improvement in his lower extremity and abdominal volume status. To promote diuresis, 25% albumin infusions were added on the second day of hospitalization. The patient received a total of four infusions (one infusion/day). The albumin dose ranged from 25 g to 1 g/kg/dose (112.5–162.5 g) at a rate of a rate of 150 mL/h. On the second day of hospitalization, he underwent paracentesis, yielding 1250 mL of thin, clear yellow fluid, which was negative for spontaneous bacterial peritonitis. A transthoracic echocardiogram (TTE) revealed normal cardiac function with Ejection Fraction 60%–65% with normal left ventricular diastolic function and normal right ventricle function, and inferior vena cava diameter is less than or equal to 21 mm with greater than 50% decrease during inspiration consistent with normal right atrial pressure.

On the third day of hospitalization, 30 min into albumin infusion, he was noted to be in acute respiratory distress with a blood pressure of 149/78 mmHg, respiratory rate of 28/min and SpO_2 of 77% on room air that improved to >94% on 6 L nasal cannula (Table 2). There were decreased breath sounds at the lung bases but no notable jugular venous distension on physical examination. At this time, albumin infusion was stopped with repeat labs demonstrating serum albumin of 4.2 g/dL up from 1.9 g/dL on admission. A chest x-ray showed scattered bilateral lung opacities.

A CT scan was negative for pulmonary embolism but revealed pleural effusion and both interstitial and airspace abnormalities concerning for pulmonary oedema (Figure 2).

The patient was managed with oxygen supplementation and increased doses of furosemide with a favourable response. On subsequent review of the record, it was noted that the patient received an albumin infusion 23 min prior to the development of acute-onset pulmonary oedema.

DISCUSSION

Albumin is a cornerstone for the management of complications related to liver cirrhosis. Albumin therapy is indicated for treating

TABLE 1 Laboratory findings on admission and development of TRALI (day 3).

	On admission	Day 1	Day 2	Day 3	Reference range
White blood count (B/L)	5.5	2.5	9.5	9.8	4.0–111.0 B/L
Haemoglobin (g/dL)	10.6	9	9.7	8.6	14.0–17.0 g/dL
Haematocrit (%)	31.6	27.5	29.2	27.2	42%–52%
Platelet count (B/L)	31	19	43	44	140–400 B/L
Sodium (mmol/L)	139	140	139	137	135–146 mmol/L
Potassium (mmol/L)	4.3	3.6	4.4	4.5	3.5–5.0 mmol/L
Chloride (mmol/L)	105	101	99	99	89–109 mmol/L
CO2 (mmol/L)	26	29	28	27	21–30 mmol/L
Anion gap (mmol/L)	8	10	12	11	6–14 mmol/L
Blood urea nitrogen (mg/dL)	10	9	17	23	10–26 mg/dL
Protein (g/dL)	4.7	5.4	5.7	6.2	6.0–8.5 g/dL
Albumin (g/dL)	1.9	3.5	3.3	4.3	3.2–4.9 g/dL
Calcium (mg/dL)	7.6	8	8.3	8.8	8.5–10.3 mg/dL
Glucose (mg/dL)	137	83	139	94	70–100 mg/dL
Creatinine (mg/dL)	0.71	0.76	1.15	1.23	0.7–1.4 mg/dL
Total bilirubin (mg/dL)	8.8	11.7	16.3	13.1	0.1–0.9 mg/dL
Alkaline phosphatase (IU/L)	140	104	125	114	29–92 IU/L
Aspartate aminotransaminase (IU/L)	75	53	61	52	7–42 IU/L
Alanine aminotransaminase (IU/L)	41	29	30	26	<45 IU/L
eGFR	118	116	82	76	≥60 mL/min/1.73 m^2
INR	2.1	2.7	2.5	2.7	0.84–1.16
Prothrombin time (s)	24	31.2	29.1	31.1	9.4–13.0 s

Abbreviation: eGFR, estimated glomerular filtration rate; INR, international normalised ratio; TRALI, transfusion-associated acute lung injury.

