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Vox Sanguinis reports on important, novel developments in transfusion medicine. Original papers, reviews and international fora are published on all aspects of blood transfusion and tissue transplantation, comprising five main sections:

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5. *Cellular Therapy* (including clinical, quality and regulatory aspects) Cell-based therapies; Stem cell sources; Stem cell processing and storage; Stem cell products; Stem cell plasticity; Regenerative medicine with cells; Cellular immunotherapy; Molecular therapy; Gene therapy

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REVIEW ARTICLE

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In vitro diagnostics for screening the blood supply: the new European regulation for IVD and the WHO IVD prequalification programme

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Blood transfusion remains a routine life-saving medical procedure that helps replace blood lost due to surgery, injury or disease. The quality of transfused blood is crucial in this process as blood donors must be free of transfusion-transmissible infections and donated blood should be compatible to that of the recipient. The quality of donated blood could be affected by the quality of in vitro diagnostic medical devices (IVDs) used in the screening process. Consequently, the need for high-quality, safe and well-performing IVDs for use in transfusion medicine arises, accompanied by the need for tight regulations in this domain. In the European Union, the new IVD Regulation will replace the existing IVD Directive within a five-year transitional period. Manufacturers of IVDs are expected to fully comply with the new Regulation by 26 May 2022. In this review, we address the major differences relating to marketing authorization and testing between this new Regulation and its predecessor. We further present the main elements of the prequalification assessment introduced by the WHO for IVDs, including disease-specific IVDs for blood screening laboratories.

Key words: blood transfusion, IVD regulation, prequalification, TTIs.

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Introduction

The delivery of healthcare today involves a huge variety of health products and technologies with wide applications ranging from disease diagnosis, prevention, treatment and monitoring. In vitro diagnostic medical devices (IVDs), also part of these health technologies, are defined as medical devices, used alone or in combination and intended by the manufacturer for the in vitro examination of specimens derived from the human body to provide information for diagnostic, monitoring or compatibility purposes [1]. IVDs play an important role in the field of blood transfusion, enabling the safety of blood and blood products through their use in the screening for transfusion-transmissible infections (TTIs) and determination of blood compatibility parameters.

Blood transfusion saves many lives worldwide. This process, if not well regulated and executed from the point of a blood donation, could be very risky due to the potential presence of TTIs [2] – the major ones being the human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), human T-cell lymphotropic virus (HTLV) and Treponema pallidum (causing syphilis). A high prevalence of TTIs in the general population would pose a considerable hazard to blood safety, which may be reduced by appropriate screening tests. Good regulation of IVDs used in the field of blood transfusion is key for assurance of suitable quality, safety and performance of these devices. According to the World Health Organization (WHO) [3], globally a significant proportion of donated blood is at a lower safety level either

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because it is not screened for all the major TTIs or the screening is not done within a quality system, as some countries do not screen in a quality-assured manner. False-negative test results may occur with test systems of suboptimal sensitivity for the pathogen strains or genotypes prevalent in the region. Likewise false-positive (or false reactive) test results may cause unnecessary deferral of healthy blood donors and contribute to blood shortage [4]. False results in assessing blood compatibility marker, for example blood groups A, B, O, testing, may cause adverse events in recipients, including life-threatening complications.

Validity of screening results is key, and IVDs of suitable quality are indispensable in guaranteeing a steady supply of safe blood and blood products.

In the global field of blood transfusion, a variety of different technologies and devices are in use, ranging from rapid diagnostic tests to fully automated systems. Serologic assays based on antigen and/or antibody detection systems to screen blood and blood products for TTIs have been available for over 50 years. With recent technological advances, nucleic acid amplification technology (NAT), such as the polymerase chain reaction (PCR), ligase chain reaction (LCR) and transcription-mediated amplification (TMA), is getting introduced into blood screening routine. These new technologies offer advantages of speed, sensitivity, specificity and accuracy in detecting nucleic acids of TTI causative agents or determining the genetic background of blood group markers.

Considering the very significant role IVDs play not only in transfusion medicine but also in general medical laboratory diagnosis, it is crucial for the design, validation, manufacture, distribution/sale and use of these devices to be thoroughly regulated. Different countries and regions around the world implement different regulations for IVDs but with the sole aim to guarantee the safety, quality and performance of these devices. In the European Union (EU), the new in vitro diagnostic medical devices Regulation (IVDR; 2017/746/EU) published in May 2017 will replace the existing in vitro diagnostic medical devices Directive (IVDD; 98/79/EC) within a five-year transitional period. The new Regulation will create a more robust, transparent and sustainable regulatory framework, recognized internationally within the EU and beyond, that improves clinical safety and creates fair market access for manufacturers [5]. The EU regulatory process for medical devices, including IVDs, begins with the correct classification of the device by the manufacturer. A quality management system implemented in all design and development phases is required for preparation of a technical documentation (TD) and documentation of compliance of the device with the 'general safety and performance requirements' stated in the Regulation.

Part of these requirements are confirmed during performance evaluation of the device covering both analytical and clinical performance. For IVDs belonging to higher risk categories, the manufacturer applies to a conformity assessment body, called Notified Body (NB), to audit the quality management system and to assess the technical documentation (design dossier). After a successful outcome, a European certificate confirming compliance of the device with the European requirements is issued and the CE mark may be affixed to the device.

Once IVDs have been certified for release into the market, post-market surveillance mechanisms should be implemented to ensure that they continue meeting the same quality, safety and performance requirements after placement on the market [6]. These post-market surveillance activities could either be reactive to a documented IVD event or proactive to a potential IVD issue. Incumbent upon the manufacturer is complaint evaluation related to compromised safety, quality or performance, and all necessary corrective and preventive measures, as needed [6]. A total lack of or inadequate oversight over IVDs could lead to test failures with devastating consequences, sometimes attracting public attention or generating scandals. Historical examples of such scandals in the medical device field include the silicone breast implants scandal in France where poly implant prothese (PIP) implants made from cheaper, industrial-grade silicone not approved for medical use were rupturing, resulting in injuries. Such incidents eventually led to major regulatory updates [7]. In the IVD field, an anti-HIV test was ordered to be withdrawn from the European market by the European Commission after its low performance had become obvious [8]. Furthermore, with a considerable number of IVD products available for purchase online, their authenticity remains questionable. A recent press report has revealed that in the past 4 years, about 12 000 fake sexually transmitted infections kits were seized in the United Kingdom [9].

Although the World Health Organization (WHO) is not a regulatory body, the WHO assists Member States through its prequalification assessments in decision-making on selecting essential health products, including IVDs. The focus of the WHO prequalification is on IVDs to be used in low-resource settings for the diagnosis of life-threatening infectious diseases (including HIV, HBV, HCV, but also Malaria). In this review, we address the new aspects, which come with the new European Union IVDR, as well as the major differences between this new regulation and its predecessor, the current IVDD. We also present an overview of the prequalification process introduced by the WHO for IVDs, including disease-specific IVDs for blood screening laboratories.

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Regulation of IVDs in the EU – IVD Directive (IVDD) and IVD Regulation (IVDR)

The EU Directive on in vitro diagnostic medical devices (98/79/EC) [10] adopted on 7 December 1998, is a legislative act that sets out regulatory requirements to which all EU Member States must adhere regarding the safety, quality and performance of IVDs. Prior to the year 2000, different national regulations on IVDs existed in some EU Member States. In Germany for instance, the Paul Ehrlich Institute (PEI) performed evaluation and approval of IVDs (for HIV, HBV, HCV, Cytomegalovirus, Rubella and blood group markers) under the German drug law. Since the year 2000 however, the IVDD had to be transposed into the national laws of all EU Member States, following a transitional period. Starting December 7, 2003, adherence to the IVDD became obligatory and since then only compliant IVDs have been permitted for sale and use in the EU. Only these have been allowed to bear the 'Communautés Européennes' (CE) mark. Summarily, amongst other rules, the IVDD prescribes essential requirements of safety, quality and performance for all IVDs in the EU market. The IVDD defines tasks performed by Notified Bodies (NBs) which are assigned by individual Member States. For so-called high-risk devices (Annex II list A and B, and self-tests), manufacturers must apply to a NB for certification of their products. The NB will assess according to predefined conformity assessment procedures. In case of the 'highest risk' IVDs (defined as list A in Annex II: HIV, HBV, HCV and blood group markers), each device will additionally undergo a thorough review of the Technical Documentation (TD) including assessment of its design and performance. Common Technical Specifications (CTS) have been specifically drafted, in derogation of the normal standardization mandate, to define minimal performance characteristics for these highest risk IVDs.

In the wake of the recent scandals with medical devices described in the introduction, a new Regulation was adopted on 5th April 2017 and entered into force on 25th May 2017. Regulation 2017/746 [11] of the European Parliament and of the Council of 5 April 2017 on in vitro diagnostic medical devices (IVDR) was instituted, repealing the IVDD 98/79/EC. Since a Regulation is directly binding law in the entire European Union, it prevents differences in national interpretations and increases harmonization between the Member States. This is in contrast to a Directive that needs to be implemented in the national law of the Member States, with some room for interpretation and thus for potential differences between Member States. The IVDR, which can be applied already since May 2017, and which is fully mandatory after a transitional period of 5 years, provides a modernized and more robust EU legislative framework to ensure better protection of public health and patient safety [12]. The IVDR is intended to lead to an improvement in the health and safety protection standards for EU citizens, and it will also ensure that the EU legislation is adapted to the significant technological and scientific progress occurring in this sector over the last 20 years [11]. With effect from 26 May 2022, the IVDD will be repealed and this same day marks the 'Date of Application' of the IVDR, with some of its provisions (e.g. regarding NB) coming into force earlier.

The IVDD and the IVDR have the same basic regulatory process with respect to their impacts on manufacturers and products. The IVDR presents important improvements, some of which include: a broader and clearer IVD definition, a new IVD classification system, augmentations to the criteria for the designation of Notified Bodies and their subsequent oversight procedures, requirements for clinical evidence to be confirmed for all IVDs, stringent rules for manufacturers regarding compliance to ensure patient safety, and the strengthening of vigilance and post-market surveillance requirements for manufacturers as well as improved coordination strategies between EU countries in this domain. The new IVD classification system is based on IVD associated risks, makes use of classification rules and replaces the predefined IVD lists of the IVDD. The classification rules differentiate devices from class A, low risk, up to class D, high risk (see Table 1 for details), with parallel increasing regulatory requirements. The intended purpose of an IVD, for example diagnosis of patients or screening of blood donors, may impact its classification and the connected regulatory burden. Therefore, for some devices manufacturers may decide not to claim 'blood screening' though the device would be suitable for this application. This approach may cause problems for blood safety, for example with only few devices developed in the beginning of new or emerging infections. The Regulation basically adds new requirements to the existing ones in the Directive. For example, a major new element of the IVDR is the obligatory inclusion of a European Union Reference Laboratory (EURL) for laboratory verification of the most relevant performance claims prior to marketing a new high-risk device (class D). The IVDR also highlights the continuous evaluation of products throughout their life cycles and requires evidence from manufacturers showing the existence of an effective quality management system. It requires more transparency regarding information on IVD devices and more explicit requirements for performance studies on high-risk devices; summaries of which have to be made available to the public. Furthermore, self-testing and near-patient testing devices will now be subject to a premarket approval approach, checking IVD

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suitability for the respective user groups. The IVDR adopted several concepts elaborated by the International Medical Device Regulators Forum (IMDRF) to achieve on a global level regulatory harmonization and convergence. The IVDR also gives no room for 'grandfathering' provisions (for devices that were already on the market prior to the new Regulation) and recommends that all currently approved IVD devices need to be recertified in accordance with the new requirements. These improvements and/or differences between Directive and Regulation are summarized in Table 1. Differences between conformity assessment procedures in the IVDD and the IVDR are outlined in Table 2.

A series of technical and clinical requirements guiding the general safety and especially, the performance of IVDs is contained in the so-called Common Technical Specifications (CTS in the IVDD) or Common Specifications (CS in the IVDR) documents. These documents outline the general safety and performance requirements and minimal requirements for performance studies to which IVD manufacturers are expected to comply. Compared with the IVDD's Common Technical Specifications (CTS), the IVDR's Common Specifications (CS) can adopt requirements with respect to the technical documentation (IVDR – Annexes II, III), performance studies or performance evaluation (IVDR – Annex XIII) and post-market follow-up (IVDR – Annex XIII). The last revision of the CTS enlists provisions for performance evaluation (sensitivity, specificity and interferences), reference materials and batch release for manufacturers [13]. The CTS also clearly provides requirements on sensitivity, specificity and sampling, for consideration during the performance evaluation of blood screening IVDs. Such IVDs typically belong to Class D, including those for blood typing and compatibility testing, and will also be covered and extended under the IVDR by respective Common Specifications. European Union Reference Laboratories (EURL) will have to verify by laboratory testing, the performance claimed by the manufacturers of all class D devices and compliance with the CS. Results of this obligatory manufacturer-independent pre-market testing have to be used for the decision of the NB. Further tasks of EURLs are batch verification testing of class D devices and advice on IVD-related questions to the European Commission, to Member States and to NBs. With the huge diversity of class D devices, it is expected that there will be different scopes (sets of markers) to be covered by different EURLs and several EURLs per scope in order to manage the expected workload. The different EURLs have to assure uniform testing approaches and decision criteria by building an EURL network; individual EURLs may make use of specific expertise present in 'national reference laboratories' which may be subcontracted. Tasks and criteria of EURLs are summarized in IVDR Art 100 and will be specified in detail by implementing acts of the European Commission.

WHO prequalification of IVDs

The WHO undertakes prequalification assessments of specific IVDs to determine whether the product meets the prequalification requirements for safety, quality and performance, subjecting individual diagnostic tests through a standardized assessment procedure [14]. The prequalification requirements have been designed in a manner to meet the needs of resource-limited settings. The WHO carries out prequalification to guide interested United Nations agencies and WHO Member States without mature regulatory assessment systems for IVDs in their product selection and procurement decisions [14]. The prequalification process is governed by eligibility principles and criteria. The scope for eligibility is defined by the global need for IVDs for a particular disease or disease state, the appropriateness of the product for use in resource-limited settings, requests from WHO Member States for particular IVDs, a recommendation in WHO disease-specific testing guidelines, and the availability of prequalified products that are of a similar assay format and/or assay principle [15]. Products manufactured by original product manufacturers, which are commercially available when submitted for prequalification assessment, are of interest to UN organizations and other procurement agencies, and products for which there exists few other prequalified products all make up the eligibility criteria used for prequalification [15]. The WHO prequalification team considers applications for prequalification from manufacturers of IVDs used in the diagnosis and infection monitoring of the major TTIs like HIV, HBV and HCV, but also from manufacturers of other pathogens like human papilloma virus (HPV), toxigenic *Vibrio cholerae*, G6PD enzyme activity and malaria parasites.

The WHO IVDs prequalification (IVD-PQ) process begins with the manufacturer applying for prequalification through a pre-submission form, providing brief information about the product, its regulatory version and details of the manufacturer [14]. This information enables the IVD-PQ team in determining whether the product is eligible for assessment. The manufacturer proceeds to compiling and submitting a product dossier in accordance with specified requirements. Summarily, the product dossier provides information about the product´s description including variants, essential principles, risk analysis and control, design and manufacturing information, product performance specifications and associated validation and verification studies, labelling, commercial history, regulatory history and a quality management system [15]. This product dossier is reviewed by an assessor assigned by

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Table 1 Summary of the major differences between the IVDD and the IVDR

Subject	IVDD 98/79/EC	IVDR 2017/746/EU
IVD definition	• Limited to reagent, calibrator, control material, kit, instrument, apparatus, specimen receptacle, equipment or system, intended for the in vitro examination of specimens derived from the human body, solely or principally for determining a physiological or pathological state, a congenital abnormality, the safety and compatibility with potential recipients, or to monitor therapeutic measures (Article 1)	• Now expanded to also include tests intended to predict a medical condition or a disease, software and companion diagnostics (to predict treatment responses or reactions) (Article 2) • Also introduces some new definitions, e.g. devices for 'near-patient' testing designed for use by health professionals outside a laboratory environment; devices used in direct combination with specific drug treatment (companion diagnostics)
IVD classification	• Classified into pre-defined high-risk devices (list A) and moderate-risk devices (list B) (both in Annex II); devices for self-testing; residual IVDs. • Main involvement of Notified Bodies for list A devices, less for list B and devices for self-testing; together comprising around 10% of all devices	• **IVD classes** (a) **class A** (low individual risk and low public health risk) (b) **class B** (moderate individual risk and low public health risk) (c) **class C** (high individual risk and moderate public health risk) (d) **class D** (high individual risk and high public health risk; 'high risk' IVDs) • CE certification of class B, C and D devices (85% of all IVDs) requires increasing levels of Notified Body involvement • CE certification of class D devices requires an EU reference laboratory for verification of performance and batch testing
'High risk' IVDs	Markers defined as list A in Annex II, difficult to extend by new markers List A: • Viruses: HIV1 and 2, HTLV I and II, HBV, HCV, HDV • Blood compatibility: ABO, Rhesus, antiKell	Class D; defined by Annex VIII Rule 1 or Rule 2, flexible for future inclusion of further markers Rule 1: Devices intended to be used for (a)safety testing of blood, tissues, cells, transplants (e.g. HIV1 and 2, HTLV I and II, HBV, HCV, HDV, HEV, *Treponema pallidum*, CMV, EBV, *Plasmodium spp*, *Trypanosama cruzii*, *Toxoplasma gondii*); (b)detection of transmissible agents causing life-threatening disease (e.g. Ebola V, Marburg V, Lassa V, SARS CoV, MERS CoV, virulent Influenza, vCJD (c)monitoring infectious load of life threatening disease in patient management (e.g. HIV, HBV, HCV)Rule 2: Blood grouping/ tissue typing systems in transfusion/ transplantation ABO, Rhesus, Kell, Kidd, Duffy
Notified Bodies	• Several roles listed in conformity assessment procedures, EC declaration of conformity, etc. (Articles 9, 15, 21 and Annexes III – VII)	• Greater scrutiny of Notified Bodies • Notified Bodies have to be designated: a 12 months process involving assessors from different national and European authorities • More stringent criteria for scientific and technical evaluation competences

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Table 1 (Continued)

Subject	IVDD 98/79/EC	IVDR 2017/746/EU
		• More rigorous surveillance expected
Device identification	• No particular requirement for a harmonized device identification system in place	• Unique device identifiers (UDIs) system now in place to improve the identification, traceability and post-market safety activities of IVDs (Article 24). • A device identifier (UDI-DI) specific to the model and packaging of the device and a production identifier (UDI-PI) to identify the manufacturing site.
Manufacturers' obligations	General obligations stated • Risk management and quality management systems; • Conformity assessment procedure; • Claiming responsibility for devices on the market (taking appropriate corrective measures, recording and reporting incidents, and providing appropriate evidence of conformity to authorities)	More elaborate and clear instructions on: (Article 10) • Risk management and quality management systems; • More rigorous clinical evidence: conduct clinical performance studies and evaluations; • Updated archive of the technical documentation; • Conformity assessment procedure; • Claiming responsibility for devices on the market (taking appropriate corrective measures, recording and reporting incidents, and providing appropriate evidence of conformity to authorities) • Common specifications with further requirements for certain devices (Article 9) • Appointing a designated person responsible for regulatory compliance who has requisite expertise in the field of IVDs (Article 15)

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Table 2 Differences between Conformity Assessment Procedures in the IVDD and the IVDR

Device category	Conformity Assessment Procedures	Notified Body Assessment of	
		Quality Management System	Technical Dossier
IVDD			
List A devices	Full quality assurance system (Annex IV)	Yes	Yes
	EC type-examination/ production quality assurance (Annexes V/VII)		
	Option for verification of manufactured batches by testing lab		
List B devices	Full quality assurance system (Annex IV)	Yes	Yes (samples)
	EC type-examination/ production quality assurance (Annexes V/VII)		
	EC type-examination/ EC verification (Annexes V/VI)		
Self-testing devices	Annex III (6)	-	Yes (design examination: studies carried out with lay persons)
All other devices	Self-certification	-	-
	EU declaration of conformity		
In-house devices	Manufactured by and used within health institution	-	-
	No specific requirements, Member States may define own requirements.		
IVDR			
Class D	Quality management system and assessment of TD (Annex IX)	Yes	Yes
	EU type-examination (Annex X)		
	Production quality assurance (Annex XI)		
	new IVDs of its kind, IVDs without CS		
	Scrutiny procedure (Competent Authority (CA) and Medical Device Coordination Group (MDCG))		
	Lab testing by EURL		
	(a) Verification of claimed performance and compliance with the applicable CS (Art 100 (2a))		
	(b) Batch verification (Art 100 (2b); Annex IX (4.12), Annex XI (5.1)		
Class C	Full quality management system (Annex IX)	Yes	
	EU type-examination (Annex X)		
	Production quality assurance (Annex XI)		Yes
	companion diagnostics		(≥1 device representative for generic device group)
	consultation of Competent Authority (CA)		all companion diagnostics
	Annex IX 5.2, Annex X 3		
Class B	Quality management system (Annex IX – Chapter I)	Yes	Yes (≥1 representative device per product category)
Class A	Self-certification and EU declaration of conformity (Annex IV)	-	-
In-house devices	Manufactured by and used within health institution; non-industrial scale;	-	-
	health institution justifies that the specific needs cannot be met by an equivalent device available on the market		
	Competent authority (CA) to be informed		
	QM system according ISO EN 15189		
	Annex I (general safety and performance requirements) applies		
	Class D IVDs: documentation of performance evaluation		

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the WHO IVD-PQ team, with the purpose of assessing evidence in support of safety and performance of the product. WHO also determines whether the manufacturer's quality management system meets the standard to warrant an inspection of the manufacturing site, and assessing the product design and manufacturing process [16].

A manufacturing site inspection is planned simultaneously to the dossier review, aiming to assess compliance of the manufacturer's quality management system as well as manufacturing practices with international standards (ISO 13485: 2016), verification of the data supporting the claims presented in the submitted pre-submission form and product dossier, and inspection of the quality management system according to the manufacturer's own requirements [17]. For the inspection to be adequately performed, the manufacturing site must be in active production of at least one of the products undergoing prequalification assessment [17]. Key personnel from the manufacturer (persons in charge of the quality management system, quality control and production line) and from the inspection team (whose competence should match all the parameters of inspection) must be present on the agreed date(s) of inspection. Inspectors from the local national regulatory authority are usually invited as observers.

In addition to the product dossier review and manufacturing site inspection, evaluation of the product in question takes place. This evaluation, which aims at assessing the operational and performance characteristics of the product, is carried out by a WHO performance evaluation laboratory [18]. At the WHO Collaborating Centre in Antwerp, Belgium, for example, test kit evaluations are carried out with the use of a WHO HIV reference panel of well characterized serum/plasma specimens of geographically diverse origin, seroconversion panels and low titre panels [18]. Similar evaluations for hepatitis B and C virus test kits are done by the WHO Collaborating Centre – Public Health England, in London, United Kingdom. A summary of the prequalification assessment is published in the prequalification public report [19]. Manufacturers of prequalified IVDs have post-market surveillance obligations to ensure that these IVDs continue meeting the same quality, safety and performance requirements.

With the increased variety and widespread use of IVDs globally, cooperation between IVD medical device regulators becomes essential. Different models of regulatory collaboration can be observed between some countries and regions around the world. Reliance is one of such processes, defined by the WHO as one wherein a regulatory authority considers or relies upon the evaluations performed by another regulatory authority or trusted institution to make its own decision about a device [20]. Through this model, under-resourced and/or less-experienced regulatory bodies can rely on decisions from more advanced regulatory institutions. However, a sound understanding of the regulatory system on which reliance is based upon is very crucial. This also comes with trust building, confidence and confidentiality on information exchange especially for mutual reliance between authorities. Recognition, a very similar model to reliance, is a process whereby certificates issued by one regulator or NB to a manufacturer is recognized or accepted by the regulatory authority of another importing country, without necessarily repeating the audit process [20].

In countries with limited access to quality health products especially IVDs, progress towards addressing high-burden diseases is seriously hampered. In addition, the prevalence and incidence of some of the major TTIs are high in these countries. Since the WHO IVD-PQ focuses on IVDs for priority diseases such as HIV/AIDS, hepatitis B and C, and their suitability for use in resource-limited settings, regulatory authorities in these countries may choose to rely on evaluations conducted by the WHO [20]. Consequently, the IVD-PQ assessments take into consideration the regulatory version of the product marketed on the global market, make sure the scrutiny level reflects individual and public health risks in resource-limited settings, and guarantee that data submitted by the manufacturer is assessed from the perspective of such settings so as to reflect the environment and user in these places – including for example, real-world transportation conditions, temperature or humidity [20].

The WHO prequalification service has the main focus on IVDs for so-called priority diseases; however, if an IVD device is submitted for prequalification with claims including for use in screening blood donors, then the IVD is assessed for this claim following WHO requirements. In this regard, several products for screening blood donors have been prequalified, and the public reports for all prequalified IVDs can be accessed online [19].