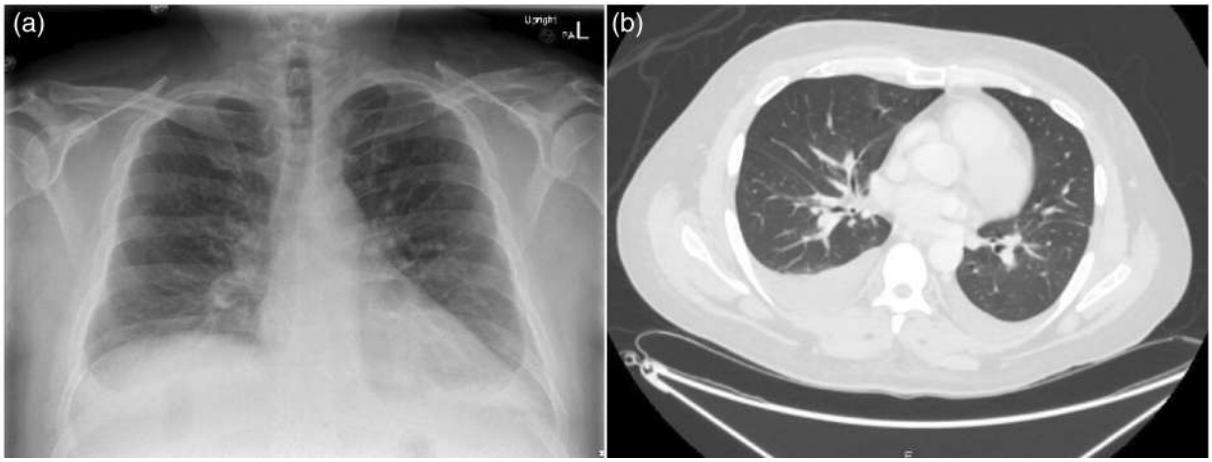

FIGURE 1 Chest x-ray (a) and computerized tomography (b) on presentation showed small effusions and underlying atelectasis at the lung bases. The cardio-mediastinal silhouette is normal.

TABLE 2 Temporal haemodynamic alteration into the acute incident.

Hospitalization day	Temperature (°F)	Pulse (beats/min)	Blood pressure (mm/Hg)	Respiratory rate (breath/min)	SpO_2%	Oxygen therapy (litres/min)
Day 1	98.1	83	168/84	18	96%	Room air
Day 2	98.8	77	160/78	19	99%	Room air
Day 3 (incident)	98.7	69	149/78	28	97%	Nasal cannula (6 L)