Summary and conclusion

Transfusion-transmissible infections remain a major concern when it comes to blood products. TTIs have diagnostic window periods, which further make their detection difficult; sensitivity of high-quality IVDs is key. Therefore, effective screening systems are required to eliminate blood donations and derivatives that may contain TTIs, to guarantee a safe supply of blood and blood products. However, this cannot be achieved without the use of highly sensitive and specific, safe, high-quality IVDs. Good quality manufacture and supply of such IVD devices can only be guaranteed through a robust regulatory process, which encompasses the product‘s life cycle.

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The European Union approached this goal by introducing the IVD Directive in 2000, together with the CTS defining requirements for devices that are important for assessing the virus safety of blood, human tissues and organs. The new IVD Regulation, which is gradually being transitioned into force since May 2017 and is expected to take full effect from May 2022, will increase respective requirements. It provides the new European regulatory basis for manufacturing, placing on the market, distribution and putting into use of IVD devices, including those for blood screening.

In resource-limited settings however, regulation of IVD medical devices is either completely absent, just being introduced or already existing but often not functioning optimally. In such instances, the WHO's IVD prequalification provides support to WHO Member States by listing prequalified IVD products for priority diseases, on which these countries can rely for product selection and procurement. It however remains important for countries in resource-limited settings to have at least basic effective regulatory systems in place for IVDs so that they can guarantee the safety, quality and performance of IVD devices circulating in their countries. Such regulatory authorities could collaborate with regulatory bodies from other countries or regions through the concept of reliance and recognition when it comes to certain products, hence reducing the burden originating from lack of resources. The WHO IVD prequalification process is known to provide a good opportunity for regulators especially in resource-limited settings to rely upon assessments of IVD medical devices already performed by this WHO service.

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COMMENTARY

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Use of COVID-19 convalescent plasma in low- and middle-income countries: a call for ethical principles and the assurance of quality and safety

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The limited clinical data available suggest that convalescent plasma (CP) may have a therapeutic benefit in COVID-19 [1]. Absent any known effective therapy and considering the potential for local production, COVID-19 CP is becoming a global priority for investigational use. High-income countries with established national infrastructures and effective regulatory oversight can produce quality and safe plasma for transfusion that complies with international standards [2] and have initiated controlled clinical studies of COVID-19 CP [3,4].

Unfortunately, in low- and middle-income countries (LMIC) safe blood collection and transfusion are the challenges in the absence of a well-organized and nationally regulated blood collection system and limitations of critical resources and manpower. Nevertheless, provision of COVID-19 CP in LMIC needs to comply with the same principles of product safety and ethics regarding collection and use as in HIC, and guidance is needed [5,6].

The preparation of COVID-19 CP in LMIC should be organized as national initiatives supervised by the Ministries of Health and coordinated by the National Blood Services (or in its absence, cooperating blood establishments demonstrably meeting quality standards) to assure that legal and ethical guidelines for human research are applied to COVID-19. COVID-19 CP should be obtained only from volunteer, non-remunerated donors with reliable clinical, virologic or serologic evidence of prior infection with SARS-CoV-2. CP can be collected without additional testing for SARS-CoV-2 at >14 days after full recovery from symptoms. However, as an additional precaution against contagion in the donor room, prior demonstration of resolution of infection by a non-reactive Nucleic Acid Test (NAT) for SARS-CoV-2 performed on a nasopharyngeal swab sample can be considered for collections of CP between 14 and 28 days after full recovery from symptoms [7]. Collecting blood or plasma only from male donors or from female donors who have never been pregnant (including miscarriages and abortions) is advised for prevention of Transfusion Related Acute Lung Injury (TRALI). Selection criteria for blood donation and blood testing procedures should meet the established local requirements and standards. Where plasmapheresis is unavailable, CP should be prepared through component separation from whole blood (WB) while selecting donors carefully to avoid causing undue red cell loss and a low haemoglobin level. Transfusing convalescent WB should be considered only if WB transfusion is clinically indicated. ABO and RhD testing are needed to ensure blood group compatibility of CP and red blood cells.

The COVID-19 epidemic is one additional wake-up call that capacity building of a sustainable national blood system integrated within the public health system is crucial to ensure adequate, accessible and safe life-saving blood products in all countries, including in emergency situations [8].

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Disclaimer

Jay Epstein's contributions to this article reflect his own views and should not be construed to represent FDA's views or policies.

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COMMENTARY

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Blood group ABO polymorphism inhibits SARS-CoV-2 infection and affects COVID-19 progression

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The ABO blood group system consists of glycan antigens A and B and polyclonal antibodies against these antigens in individuals who do not express the antigens (Landsteiner's law). A and B antigens can also be expressed on other types of cells than RBCs, including epithelial cells of the gastrointestinal and respiratory tracts and endothelial cells lining the blood vessels. Therefore, ABO matching is also crucial for cell/tissue/organ transplantation, in addition to RBC transfusion.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for the ongoing pandemic of coronavirus disease 2019 (COVID-19). Although most infected people show no or mild symptoms, some progress to severe pneumonia, multiple organ failure and even death [1]. Older people are at high risk [2]. Eighty per cent of deaths have occurred in people with at least one underlying comorbidity, particularly cardiovascular disease/hypertension, overweight/obesity and diabetes. Male patients tend to have a worse prognosis than female patients with a greater risk of being hospitalized in the ICU and subsequent death. SARS-CoV-2 exhibits extensive organotropism, infecting and proliferating in epithelial cells of the respiratory and digestive tracts. An overreaction of the immune system by cytokine storm can attack the tissues and transform COVID-19 into a multi-organ disease.

Similar to the SARS-CoV responsible for SARS, SARS-CoV-2 is encapsulated with a cell membrane. Spike (S) glycoproteins embedded in the membrane mediate viral association with the cell surface receptor, angiotensin-converting enzyme 2 (ACE2). S proteins express A and/or B glycan antigens, reflecting the ABO phenotype of the cells where viruses are produced. In an experimental SARS cell model, the physical interaction between viral S proteins carrying A antigens and cellular ACE2 proteins was inhibited using mouse monoclonal or human polyclonal anti-A antibodies [3]. Similar observations have been made with HIV and measles viruses expressing A or B antigens [4, 5].

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Association of ABO blood groups and SARS-CoV-2 infection/COVID-19 disease

The ABO blood group polymorphism was previously shown to influence the susceptibility to SARS with individuals in groups A and O having a higher and lower risk, respectively [6]. Since 11 March 2020, several papers reported the association between ABO blood groups and SARS-CoV-2/COVID-19. These include papers by Zhao, *et al.* [7], Zietz *et al.* [8], and Zeng *et al.* [9], which were initially posted on *medRxiv*, the preprint server for health sciences. Li, *et al.* [10] just published an article in *Br. J. Hematol.* The authors compared ABO blood group distribution among 265 SARS-CoV-2-infected patients and 3,694 healthy controls. The proportion of group A in patients was significantly higher than in healthy controls (39·3% vs. 32·3%, $P = 0{\cdot}017$), while the proportion of group O in patients was significantly lower than in healthy controls (25·7% vs. 33·8%, $P < 0{\cdot}01$). The distribution proportions of groups A and O within various ages and genders were almost consistent with the trend. A higher and lower infectious risk of group A and O individuals, respectively, was also observed in the other studies [7–9].

On 8 June, the genetic testing company 23andMe released preliminary unpublished data from its ongoing COVID-19 genetic study, using the survey of more than 750 000 participants [11]. The percentage was calculated from individuals with different ABO groups who reported COVID-19: 1·3%, 1·4%, 1·5% and 1·5% for groups O, A, B and AB, respectively, among all participants, and 3·2%, 3·9%, 4·0% and 4·1% among health professionals. The protective effect of group O against acquisition (OR = 0·86, $P < 0{\cdot}0001$) and hospitalization (OR = 0·81, $P = 0{\cdot}05$) was observed in the entire population and also among health professionals (OR = 0·81, $P < 0{\cdot}0001$ to acquire). The results showed the power of this novel approach to study genetic associations based on genome sequencing and survey questions.

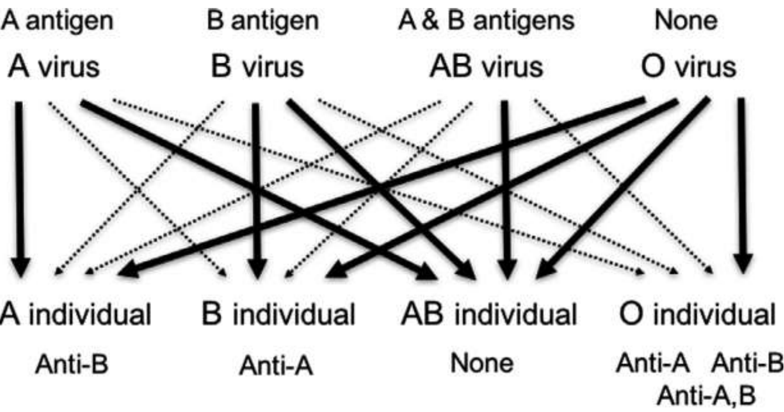

Fig. 1 Differential inhibition of infection between SARS-CoV-2 viruses exhibiting different ABO phenotypes and individuals of groups A, B, AB and O.

Another important advance was achieved in the genome-wide association study (GWAS) published in *N Engl J Med* [12]. The authors analysed 8 582 968 single nucleotide polymorphism (SNP) sites from 835 patients with severe COVID-19 disease defined as respiratory failure and 1255 control participants from Italy and 775 patients and 950 control participants from Spain. Significant associations were observed with SNPs on chromosome 3p21.31 and on 9q34.2. Furthermore, the frequency of the risk alleles of the lead variants in 3p21.31 and 9q34.2 was higher in patients with mechanical ventilation compared to those who received oxygen supplementation. The association on 9q34.2 was mapped to the ABO locus. The group-specific analysis showed a higher risk for group A (OR = 1·45, $P = 1{\cdot}48 \times 10^{-4}$) and a protective effect for group O (OR = 0·65, $P = 1{\cdot}06 \times 10^{-5}$).

Natural antibodies against SARS-CoV-2 infection

The SARS-CoV-2 viruses produced in individuals of groups A, B, AB and O express A, B, A and B antigens, and none, respectively. People in groups A, B, AB and O have anti-B, anti-A, none and anti-A/anti-B/anti-A,B antibodies, respectively. Therefore, these antibodies can react to the corresponding antigens and inhibit, at least partially, interpersonal infection between certain individuals with different ABO phenotypes [13]. This situation resembles 'matched' and 'mismatched' combinations in blood transfusion. For example, SARS-CoV-2 viruses produced in group A individuals may express A antigens and infect group A or AB individuals without such antigen–antibody reactions. However, infection in group B or O that possess anti-A antibodies may be somewhat inhibited. Similarly, group B SARS-CoV-2 viruses can infect individuals from group B or AB. However, infection in group A or O individuals possessing anti-B antibodies may be somewhat limited. SARS-CoV-2 infectivity is shown schematically in Fig. 1. Solid and dotted arrows indicate infectivity without and with inhibition, respectively. Inhibition may or may not be 100% efficient. Once infection is established, newly produced SARS-CoV-2 viruses exhibit the same ABO phenotype as the infected individual, and these antibodies no longer inactivate them. Therefore, natural antibodies seem to be only relevant for the initial attack rate and not for the subsequent productive infection. Ironically, group O individuals with the lowest risk of becoming infected by SARS-CoV-2 can produce group O SARS-CoV-2 viruses capable of infecting individuals with any ABO phenotype efficiently. Consequently, countries with the highest frequency of O alleles, such as Ecuador (75%) and Peru (70%), also suffer from the COVID-19 pandemic. It should be noted that infectivity is directional and depends on matched/mismatched ABO phenotypes of SARS-CoV-2 and host cells.

Individuals with anti-A antibodies (groups O and B combined) were represented less in patients with COVID-19 than individuals lacking anti-A antibodies (groups A and AB combined) [14]. Furthermore, patients in group O were underrepresented, whereas group B patients were overrepresented, suggesting a greater protective effect of anti-A antibodies in group O than anti-A antibodies in group B ($P < 0{\cdot}001$). Anti-A, anti-B and/or anti-A,B antibodies of the IgA class may be primarily responsible for mucosal immunity, although natural antibodies of other classes, especially of the IgG class, may also function. Inhibition results in a decrease of R0, the expected number of cases generated directly by a case. Furthermore, inhibition may be more effective in ABO-heterogeneous populations than in ABO-homogeneous populations.

Group O individuals have 25% lower serum levels of von Willebrand factor (vWF) and factor VIII (FVIII) essential for platelet adhesion, aggregation and fibrin clot formation. vWF, a transporter and stabilizer protein for

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FVIII, is mostly synthesized in vascular endothelial cells and released into plasma. Group non-O individuals have an increased risk of thrombosis, pulmonary embolism and venous thromboembolism [15]. The dysregulation of vascular tone and permeability and the induction of cytokine storm and redox stress are therefore somehow associated with the ABO polymorphism. Consequently, the ABO polymorphism can differentially affect the progression of COVID-19 disease by the molecular mechanism that does not involve natural antibodies. However, the disease progression also depends on other more underlying relevant factors. And several of them have much higher ORs than the ABO polymorphism. For example, people over the age of 85 have ORs of 13 and 630 for hospitalization and death, respectively, compared to people between 18 and 29 [2]. In these circumstances, the ABO effects on clinical outcomes of severity and mortality can be easily masked, which may provide an explanation for the controversy about the presence/absence of association [16, 17]. The new studies that are coming will help us to better clarify this and many other aspects of the ABO involvement in the SARS-CoV-2 infection and the COVID-19 progression.

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Guidance for the procurement of COVID-19 convalescent plasma: differences between high- and low-middle-income countries

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Vox Sanguinis

Abstract

Background and objectives COVID-19 convalescent plasma (CCP) has been used, predominantly in high-income countries (HICs) to treat COVID-19; available data suggest the safety and efficacy of use. We sought to develop guidance for procurement and use of CCP, particularly in low- and middle-income countries (LMICs) for which data are lacking.

Materials and methods A multidisciplinary, geographically representative group of individuals with expertise spanning transfusion medicine, infectious diseases and haematology was tasked with the development of a guidance document for CCP, drawing on expert opinion, survey of group members and review of available evidence. Three subgroups (i.e. donor, product and patient) were established based on self-identified expertise and interest. Here, the donor and product-related challenges are summarized and contrasted between HICs and LMICs with a view to guide related practices.

Results The challenges to advance CCP therapy are different between HICs and LMICs. Early challenges in HICs related to recruitment and qualification of sufficient donors to meet the growing demand. Antibody testing also posed a specific obstacle given lack of standardization, variable performance of the assays in use and uncertain interpretation of results. In LMICs, an extant transfusion deficit, suboptimal models of donor recruitment (e.g. reliance on replacement and paid donors), limited laboratory capacity for pre-donation qualification and operational considerations could impede wide adoption.

Conclusion There has been wide-scale adoption of CCP in many HICs, which could increase if clinical trials show efficacy of use. By contrast, LMICs, having received little attention, require locally applicable strategies for adoption of CCP.

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the cause of coronavirus disease 2019 (COVID-19), has spurred a modern pandemic. Passive antibody administration through transfusion of plasma collected from donors who have recovered from COVID-19 has emerged as a promising therapy for the treatment of COVID-19 [1]. This stems from early reports from China where favourable outcomes were observed following administration of convalescent plasma to patients with severe and/or life-threatening COVID-19 [2–4]. Convalescent plasma is not a novel therapeutic approach: it has been used for over a century to treat a variety of infectious diseases, including other coronaviruses (e.g. severe acute respiratory syndrome [SARS], Middle East respiratory syndrome [MERS]) [5–9]. The efficacy data supporting early use of convalescent plasma to treat COVID-19 were limited and largely gleaned from small, uncontrolled case series whereby interpretation of the data was complicated by the presence of concurrent therapies and severity of illness [2, 3, 10]. More recent data both from a matched controlled study and a randomized clinical trial suggest benefit of CCP, even in the setting of severe COVID-19 [11, 12]. At least one study does question its value whereby the mortality was not observed to be significantly different between recipients of CCP and that of controls [13]. However, adverse events have been few to date, suggesting that the risk is comparable to that of non-immune plasma [10, 14]. Rigorously controlled studies – including clinical trials – are already underway and should provide the necessary means to guide practice, definitively [1]. Until those data become available, convalescent plasma is one of only a few available options to contend with COVID-19, providing a stopgap ahead of the possible development of targeted treatment (e.g. direct acting antivirals, plasma-derived SARS-CoV-2 hyperimmune immunoglobulins, monoclonal antibodies,) and/or

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preventive strategies (e.g. vaccines). There could also be scope where CCP could be used as a longer-term treatment option, particularly in low- and middle-income countries (LMICs) where resource constraints could bar access to novel treatments, even once available.

We sought to describe the challenges to the convalescent plasma workflow, which span donor identification, recruitment, collections, blood product processing and distribution with an ultimate view to addressing patient needs. Further, while attention to the pandemic has largely focused on high-income countries, it is important to note that the COVID-19 disease burden extends to LMICs that lack comparable resources to contend with the pandemic. This includes to the procurement of convalescent plasma; specifically, the challenges of scaling up this intervention are likely to be affected by the local environment and associated resource constraints. LMICs suffer from a host of systemic challenges that impact their ability to contend both with the health crisis at large and adoption of COVID-19 convalescent plasma (CCP) [15]. This requires careful consideration if to devise solutions that are locally or regionally applicable.

Materials and methods

The International Society of Blood Transfusion (ISBT) established a working group (WG) to develop a guidance document pertaining to the use of CCP as a treatment for COVID-19. The WG comprises 41 members with expertise spanning transfusion medicine, infectious diseases, adult and paediatric haematology. Many of the invitees were members of other ISBT Working Parties (WPs) including clinical transfusion, global blood safety hemovigilance and transfusion-transmitted infectious diseases; most of the invitees were actively engaged in CCP initiatives. In addition to ISBT, members were also aligned with AABB (formerly American Association of Blood Banks) and the Asian Pacific Blood Network. The members represent the Americas, Europe, Africa, Asia and Australia. Three subgroups were established based on interest and expertise related to the donor, product and patient. A series of questions pertaining to each domain was devised and addressed by the subgroups (April to May 2020), based on the best available evidence. Donor- and product-related content was combined into a single document. The content of the guidance document was informed both by expert opinion and a survey, which was administered to members of the ISBT Convalescent Plasma Working Group. In selected cases where there was insufficient geographic representation within the group, the survey was shared with outside members of ISBT. Discussion points were cross-referenced drawing on available evidence at the time (e.g. pre-print and published peer-reviewed data), coupled with government institutional (e.g. European Commission, US Food and Drug Administration) or professional society guidelines. The approach was primarily descriptive yet the findings were used to guide practice through anticipation of potential challenges, particularly in LMICs.

The content areas that are summarized here include donor selection criteria for CCP collection, pre-donation qualification of CCP donors (including antibody testing) and operational considerations pertaining to collection, storage and distribution of CCP. A separate paper that focuses on clinical use of CCP and related concerns has been prepared.

Donor eligibility

All donors require evidence of COVID-19, either by a molecular test for SARS-CoV-2 (i.e. typically undertaken during active infection), or the presence of antibodies against SARS-CoV-2 (following resolution of symptoms resolution) [16]. It is recommended that blood collectors review documentation of infection rather than rely on verbal account alone. In some countries, a history of symptoms consistent with COVID-19 may also permissible in lieu of laboratory testing [17] (Table 1).

Donors need to have recovered (i.e. be free of symptoms) at time of donation. Definition of 'recovery' is somewhat variable (Table 1). A minimum of 14 days following resolution of symptoms is consistently applied across countries [18]. However, countries differ in regard to their requirements for repeat negative testing for SARS-CoV-2. Between 14 and 28 days, some countries require a negative molecular test (e.g. of nasopharyngeal swab) before allowing donation. This requirement was largely informed by the perceived risk to collections staff rather than concern of transfusion transmission of SARS-CoV-2 (RNA-aemia is rare in the absence of symptoms) [19]. Nonetheless, some countries maintain stringent requirements for repeat testing, in some cases requiring paired negative tests (e.g. throat and nasopharyngeal swabs [NP] 24 h apart) to confirm viral clearance [20]. The requirement for negative testing has been questioned given limited capacity to perform tests coupled with challenges surrounding the interpretation of those results. Specifically, a high proportion of individuals are still positive for RNA on repeat NP swabs following resolution of symptoms. RNA positivity does not necessarily correlate with infectivity, further confusing the determination of donor suitability. By 28 days after being symptom-free, most countries allow for donation even in the absence of repeat negative testing.

It is important to note that donors of CCP are still required to satisfy all eligibility criteria for community

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Table 1 Risks, challenges and potential strategies pertaining to determination of donor eligibility, recruitment and qualification for CCP donation.

Donor considerations	Approach	Challenges
Donor awareness	Education/awareness about the process of becoming a blood donor (and thus a CCP donor)	A high proportion of convalescent plasma donors are expected to be first-time donors • Low familiarity with eligibility criteria and donation process • First-time donors are high risk for transfusion-transmitted infections and higher risk for donation related adverse events than repeat donorsDonors of CCP need to satisfy same eligibility criteria as community blood donors • Attestation from a licensed physician as an accepted donor is needed in some settings • In case of a deferral: need 2to properly communicate reason for deferral/ ineligibility including test results, for example infectious disease results.
Donor eligibility	Standardization of donor eligibility criteria	Lack of uniformity in donor eligibility criteria with respect to: • Ascertainment of diagnosis*Molecular testing* at time of symptomatic disease vs. *Evidence of antibodies* against SARS-CoV-2 following resolution vs. *Symptoms* consistent with COVID-19 in absence of testing • Time since resolution of symptoms to be eligible to donate (e.g. 14 days vs. 28 days) • Requirement for negative SARS-CoV-2 testing prior to donationThe criteria for eligibility are continually evolving as more information is known • Lack of consensus
	• Medical Director use discretion to qualify donors • Relaxing of selected eligibility criteria e.g. donation frequency	• Need to preserve donor safety and comply with national/local regulations
Donor identification	• Self-identification • Hospital-based referral • Mining electronic medical records and patient registries	• Donor education: A high proportion of those who self-identify will not qualify*Variable reliability of self-referrals* • Motivation of donors may alter information to secure early donation to aid a friend/family member in need; anticipated/promised reimbursement • Recall: timing of symptom resolution • Test-seeking to confirm immune status • Individuals may not be able to provide documentation attesting to confirmed infection • Some donors may not have internet access or be internet savvy • Donors may be wary of telemarketers and are unwilling to answer phone calls or and scheduling online • Same donor may be associated with multiple hospitals/blood centres
Donor recruitment	• Community and hospital outreach • Social media • Professional websites • Formal news outlets • Reflex patient notification following positive test • Health departments	• Lockdown policies restrict access to eligible individuals • Donors may not be adept with technology, limiting uptake of websites and online applications • Donors may be contacted by multiple organizations • Motivators for and deterrents against blood donation not well studied in LMICs • Electronic medical records and patient registries not widely available in LMICs

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Table 1 (Continued)

Donor considerations	Approach	Challenges
Pre-donation qualification	• Pre-donation screening and administration of donor history questionnaire	*CCP donors need to meet all the same eligibility criteria as community blood donors* • Individuals who satisfy criteria for CCP donation may be deferred for unrelated reasons, for example travel and MSM
	Gender and parity-based screening	Depending on country/blood establishment policy, parous females may be deferred from blood donation as part of TRALI mitigation • In some countries, parous females may be subject to HLA antibody screening
Compensation/ reimbursement	Donor compensation	Policies regarding compensation vary widely by country Expectation of replacement and/or paid donation is common in low and low-middle-income countries. • Confers risk of TTIs • Limited reimbursement for travel and small gifts that cannot be monetized may be permissible in some high-income countries • Donors may be allotted special bonus points/blood centre non-monetary currency for CCP donation • COVID antibody testing may motivate incentivize donation • Active recruitment of donors at paid plasma collection sites to support hyperimmune globulin and vaccine development could result in competition between community blood centres and dedicated plasma collection sites for eligible donors
	Community organizers	• Community organizers may expect compensation for identification/referral of potential donors. • The ISBT Code of Ethics does not support compensating community organizers for identifying/referring potential donors, outside of traditional compensation mechanisms for the appropriate reimbursement of tests performed
Donor Privacy	Informed consent	Loss of privacy and confidentiality • Balancing respect for privacy and confidentially with need to access donor medical records to identify eligible donors for CCP • Data sharing via email or other electronic means between referring hospitals and health agencies with donor centre • Unintended release of private material (e.g. donor pictures, videos and clinical stories/histories) on social media without consent.
Donor safety	Procedural risks	• First-time donors are higher risk of donation-associated adverse events than repeat donors, for example vasovagal reactions • Risk and complications from the venipuncture and apheresis procedure, for example hypocalcemia during apheresis • Some donors may be more comfortable with a whole blood donation versus apheresis procedure
	Repeat donations	• Adverse effect on immunity following repeated donations has NOT been shown
	Psychological duress to donors	*Donors may feel obligated to donate* Societal pressure/expectation. • May discourage admission of high-risk behaviour impacting risk of TTIs*Risk of repeated quarantine* • A high proportion of individuals have positive PCR tests from nose or throat swabs 14–27 days post-symptom resolution conferring risk of quarantine until PCR negativeThe interpretation of persistent PCR-positive test result is unclear, that is whether testing represents active infection (live virus)

CCP, COVID-19 convalescent plasma; ISBT, International Society of Blood Transfusion; LMICs: low- and middle-income countries; MSM, men who have sex with men; PCR, polymerase chain reaction; TTI, transfusion-transmitted infections; TRALI, transfusion-related acute lung injury.