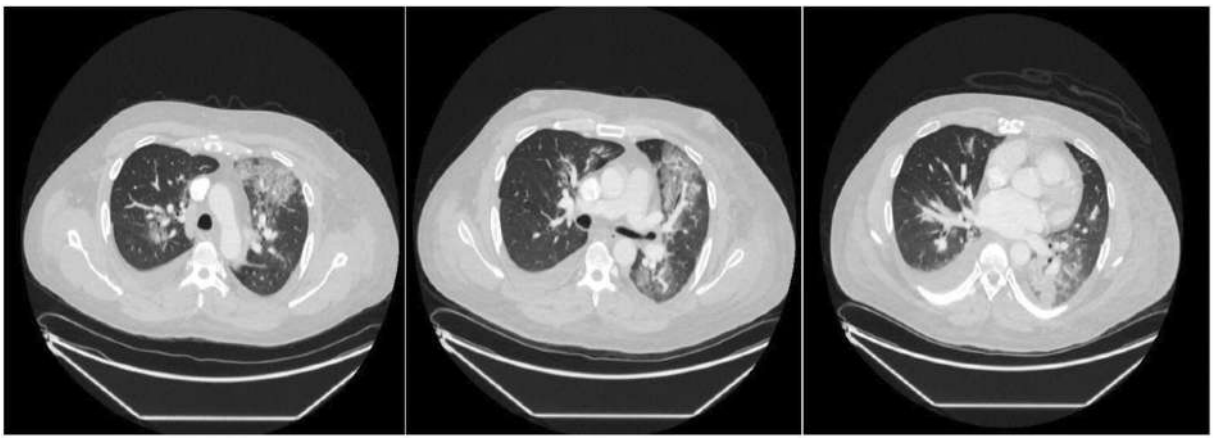

FIGURE 2 Computerized tomography of the chest showed no evidence of acute pulmonary embolus—widespread Interstitial and airspace opacities with pleural effusions. The constellation of findings suggests pulmonary oedema with asymmetric distribution of airspace oedema anasarca with subcutaneous oedema.

spontaneous bacterial peritonitis, large-volume paracentesis and diuretic-refractory ascites [3]. The favourable effect of albumin is mainly derived from the ability to maintain colloid osmotic pressure. Patients with impaired hepatic synthetic function may present with signs and symptoms attributed to albumin deficiency, and treatment with albumin has been shown to improve outcomes and reduce mortality accordingly [4].

Clinical use of albumin, however, is not without risk. In albumin-deficient patients, treatment with albumin may acutely alter one or more of the Starling forces, differently affecting various organs' haemodynamics. Of note, the pulmonary capillaries appear to have a prominent baseline permeability to albumin compared with other capillaries and, therefore, may lead to undesirable effects in the event of acute alteration of serum albumin [5, 6].

Our patient presented with a markedly decreased albumin level and was treated with albumin infusions, giving rise to acute albumin level alteration. On the third day of hospitalization, he developed TRALI thought to be related to the acute rise of albumin level. A previously reported case described a patient who developed acute pulmonary oedema following albumin infusions, leading to circulatory overload [7]. The authors justified the observed findings due to a faster albumin infusion rate with resultant acute pulmonary oedema within 30 min of administration.

Although transfusion-associated circulatory overload (TACO) may exhibit similar symptoms, it arises from elevated hydrostatic pressure in the lung capillaries. In contrast, the primary physiological abnormality causing pulmonary oedema in TRALI is an increase in capillary permeability, whereas, in TACO, oedema is primarily thought to stem from heightened capillary hydrostatic pressure (non-permeability oedema) [1]. Interestingly, Djalali et al. reported that rapid albumin infusion could serve as a potential intervention for severe TRALI characterized by significant pulmonary capillary leak during the acute stage [8]. Nevertheless, our report suggests albumin may itself induce TRALI, perhaps when the albumin state is particularly deficient by acutely impairing driving pressure homeostasis and causing alveolar space volume overload [2]. Our management of this patient was supportive with close monitoring, oxygen supplementation and successful diuresis.

In conclusion, albumin is a widely used treatment option for patients with liver disease. Treatment can be associated with identifiable risks, including TRALI. To our knowledge, this is the first case report describing this adverse effect. We aim to increase awareness of this potential adverse effect and to encourage further studies to determine safe clinical application. Further research is warranted further to understand the effect of albumin treatment on haemodynamics.

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H.A., M.B., K.O. and S.N. wrote the main manuscript text. S. N. performed a literature review. H.A. prepared all the figures. All authors read and approved the final manuscript.

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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EVENTS

See also https://www.isbtweb.org/events.html	
28 August 2024	E-learning in Transfusion Medicine ISBT webinar. https://www.isbtweb.org/events/e-learning-in-transfusion-medicine.html
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27–29 September 2024	ESPGI 2024—Platelet and Granulocyte Immunobiology. https://sanquinacademy.nl/en/offers/espgi-2024/
23–26 October 2024	Brazilian Congress of Hematology, Hemotherapy and Cell Therapy (Hemo 2024). https://www.hemo.org.br/2024/index.ingles.php
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