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blood donation [18]. Those requirements are intended to preserve donor safety while protecting against risk of transfusion transmissible infections (TTIs). This needs to be integrated into pre-donation qualification to avoid deferral at time of donation despite having satisfied eligibility criteria to serve as a CCP donor. Some criteria for community blood donation have been relaxed with the advent of COVID-19 crisis, including the deferral period following travel, minimum haemoglobin levels, and deferrals pertaining to variant Jacob–Creutzfeldt disease (vCJD) and men who have sex with men (MSM) [21].

The donor eligibility criteria for CCP vary widely by country or even by institution within a given country (Table 2). A determination of donor eligibility is a formidable challenge in LMICs. Capacity for SARS-CoV-2 testing is low in LMICs, even for acutely symptomatic patients. This is ascribed to limited laboratory infrastructure, availability of testing kits and technical expertise, all of which are necessary to execute large scale molecular testing and surveillance. Without testing, the pool of eligible CCP donors remains uncertain. At time of writing, most LMICs report less than 10 000 cases of COVID-19 with most reporting tens to hundreds of cases, questioning whether there is as yet a critical mass of tested individuals and – broadly – whether the burden of COVID-19 is being severely underestimated [22].

Donor recruitment

A variety of approaches have been used successfully to recruit donors for the international CCP initiative. Both formal (e.g. news outlets) and social media have raised public awareness about COVID-19 and the potential efficacy of CCP. This has helped to spur self-identification, whereby recovered patients have been volunteering to donate. There are also parallel active recruitment efforts by blood centres and hospitals, through identification of patients either during admission or testing. Both testing sites and community public health surveillance initiatives can also be used effectively to identify potential donors. For example, recruitment materials can be shared with those who test positive for SARS-CoV-2.

At time of recruitment, information about CCP is provided to prospective donors including the eligibility for donation, the intended application of use (e.g. investigational vs. compassionate use), method of collection and the donation process itself. Many – if not most – CCP donors are expected to be first-time donors and will be unfamiliar with the donation process. First-time donor status introduces considerations of risk: first-time donors are higher risk of TTIs and donation-associated adverse events than repeat donors [23–27]. Repeat donation selects for healthier individuals as criteria for donation (e.g. notably the absence of social and medical risk factors for infections) need to be met at each donation. Donor status is a notable concern in LMICs: while robust infectious marker screening in HICs and residual risk of TTIs is low, this is not universally the case in LMICs where high incidence and prevalence of TTIs, near exclusive reliance on antibody testing and suboptimal quality systems contribute to risk of TTIs. In short, the theoretical benefits from CCP need to be weighed against the real risks of TTIs. Given the relaxation of some temporary deferral periods during the pandemic, there may be additional risk that has not yet been quantified. Pre-donation qualification and donor informed consent are routine safeguards required to ensure privacy and confidentiality of donors.

There are additional considerations that are specific to CCP. First, given that CCP is still of largely unproven efficacy, some countries have only allowed recruitment of donors for CCP as part of approved clinical trials (e.g. Italy and South Africa). The latter are planned or already underway to evaluate efficacy. Second, given parallel efforts to produce hyperimmune globulin, there is potential competition for eligible donors for convalescent donors, particularly given the ability to compensate donors at plasma collection centres in some countries (e.g. USA). By contrast, community blood centres – at least in most high-income countries – are bound by stringent regulations that limit or preclude financial compensation. Instead, only gifts that are unable to be monetized or reimbursement for travel are allowed.

There is enormous heterogeneity among LMICs with respect to capacity for donor mobilization, collections and distribution [28, 29]. Indeed, some LMICs are able to sustain their blood supplies using voluntary non-remunerated donors (VNRBDs), exclusively. Nonetheless, donor eligibility and mobilization is likely to be a major challenge in the majority of LMICs. Independent of COVID-19, there is an unmet need for blood products in LMICs [30]. In large part, this stems from a low proportion of the eligible population that donate. Recruitment of voluntary non-remunerated donors (VNRBDs) is complex and relatively expensive in LMICs. Ideally, recruitment of VNRBDs is guided by local or regional knowledge of the motivators for and barriers against donation. Such is largely lacking in LMICs. Instead, there is reliance on replacement (e.g. friends and families of the intended recipient) and/or paid donation in decentralized transfusion services in LMICs. Recruitment in HICs has relied, primarily, on prosocial motivation ('altruism'), whereby donors self-identify as being willing to contribute. It is uncertain to what extent that this approach to recruitment applies to the replacement and paid donation – models and how that might impact the CCP initiative in LMICs. Most of the research to guide donor recruitment

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Table 2 Regional variation in criteria for COVID-19 convalescent plasma procurement

Geographical distribution	Country	Definition of diagnosis*	Definition of donor recovery for eligibility						
			At least 14 days since resolution of symptoms without additional testing	14–28 days from resolution of symptoms with negative results for COVID-19 on donated plasma	>28 days post symptom resolution	>28 days post-symptom resolution OR >14 days negative result of a NAT testing on NP swab	>14 days post symptom resolution and 1 negative results for SARS-CoV-2 PCR or by a molecular diagnostic test from blood	Symptom free for more than 14 days AND 2 negative SARS-CoV-2 PCR tests on 2 different days	Negative result of a NAT testing on NP swab and molecular diagnostic test from blood, performed 14 days after the first test
AMERICAS	United States	√			√		√		
	Canada	√	√		√^				
	Brazil	√					√		
EUROPE	Italy	√						√	√
	UK	√			√				
	Netherlands	√	√						
	France	√	√						
	Spain	√						√	
	Germany	√				√			
	Belgium	√			√				
Asia	Singapore	√			√				
	Hong Kong	√			√				
	China	√					√		
	Taiwan*****	√			√		√		
	India(fx)	√			√			√	
	UAE	√			√			√	
	Oman	√			√		√		
	Saudi	√			√			√	
	Qatar	√			√				√
Africa	South Africa	√			√		√		
	Nigeria	√			√		√		
Australia	Australia	√			√				

Pathogen inactivation is NOT intended for the SARS-CoV-2 inactivation.
(fx) In India: donors who have had COVID diagnosis more than 4 months will be excluded from donation.
*Prior diagnosis of COVID-19 documented by a PCR test at time of infection OR by positive anti-SARS-CoV-2 serology following infection.
**Neutralizing antibody titre >1:80 by AABB). A titre of 1:80 may be considered acceptable if an alternative matched unit is not available (per FDA)
***Cut-off for sero-positivity will be set as the mean value +3 SD of the ELISA signal obtained with SARS- CoV-2 negative plasma (pool of plasma samples collected before 2020) at a 1:100 plasma dilution. NAT will not be used as a criteria to release CP (%%) Every 7 days as permitted by allogeneic donor eligibility criteria. Maximum number of donations are limited by the annual limit on volume of donation.
****France: testing has evolved over time: initially a systematic seroneutralization titre (+ an ELISA), more recently a systematic ELISA and seroneutralization titre when ELISA values are within a range of values associated with insufficient negative or positive predictive value a seroneutralization titre >40.
*****Data for Taiwan are based on optimal understanding of the situation as the low number of cases did not justify so far the transfusion of convalescent plasma.
†Highlights practices for Canadian Blood Services versus Hema-Quebec.
‡Performed neutralising antibody titres and now performs Euroimmun tests that equate to a neutralising antibody titre of >1:100.

practices stems from HICs [31]; many of those practices may not be applicable to LMICs, underscoring the need for research that is conducted locally or regionally [32–34]. While more readily accessible and lower cost to recruit replacement and paid donors, these are regarded as higher risk for TTIs [35].

Available recruitment approaches differ between high- and LMICs. For example, social media and formal news outlets could be applied broadly. By contrast, proposed strategies to mine patient records are difficult in LMICs given largely absent electronic medical records and variability in patient registries [34, 36].

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Acceptable Cut off for sero-positivity OR SARS-CoV-2 Antibody titres (if applicable)						Accepted interval for Repeat Donation and Total Number of Donations				Gender-Specific Criteria as TRALI mitigation strategy			Pathogen Inactivation
NAT titre >1:160	NAT titre >1:80	NAT titre >1:40	ELISA signal specification only	positive testing for anti-SARS-CoV-2 antibodies	No antibody tests approved currently, samples will be stored for retrospective testing	Every 7 days (%)	Every 2 weeks	Every 4 weeks	min. 2 donation free days between 2 plasmaphereses; max. 60 plasmaphereses per year;	Male donors or female nulliparous donors or negative for HLA antibodies	Male donors or nulliparous female donors	Male Donors Only	
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Pre-donation screening and testing of potential CCP donors

Pre-donation screening is intended to vet potential donors to ensure that they satisfy criteria specific to CCP as well as community donation. Given proximity to illness, some of the pre-donation screening may be undertaken over the phone or electronically (e.g. email). Screening questions address eligibility (e.g. dates of symptom onset and resolution) and donor health. Depending on the regulatory requirements, there may a need for the donor to provide formal documentation of testing.

In regions/countries where SARS-CoV-2 molecular testing is not routinely performed prior to hospital discharge or de-isolation, a minimum time period is required following resolution of symptoms prior to becoming eligible to donate

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CCP and other blood products. Most countries adhere to 14–28 days following resolution of symptoms (Table 2). Longer time periods (e.g. ≥28 days) offer dual benefit, ensuring that potential donors are no longer infectious (i.e. affording protection to the collections staff) while also allowing for sufficient time for adequate seroconversion.

Some establishments – albeit a minority – require pre-donation SARS-CoV-2 molecular testing of the blood in addition to negative testing by nasopharyngeal swabs [18, 37].

A central element to pre-donation screening is the demonstration of antibody formation. Not only is this needed to demonstrate recovery, but also the antibodies are postulated to exert efficacy against SARS-CoV-2. Unfortunately, there are multiple challenges pertaining to SARS-CoV-2 antibody testing. At time of writing, there is enormous variability in testing with little standardization to date. This relates to the assays in use, the settings in which they are being deployed (e.g. clinical vs. research laboratories) and what constitutes an acceptable threshold for donation (e.g. optical density, titre, antigenic specificities), particularly given that antibody profiles in the context of CCP treatment have not yet been correlated with clinical outcomes. The immune response in COVID-19 is complex, highly heterogeneous and is as yet not well understood [38, 39]. Neutralizing antibodies have been assumed to be desirable yet titres are variable, particularly in those who with mild to moderate infection [16, 40, 41]. Formal neutralization assays (e.g. plaque reduction neutralization tests [PRNT]) are not amenable to high throughput testing, requiring Biosafety level 3 laboratories and incurring long turnaround times, offering results in 5–7 days of initiation [42]. The assays themselves are also technically demanding accounting for considerable variation in results between laboratories. Therefore, neutralization assays are performed in relatively few laboratories and most institutions do not have ready access to neutralizing assays to determine antibody titres. Even for those that do there is lack of agreement as to what is acceptable. For example, the FDA and European commission recommend that titres are optimally ≥160 or ≥320, respectively; however, both regulatory bodies allow for lower titres (e.g. 80) if unable to meet the optimal titres or simply evidence of antibodies using a qualitative serological test [16, 17]. Comparative analyses between the various neutralization tests performed in different laboratories are already underway.

Given the challenges surrounding neutralization assays, most are relying on enzyme immunoassays (i.e. ELISAs) to qualify donors. While increased numbers of assays are becoming available, typically targeting spike protein, receptor-binding domain and nucleocapsid protein [42–44], there is still uncertainty as to which isotype (e.g. IgM vs IgG) and/or subclass (e.g. IgG1 vs IgG2 vs IgG3) of antibody is most informative. Nonetheless, there appears to be good correlation between spike-binding antibodies as detected by ELISA and neutralization antibodies [44, 45]. Further, there appears to be low cross-reactivity, notably against other coronaviruses [42].

Given the collective uncertainty of interpretation and logistical barriers to antibody testing, some countries have not been prescriptive about testing, instead encouraging retention of samples such that post hoc analysis may be undertaken when testing does become more standardized. This approach will be informative but does little for immediate patient care [16]. Further, there are ethical considerations behind transfusing a blood product of already uncertain efficacy, when one cannot even guarantee that its most basic definition (i.e. the presence of SARS-CoV-2 antibodies) is satisfied or verified. The European Union guidance on CCP collection and transfusion recommends that if the measured neutralizing activity in the collected plasma is considered to be too low for use as COVID-19 CCP, the plasma should be made available for other use (ideally fractionation) [17].

Finally, there are enormous challenges for pre-donation qualification in LMICs given limited laboratory capacity to conduct antibody testing for SARS-CoV-2. Further, in some countries stringent 'lockdown' policies that severely restrict travel may discourage or impede potential donors.

Collection facilities

Collection of CCP is no different from other plasma components (Table 3). Therefore, there is no need for a dedicated policy or procedures specific to CCP. The same sites that collected plasma (using whole blood collection or apheresis) prior to COVID-19 would undertake CCP collections. Collection may be undertaken by a centralized blood service (national or regional) or by hospitals that have the necessary expertise and infrastructure to perform collections. All certified blood centres or hospitals must be licensed (i.e. to collect plasma) and need to conform to the appropriate state or national regulatory requirements for blood collections. Those requirements – which are specific to each country – span donor eligibility criteria and donor qualifications for blood donation in general; in addition, there may be requirements that pertain to CCP specifically.

It is prudent to defer mobile collections given the potential infectious risk to collection staff. This pertains to collections for general blood needs as well as CCP. Fixed sites are easier to control from an infectious standpoint particularly given the greater ease of social distancing. By contrast, mobiles (e.g. collection vans) present confined spaces. Nonetheless, if sufficient time has elapsed since an outbreak, dedicated mobile collections could serve as an

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Table 3 Summary of collection practices and product characteristics in high- vs low- and middle-income countries

Product characteristics		HICs	LMICs
Collection facility		• Licensed/accredited sites to collect plasma under the same regulatory framework that preceded COVID-19 *Fixed sites* • Centralized blood service (national or regional) certified by FDA or a competent regulatory agency • Licensed hospital-based collection site*Mobile sites* • Not being used given infectious risk to collection staff	• Same as HICs • Sites need to comply with state or national regulatory requirements for blood collections
Mode of donation	Apheresis	*Blood centres* • Major mechanism for collection; highly efficient • If apheresis in use for platelet collections, this can be adapted for plasmapheresis (including CCP)*Sourced plasma collection sites for fractionation* • Potential competition as donors are diverted contribute towards hyperimmune globulin development	• Limited access given high cost, availability of apheresis kits and requirement for technical expertise
	Whole blood	• Has not been a major collection mechanism in HICs to date • Longer inter-donation intervals (8-12w) than apheresis collections (once to twice per 7-day period) • Minimum haemoglobin requirement applies	• Major mechanism for collection; low efficiency but inexpensive • Inter-donation interval could be relaxed (e.g. weekly) as long as minimum haemoglobin requirements is met
Donor gender		• Any female who reports a history of pregnancy should ideally be screened for antibodies against human leucocyte antigen (HLA) and human neutrophil antigens (HNA); this is recommended to mitigate against Transfusion Related Acute Lung Injury (TRALI) • Never transfused male donors and female donors who test negative for HLA and HNA antibodies accepted	• HLA and HNA antibodies not routinely undertaken given cost and laboratory complexity • In absence of testing, only males or nulliparous females recommended as plasma donors.
Volume per component (ml)	Minimum	• Most units (post-aliquoting for apheresis derived units) are between 200–250 ml • Average volume per unit is 200 ml (can be 150 ml). • ~200 to 250 ml if derived from whole blood	• ~200 to 250 ml if derived from whole blood
	Maximum	• Apheresis: 600ml-800mL (based on body weight) • 200–250 ml if derived from whole blood	See HICs
	Pooling	• Not applicable	• Data not available

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Table 3 (Continued)

Product characteristics		HICs	LMICs
Number of units per collection		• Average 3–4 units per collection	• One unit per whole blood collection
Required testing for unit	Standard guidelines	All standard testing requirements for blood donation apply • ABO blood group • Red cell antibody screening • TTI testing per local/country requirements, for example HIV, HBV, HCV, T. pallidumHLA (±HNA) antibodies (parous females only)	All standard testing requirements for blood donation apply, for example, • ABO blood group • TTI testingNote: testing for TTIs varies by country with respect to • Level of standardization • Assays in use • Testing algorithms • Availability of molecular testing (uncommon for routine donor screening in LMICs) • Quality assurance
	Specific tests	Antibody testing for SARS-CoV-2 • Approaches vary widely with respect to assays in use and recommendations for titering*Testing* • Neutralizing antibody titre (n-Ab) or validated immunoassay where the assay has been correlated with n-Ab; • If testing is not readily available, some countries have allowed for banking of sample with post hoc testing when available • Solid-phase ELISA assay against SARS-CoV-2 S, RBP and N proteins are available*Titres* • Neutralizing antibody titre for SARS-CoV-2 (Range 1:80 to minimum 1:320 and 1:640 in clinical trials)	• Neutralizing Ab testing may not be available • Donor selection may be determined by reactivity in a serologic assay for anti-SARS-CoV-2 antibodies • Recommend banking samples for neutralizing antibody testing • If no testing available, recommend collection from known convalescent individuals without antibody testing
Cellular contamination		Similar to regular FFP unit: • <1 × 10e6 WBC • <50 × 10e9 plt/unit • <1 × 10e8 RBC	See HICs
Pathogen reduction		• Licensed and approved technologies are available (e.g. photochemical inactivation) • Not widely adopted • Not mandated for CCP • Recommendation to perform PR if already routine practice; • It is not recommended to implement PR specifically for CCP	• PR not in use in most LMICs likely given high cost and technical complexity of use
Time between collection and freezing		• 8–24 h	• 8–24 h

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Table 3 (Continued)

Product characteristics		HICs	LMICs
Storage	Liquid	• If plans for infusion soon after collection, store at 1–6°C after as allowable by guidelines for maximum of 5 days • If no plans for infusion soon after collection, store at room temperature and freeze at −18°C within 24 h of collection	• 24 h storage at 1–6 C permitted after thaw • For liquid plasma - 1°C and 6°C for up to 40 days.
	Frozen	• ≤−18°C within 24 h of collection until administration • Expiration: 1 year at −18°C	• 24 h storage at 1–6 C permitted after thaw • For liquid plasma - 1°C and 6°C for up to 40 days.
Labelling		• ISBT-128: ICCBBA has issued a range of description codes for CCP • There is an ISBT128 label specific to CCP • Alternatively, there should be a text label with 'Convalescent Plasma' and/or using tag on CCP units • Special labelling as an investigational product for treatment of COVID-19 may be needed-Note: Integration of the new product codes into existing IT systems may be challenging	See HICs
Traceability		• Full traceability, as per all blood products. • Compliance with national or local regulations	See HICs
Release		• Compassionate use • Research (e.g. clinical trials) • Expanded access programmes (i.e. clinical use with data reporting requirements)CCP testing requirements (e.g. antibodies) vary based on intended use;	• Currently, most CCP administered through clinical trials • Compassionate use is also available
Expiration		• Thawed: 5 days for thawed plasma. • 12 months if frozen (same as for standard frozen plasma)	See HICs
Other products/ derivatives		CCP will likely only serve as a supportive therapy (and not the main therapy) in the future for HICs. *Hyperimmune gamma globulin* • Alliance of manufacturers has been established to accelerate development of a plasma-derived hyperimmune globulin therapy against COVID-19 • Promise of greater standardization of dosing than CCP • Differences in collection and donor eligibility requirements than CCP; donor who do not meet apheresis plasma donation criteria may still meet criteria for plasma fractionation (e.g. vCJD risk)	• Unknown at time of writing

CCP, COVID-19 convalescent plasma; FFP, fresh frozen plasma; HBV, hepatitis B virus; HCV, hepatitis C virus; HICs, high-income countries; HIV, human immune deficiency virus; HLA, human leucocyte antigen; HNA, human neutrophil antigens; IT, information technology; LICs, low-income countries; n-Ab, neutralizing antibody; PR, pathogen reduction; RBC, red blood cell; T. pallidum, treponema pallidum; TRALI, transfusion-related acute lung injury; TTI, transfusion-transmitted infection; vCJD, variant Creutzfeldt–Jakob disease; WBC, white blood cell count.

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efficient means to collect CCP in communities that have been impacted and since recovered from COVID-19.

Blood centre policies are developed around universal precautions. Nonetheless, specific policies and associated measures may be needed to optimize employee safety and/or preserve employees' confidence in their safety. These apply to general blood and CCP collections alike. This may require screening donors and employees (e.g. inquiry about symptoms and signs of COIVD-19, obtaining temperatures) prior to entering the collection facility. Social distancing of at least 1·5 m (6 feet) can be accommodated for part of collection process; however, during the confidential donor interview process and collection process, employees and donors will be closer than 1·5 m for some time. Some countries have broadly mandated routine wearing of masks in public, while other have focused on asking phlebotomy staff and CCP donors to wear masks during the collection process. Recommendations regarding the need for personal protection equipment (PPE) use have evolved over the course of the pandemic [46, 47]. Routine use of PPE was not initially recommended; with evidence of transmission of SARS-COV-2 from otherwise asymptomatic individuals, most guidelines, at least in HICs, recommend at least some form of face coverings for donors and blood centre staff. Access to PPE varies greatly but is generally limited, particularly in LMICs, resulting in an increase use of home-made masks, which may be of variable efficacy.

Mode of collection

Plasma collection using apheresis technology is the ideal, offering a highly efficient mechanism to collect large volumes of plasma. A single donor can contribute as many as 3 or 4 units (~600 to 800 ml) of plasma. Apheresis is the major mode of collection in HICs for CCP. Nonetheless, there are barriers to its expanded use, particularly in LMICs including high cost, technical expertise and availability of apheresis kits. Therefore, apheresis is not available in some countries. If apheresis is already in use for routine platelet collections, there are ways to adapt those existing technologies to plasma (including CCP) collection.

For countries that do not have apheresis equipment there is the option to recover plasma from a whole blood collection, whereby the parent product is separated into components (i.e. plasma and red blood cells) after collection. However, whole blood collections to produce CCP raises some concerns in LMICs. First, anaemia is highly prevalent in LMICs, and many potential donors may not meet the minimum haemoglobin threshold for donation [28]. Whole blood donations also confer longer deferral periods (e.g. 8–12 weeks) than plasma. In some circumstances (e.g. for fractionation), plasma donors are allowed to donate as frequently as twice a week yet adverse effect is rare. Therefore, apheresis optimizes efficiency and frequency of collections [15]. Specific to CCP, obvious potential donors are those who have been acutely ill; given comorbid risk factors for severe disease (e.g. advanced age, cardiorespiratory disease, diabetes e), these individuals may not be ideal candidates for donation given concerns over donor safety. It is important to note that plasma preparation from whole blood collections is not unique to LMICs; in addition, there has been relaxing of the inter-donation intervals whereby whole blood donors could – conceivably – be allowed to donate frequently as long as the donors still meet minimum haemoglobin thresholds. Similarly, status as an LMIC does not bar apheresis as was shown during the 2014 Ebola outbreak in West Africa where logistical barriers were overcome and CCP was collected successfully [48].

Product characteristics

The manufacturing of CCP units is similar to units of either recovered plasma or concurrent/apheresis plasma depending on whether the plasma is derived from a whole blood or apheresis collection, respectively. The volume of the product collected by apheresis may vary, depending on the gender, body weight and height of the donor, as well as the device that is used; in some cases, the collection volume can exceed 800 ml [49]. Following apheresis collection, the CCP follows the same manufacturing process as transfusable apheresis plasma products; it is separated into an appropriate number of products based on the collection volume after which it is typically frozen within 8–24 h of collection. The volume of the units is uniform (but not exact). For example, the minimum volume in the US is ~200 ml (although it can go down to 150 ml); therefore, larger product volumes that are collected using apheresis devices are split into multiple products, each containing at least the minimum designated volume. One needs to pay attention to the maximum volume as many CCP protocols limit the total volume of CCP which can be transfused into a patient. Although many blood centres are performing antibody testing of CCP units, the test results have not been uniformly required as a release criterion for CCP units with a view to compassionate use. By contrast, many research (i.e. clinical trial) protocols require characterization of the CCP (i.e. determination of antibody titres) units prior to use.

Testing

COVID-19 convalescent plasma needs to satisfy the same requirements as community blood donation as are locally

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in effect in the country or state of operation. Those testing requirements must be met prior to release of the CCP. Testing is primarily focused on TTIs, ABO isohemagglutinin titres, red cell and HLA antibodies. In some cases, testing for neutrophil antibodies is also undertaken. In most HICs, molecular and/or antibody testing is performed to detect the major TTIs (e.g. HIV, HTLV, hepatitis B and C viruses, syphilis). In many LMICs, TTI testing is more variable, both with respect to the assays, algorithms and quality assurance in use [50, 51]. In countries with high rates of TTIs, quarantine systems or pathogen reduction (PR) of the plasma is recommended but are rarely feasible. However, a quarantine system (i.e. fresh frozen plasma from whole blood being stored until the donor returns and provides a subsequent donation) is logistically challenging especially for CCP. In a few clinical trials, additional tests have been undertaken such as hepatitis E virus (HEV), hepatitis A virus, parvovirus B19, even if these were not tested for routinely prior to COVID-19. Infectious marker screening for these pathogens is typically applied to sourced plasma collections (i.e. for fractionation) [52]. HEV screening of community blood donors is routine in some countries (notably in parts of Western Europe and Japan) given evidence of transmission and risk – albeit rare – of transfusion-associated morbidity [53–55]. In the case of CCP, it is unclear why additional infectious marker testing was adopted specifically; one could speculate that those tests were added out of an abundance of caution [56, 57].

Testing is typically undertaken after collection. To that end, one might consider pre-donation testing, particularly in the event that apheresis is being used given the high cost of the collection kits. HLA antibody testing is routinely employed as a mitigation measure against transfusion related acute lung injury (TRALI) in mostly HICs. Pre-donation HLA antibody testing may be worthwhile in parous females given that up a third of women who report having been previously pregnant have HLA antibodies [58]. In countries where HLA and HNA antibody testing is prohibitive, eligibility to donate CCP may be restricted to males and nulliparous females.

Unlike other blood components (e.g. red blood cells, platelets and cryoprecipitate), quality indices are not typically required for plasma; this is currently the case for CCP units. For situations where antibody testing is not readily available, collection of a retention tube for later qualification of the transfused CCP is recommended.

Labelling

In general, it is recommended that labels and coding adhere to ISBT-128 standards. All units of CCP should be labelled specifically as COVID CP or Blood (Ref 11 and 'Recommendations for Investigational COVID-19 Convalescent Plasma | FDA; 1 May 2020'). For ISBT-128 users, there are a range of product codes that have been generated by the international standards organization which is responsible for the management and development of the ISBT 128 Standard (ICCBBA). Additional requirements are country specific. For example, in the USA, all CCP must also include following statement, 'Caution: New Drug--Limited by Federal (or United States) law to investigational use'. Challenges specific to CCP pertain to integration of the new product codes into existing IT systems. However, the base label is the same as regular plasma for transfusion.

Storage

It is recommended to freeze the plasma at −20°C or preferably colder within 24 h of the end of the collection. Plasma should be stored frozen at constant temperature below −20°C until administration. In settings without access to −20°C freezers (e.g. some LMICs), plasma can be frozen at −18°C or colder within 24 h after blood collection. Under specific circumstances when freezing is not available, liquid plasma may be stored between 1°C and 6°C for up to 40 days.

Frozen plasma can be stored for up to 12 months. Longer periods of storage should be shown not to have altered the therapeutic efficacy of CCP. CCP (like other plasma components) must be transfused ideally as soon as possible after thawing, but definitely within five days of thawing.

Pathogen reduction

Pathogen reduction (PR) refers to a variety of emerging technologies (e.g. photochemical inactivation, solvent detergent treatment) that act directly on the blood product, mitigating risk against a range of pathogens rather than a single or a few pathogens (i.e. the case with infectious marker testing). There are already licensed technologies for treatment of plasma that have been shown to be effective against coronaviruses (e.g. SARS, MERS and SARS-CoV-2) [59–61]. Independent of COVID-19, PR offers the ability to contend with emerging and re-emerging pathogens. Nonetheless, the benefit of PR in the context of SARS-CoV-2 is unclear. For one, RNA is rare in the blood of symptomatic individuals with COVID-19 [20]. While it has been detected rarely in asymptomatic (i.e. recovered) individuals, respiratory viruses are not known to be transfusion transmissible, or at least to result in clinical infection if transmitted [19]. This is tempered by the uncertainty surrounding pathogenesis of a novel virus. PR would allay concerns related to viral

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transmission from CCP. Pertinent to LMICs, CCP donors are more likely to be first-time donors and thus have a higher risk of TTIs [23, 25–27]. In countries that issue recovered FFP through a quarantine system routinely, PR would address the risk of a the major TTIs (e.g. HIV, HBV and HCV) [37, 62], increasing the overall availability of donor CCP [18]. At time of writing, efforts are underway to evaluate the impact of PR on antibody levels, the safety of PR plasma already in use (i.e. in some countries in Europe and North America) and formal evaluation of PR on the SAR-CoV-2 virus.

There are barriers to the wide adoption of PR. Foremost is cost, which may be prohibitive for most LMICs. PR also requires equipment and skilled personnel to perform. Therefore, most countries have elected not to implement PR specifically for CCP.

Limitations

There are several limitations to this guidance document. Foremost, the data are subject to change: we have tried to refrain from being too prescriptive, acknowledging that publication of new findings is occurring rapidly, and may alter the practices as currently written. Donor eligibility and pre-donation qualification criteria are two examples, where there has been significant overhaul since initial proposal. Second, there was under-representation of contributors from LMICs. At time of writing, most of CCP procurement was focused in HICs, notably the United States and Western Europe given the scale of their regional epidemics. Third, the data, particularly those depicted in the tables, are not regionally representative and in some cases may not represent all practices within a given country. There is variation in practice; this document is intended to impart a framework to contextualize one's own CCP programme if already established or to guide adoption of CCP if still being planned. It is not an exhaustive review of all countries' practices.

Conclusion

Following the advent of COVID-19, there has been remarkable scale-up in the collection and distribution of CCP. Observational data – albeit with very low level of evidence – suggest efficacy of CCP and the rates of associated adverse events are few [2, 3, 14]. Further, clinical trials are underway to evaluate the efficacy of use as post-exposure prophylaxis and treatment of COVID-19 in adult and paediatric populations alike. If CCP is shown, definitively, to work there could be an unprecedented demand for CCP both for clinical treatment and for fractionation into hyperimmune immunoglobulins. Pre-emptively and in a relatively short time, blood centres have responded to the growing demand for CCP. The eligibility criteria and recruitment strategies for CCP donors have been formalized, and collections have increased to the point that unmet need – in selected HICs – is diminishing. Nonetheless, challenges remain particularly with respect to the characterization of units of CCP and if – or how – the antibodies that are being detected, impact clinical outcomes. LMICs have been relatively neglected in the pandemic; this extends to their capacity to procure CCP [63]. There are also ethical questions pertaining to CCP, not least of which is whether it is appropriate to recommend diversion of resources towards an unproven therapy, when the existing resources in most LMICs are already insufficient to ensure a safe and adequate blood supply to meet clinical demand [64]. In the case of COVID-19, there has been a temporary decline in blood collections, potentially exacerbating the transfusion deficit. While the impact is off-set – in part – by the reduced demand for transfusion given cancellation of elective surgeries and a decline in trauma, there are still a host of challenges spanning recruitment to collections. If CCP is to be adopted in LMICs, approaches need to be tailored to local resource constraints.

Conflict of interest

EMB reports personal fees and non-financial support from Terumo BCT, personal fees and non-financial support from Grifols Diagnostic Solutions, outside of the submitted work; EMB is a member of the United States Food and Drug Administration (FDA) Blood Products Advisory Committee. Any views or opinions that are expressed in this manuscript are that of the authors, based on his own scientific expertise and professional judgement; they do not necessarily represent the views of either the Blood Products Advisory Committee or the formal position of FDA, and also do not bind or otherwise obligate or commit either Advisory Committee or the Agency to the views expressed. PPY serves on the advisory board of Fresenius Kabi and Creative Testing Solutions.

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Syncopal-type reactions tend to be delayed and result in falls among elderly blood donors

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Background Delayed syncopal-type complications are infrequent among blood donors, but sometimes have critical consequences, such as severe injury. We retrospectively investigated the characteristics of donors with delayed syncopal-type complications or falls.

Study design and methods We defined a delayed reaction (DR) as syncopal-type complications occurring >20 min after needle removal. Subjects were stratified by sex, age, estimated blood volume (EBV), body mass index (BMI) and frequency of donation. Multiple logistic regression analysis and propensity score weighted M estimation were performed to evaluate the covariate-adjusted risk of syncopal DRs among donors giving 400 ml of whole blood (WB). The DR rate was calculated as the number of DRs divided by the number of all syncopal-type reactions after needle removal. The risk of falls was assessed similarly. Donors who discontinued before completing phlebotomy (donation of 400 ml) were excluded.

Results Among 3818 syncopal-type reactions after needle removal, there were 359 DRs and 93 falls. Elderly donors and female donors with syncopal-type reactions had a significantly higher risk of DRs (P for trend < 0·001). Elderly donors with syncopal-type reactions also had a higher risk of falls (P for trend < 0·001). Among all donors with syncopal-type reactions, the risk of DRs or falls was not correlated with EBV, BMI or donation frequency.

Conclusion In female donors and elderly donors (donating 400 ml of WB), syncopal-type reactions tended to be delayed. Elderly donors with syncopal-type reactions had a significantly higher risk of falls.

Key words: blood donor, syncopal-type reaction, delayed reaction, fall.

Introduction

The prevalence of syncopal-type complications among donors of whole blood (WB) is reported to be 271·5/10 000 [1]. Most donors recover rapidly without sequelae, but some suffer severe injury when loss of consciousness (LOC) occurs after leaving the donor chair. In fact, two fatal head injuries caused by falls after blood donation have been reported in Japan [2], so preventing delayed reactions (DRs) is important.

Risk factors for IR (IRs) have been reported to be young age, female sex and first-time donor [3]. On the other hand, risk factors of DR have been reported to be different from those for immediate reactions. Kamel et al. [4] reported that women are three times more likely to have DR than man compared to no reactions, and the magnitude of the OR was different from that of IR. In the same study, donation site, sex, race/ethnicity and donation history were associated with having DR compared to IR. Also, Narbey et al. reported that female and underweight male were strongly associated with DR occurrence [5].

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However, the characteristics of donors with DRs have not been clarified sufficiently to establish an efficient strategy for preventing these reactions.

Accordingly, we evaluated the characteristics of DR donors and donors who suffered falls among all donors with syncopal-type reactions to clarify the types of donors having an increased risk of DRs or falls.

Materials and methods

Study population

The Japanese Red Cross Society is exclusively responsible for blood collection, testing donated blood and manufacture of blood products in Japan. During the study period, 11·6% of nationwide donations were accepted in Tokyo.

This analysis focused on donors providing 400 ml of WB in Tokyo between 1 April 2014 and 31 March 2018 who had syncopal-type complications of any severity after needle removal. Analysis for this study started in 2018, and 4-year period is required to obtain enough number of falls.

Donors who did not complete phlebotomy and did not provide 400 ml of WB were excluded. In Japan, donors are excluded from 400 ml WB donation if their weight are less than 50 kg, and they are preferably directed to 200 ml WB or apheresis donations. Sixteen-year-old female donors are also excluded from 400 ml WB donation [6].

Additional 2 ml of blood is drawn for pre-donation test, and 25 ml is diverted into the sample pouch.

Study design

We defined DRs as syncopal-type complications that occurred >20 min after needle removal, because donors are encouraged to stay at Tokyo blood donation sites for at least 20 min after removal of the needle. Syncopal-type complications occurring within 20 min of needle removal were defined as IRs.

We evaluated the percentage of DRs among all syncopal-type complications after needle removal. In other words, rather than calculating the incidence rate of DRs, we assessed the probability of syncopal-type complications with a delayed onset occurring among all syncopal-type complications after needle removal.

All donors who complained of mild to severe pre-syncopal or syncopal symptoms were recorded as having syncopal-type complications, together with the onset time and the time for the end of the blood donation procedure, which usually corresponded to the time of needle removal. Off-site complications were only recorded if donors notified us voluntarily.

The following factors were evaluated to assess the relation with occurrence of DRs: sex, age, body mass index (BMI), estimated blood volume (EBV) and donation frequency. Factors associated with DRs that led to falls were evaluated similarly.

Estimated blood volume is calculated with the equation proposed by Ogawa et al. [7].

Statistical analysis

We used multiple logistic regression analysis to identify the risk of DRs with adjustment for covariates. Propensity score weighted M estimation [8] was employed to increase the reliability of the covariate adjustment procedure. Propensity score adjustment, in which multiple covariates are unified into a variable called 'propensity score', is one of the widely employed covariate adjustment methods. Propensity score adjustment is well known to robust to misconfiguration of the model due to its unification of the covariates. On the other hand, logistic regression analysis has the advantage that when the regression function is correctly specified, the variation of the covariate is removed, resulting in improving the power of the test.

The sex, donation frequency (first time, ≤2 previous donations or ≥3 previous donations) and age group (16–19, 20–29, 30–39, 40–49 or 50–69 years) were used as categorical variables. BMI and EBV were divided into quartiles and were also used as categorical variables.

The risk of falls associated with DRs was analysed similarly.

Results

During the study period, 2 253 584 blood donations were accepted under the jurisdiction of the Tokyo Metropolitan Blood Center, including 1 446 733 donations of 400 ml of WB. In addition, 6611 syncopal-type complications were recorded among donors giving 400 ml of WB. Of those complications, 2793 occurred before needle removal, 3818 occurred after needle removal, and 359 occurred more than 20 min after needle removal (DRs). Ninety-three falls were recorded. Among those, 186/359 DRs (51·8%) and 29/93 falls (31·2%) were off-site reactions.

The characteristics of the subjects are shown in Table 1.

Risk factors for DRs

The results of assessing risk factors for DRs by unadjusted chi-squared test (sex) or Cochran–Armitage test for trend (age, EBV, BMI, donation history) are shown in Table 1. The correlation between DRs and sex, age, EBV, and BMI

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Table 1 Characteristics of 400 ml WB donors experienced syncopal-type reactions after needle removal.

	All subjects		Donors with DRs		Donors with falls	
Characteristics	***n* (%)**	**Mean ± SD**	***n* (%)**	**Mean ± SD**	***n* (%)**	**Mean ± SD**
Sex				*P* < 0·001		*P* = 0·012
Male	1863 (48·8)		69 (19·2)		33 (35·5)	
Female	1955 (51·2)		290 (80·1)		60 (64·5)	
Age		30·02 ± 11·49		35·70 ± 14·10		35·28 ± 13·95
Age group				*P* for trend < 0·001		*P* for trend < 0·001
16–19	618 (16·2)		35 (9·7)		9 (9·7)	
20–29	1682 (44·0)		124 (34·5)		35 (37·6)	
30–39	710 (18·6)		61 (17·0)		15 (16·1)	
40–49	498 (13·0)		65 (18·1)		17 (18·3)	
50–69	310 (8·1)		74 (20·6)		17 (18·3)	
EBV (L)		4·34 ± 0·68		4·04 ± 0·57		4·06 ± 0·55
				P for trend < 0·001		*P* for trend = 0·004
3·203–3·760	853 (22·3)		78 (21·7)		31 (33·3)	
3·761–4·155	937 (24·5)		77 (21·4)		22 (23·7)	
4·156–4·663	1031 (27·0)		96 (26·7)		19 (20·4)	
4·664–8·632	997 (26·1)		108 (30·0)		21 (22·6)	
BMI (kg/m^2)		22·43 ± 2·82		23·0 ± 2·93		22·68 ± 2·61
				P for trend = 0·04		*P* for trend = 0·82
15·78–20·42	770 (20·1)		52 (14·5)		15 (16·1)	
20·43–21·79	984 (25·8)		90 (25·0)		18 (19·4)	
21·80–23·50	983 (25·7)		83 (23·1)		28 (30·1)	
23·51–40·90	1081 (28·3)		134 (37·3)		32 (34·4)	
Number of prior donations		4·16 ± 9·83		9·29 ± 16·0		10·25 ± 17·84
				P for trend = 0·27		*P* for trend = 0·045
0	1621 (42·5)		163 (45·4)		35 (37·6)	
1 or 2	1138 (29·9)		102 (28·4)		22 (23·7)	
3–	1059 (27·7)		94 (26·2)		36 (38·7)	

were significant with unadjusted analysis (*P* < 0·001, *P* for trend < 0·001, <0·001, =0·04, respectively). However, correlation between donation frequency and DRs was insignificant (*P* for trend = 0·27).

The results of assessing risk factors for DRs by adjusted multiple logistic regression analysis and by M estimation with propensity score matching are listed in Table 2.

Adjusted multiple regression analysis showed that the risk of DRs was higher for female donors (odds ratio [OR], 4·96; 95% confidence interval [CI], 3·50–7·03; *P* < 0·001) and for elderly donors (*P* for trend < 0·001). Propensity score weighted M estimation yielded similar results for the influence of sex and age.

The correlation of EBV and DR become insignificant after adjustment by multiple regression analysis (*P* for trend = 0·78) and by propensity score weighted M estimation. Larger BMI was associated with a lower risk of DRs by multiple regression (*P* for trend = 0·04), but propensity score weighted M estimation did not detect a significant correlation between BMI and DR. After adjustment, the correlation between donation frequency and DR remained insignificant (*P* for trend = 0·28).

Risk factors for falls

The results of assessing risk factors for falls by unadjusted chi-squared test (sex) or Cochran–Armitage test for trend (age, EBV, BMI, donation history) are shown in Table 1. With unadjusted analysis, correlation between fall and sex, age, EBV, and donation frequency were significant (*P* = 0·012, *P* for trend < 0·001, =0·004, =0·045, respectively). However, correlation between BMI and fall was insignificant (*P* for trend = 0·82).

The results of assessing risk factors for falls by multiple logistic regression analysis and propensity score weighted M estimation are displayed in Table 3.

Both analytical methods showed that elderly donors had a higher risk of falls (*P* for trend < 0·001).

Unlike DRs, female sex did not show significantly higher risk of falls (*P* = 0·61). After adjustment, higher

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Table 2 Risks of DRs among 400 ml WB donors

		Multiple logistic regression				Propensity score weighted M estimation method		
	Number of donors	OR	95% CI	*P* value	*P* for trend	DR rate	95% CI	*P* value
Sex								
Male	69	1				0·03	0·02–0·05	
Female	290	4·96	3·50–7·03	<0·001		0·14	0·12–0·17	<0·001
Age								
16–19	35	1			<0·001	0·06	0·04–0·08	
20–29	125	1·28	0·86–1·89	0·22		0·07	0·06–0·08	0·29
30–39	60	1·72	1·10–2·67	0·02		0·10	0·07–0·12	0·01
40–49	65	2·77	1·78–4·31	<0·001		0·13	0·10–0·16	<0·01
50–69	74	5·10	3·28–7·93	<0·001		0·23	0·18–0·28	<0·01
EBV (ml)								
3203–3760	78	1			0·78	0·09	0·07–0·10	
3761–4155	77	1·07	0·81–1·42	0·61		0·10	0·08–0·12	0·31
4156–4663	96	0·88	0·61–1·26	0·49		0·08	0·06–0·10	0·79
4664–8632	108	1·24	0·80–1·90	0·33		0·10	0·05–0·15	0·60
BMI (kg/m^2)								
15·78–20·42	52	1			0·04	0·11	0·09–0·13	
20·43–21·79	90	0·87	0·64–1·18	0·38		0·10	0·08–0·12	0·42
21·80–23·50	83	0·75	0·55–1·03	0·08		0·09	0·07–0·10	0·10
23·51–40·90	134	0·75	0·54–1·03	0·07		0·09	0·07–0·10	0·10
Number of prior donation(s)								
0	78	1			0·28	0·10	0·09–0·12	
1 or 2	77	0·87	0·66–1·14	0·3		0·09	0·07–0·11	0·30
3–	204	0·86	0·65–1·14	0·29		0·09	0·07–0·11	0·34

EBV was associated with a significantly lower risk of falls by multiple regression analysis ($P = 0{\cdot}03$), but propensity score weighted M estimation did not show a significant correlation of EBV with falls. After adjustment, donation frequency was not correlated with the risk of falls by both analytical methods. BMI also did not have significant correlation with fall.

Discussion

This study demonstrated that elderly WB donors who developed syncopal-type reactions had a higher risk of both DRs and falls. In the case of female donors with syncopal-type reactions, the risk of DRs was higher, but not the risk of falls. Other factors that we investigated (EBV, BMI and donation frequency) were not significantly correlated with the risk of either DRs or falls.

The rate of syncopal-type reaction among our study population (0·46%, 6611/1446733) was lower than previously reported (2·7%) [1]. However, a report from our institution showed that overall rate among all donation types was 0·65% [9], which is consistent with the rate among subjects of this study. One of the reasons of this discrepancy might be Japanese eligibility criteria of 400 ml WB [6]. With Japanese criteria, donors are excluded from 400 ml WB donation if their weight are less than 50 kg, and they are preferably directed to 200 ml WB or apheresis donations. Sixteen-year-old female donors are also excluded. As a result, donors with high risk of syncopal-type reactions are excluded from 400 ml WB donation. Kamel et al reported that African American and Hispanic donors were more likely to have DR compared with Caucasian donors [4]. Also, rate of syncopal-type reaction among 17 French blood centres were reported heterogeneous and 3 of them are very low compared with others [5]. Two of the 3 centres are located in French overseas departments. Although it is not clear if Asian donors are less likely to have syncopal-type reaction, ethnicity might affect our low incidence of syncopal-type reaction.

Previously, higher risk of DR among female donors was reported [4, 5]. In this study, female donors were at higher DR risk even among donors with syncopal-type reactions.

A recent systematic review showed that young age was a risk factor for syncope during and after blood donation in all seven studies reviewed [10]. Previously from our institution, one of the reported risk factors of IR was

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Table 3 Risks of falls among 400 ml WB donors

	Number of donors	Multiple logistic regression				Proprensity score weighted M estimation method		
		OR	95% CI	*P* value	*P* for trend	Fall rate	95% CI	*P* value
Sex								
Male	33	1				0·02	0·01–0·03	
Female	60	1·15	0·66–2·00	0·61		0·03	0·02–0·04	0·27
Age								
16–19	9	1				0·016	0·005–0·028	
20–29	35	1·46	0·70–3·08	0·32	<0·001	0·019	0·013–0·026	0·63
30–39	15	1·68	0·73–3·90	0·22		0·021	0·010–0·032	0·52
40–49	17	2·84	1·24–6·49	0·01		0·037	0·019–0·056	0·06
50–69	17	4·19	1·83–9·58	<0·001		0·054	0·028–0·080	0·01
EBV (ml)								
3203–3760	31	1			0·03	0·03	0·01–0·05	
3761–4155	22	0·91	0·53–1·55	0·72		0·033	0·02–0·04	0·83
4156–4663	19	0·68	0·35–1·32	0·25		0·023	0·01–0·03	0·60
4664–8632	21	0·41	0·18–0·94	0·03		0·03	0·00–0·06	0·98
BMI (kg/m^2)								
15·78–20·42	15	1	1		0·873	0·027	0·02–0·04	
20·43–21·79	18	1·02	0·57–1·80	0·96		0·026	0·02–0·04	0·90
21·80–23·50	28	0·65	0·34–1·22	0·18		0·017	0·01–0·03	0·15
23·51–40·90	32	1·09	0·62–1·91	0·78		0·028	0·02–0·04	0·85
Number of prior donation(s)								
0	20	1				0·021	0·014–0·029	
1 or 2	16	0·92	0·54–1·59	0·78	0·058	0·019	0·011–0·028	0·74
3–	57	1·66	1·02–2·69	0·04		0·036	0·024–0·048	0·04

younger age [11]. Indeed, overall syncopal reactions were more frequent among younger donors compared with all donors in the Tokyo area during our study period. The number of syncopal-type reactions/blood donations in each age group calculated with our institutional data was as follows: 2109/116293 (1·8%) for 16–19 years, 6263/490102 (1·3%) for 20–29 years, 2775/470958 (0·6%) for 30–39 years, 2102/628238 (0·3%) for 40–49 years, 1122/430415 (0·3%) for 50–59 years and 334/131107 (0·3%) for 60–69 years (P for trend < 0·001).

On the contrary, DR risk was higher among elderly in present study. The discrepancy of the results might be explained by the difference of study design. In this study, DR rate was calculated as the number of DRs or falls divided by the number of all syncopal-type reactions after needle removal, instead of the number of all donors including those who did not have any reactions. Bravo et al reported youth were associated with fainting; however, the association was most significant during phlebotomy than after phlebotomy [12].

Our study focused on the frequency of DRs and falls among all syncopal-type reactions after needle removal, in order to determine how likely such complications were in donors with syncopal-type reactions. We found that elderly donors tended to have delayed syncopal-type reactions that were more likely to result in falls.

It was reported that elderly persons show reduced vasomotor sympathetic responsiveness [13]. Impaired balance is one of the well-known problems of ageing, but this is not considered to be an issue in the present study because the upper age limit criterion for blood donors excludes very elderly persons. On the other hand, age itself may not be an appropriate independent exclusion criterion for blood donors. It has been reported that donors aged over 70 can continue to donate blood safely [14]. Also, Eder et al reported that overall complication rate was lower among elderly (>60 years) than that of younger age group [1]. The contribution of elderly donors is highly appreciated, but their risk of DRs and falls associated with syncopal-type reactions should be carefully evaluated.

Our study has several limitations. First, the frequency of adverse reactions might have been underestimated because DRs and falls occurred outside the blood donation sites were only recorded when donors voluntarily reported the incidents. Among our subjects, 186/359 DRs

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(51·8%) and 29/93 falls (31·2%) were off-site reactions. Second, we could not evaluate the risk of DRs and falls among donors over 70 years old because such donors are not accepted according to the Japanese Red Cross Donor Eligibility Criteria [6]. Third, since there were multiple donations from many donors, and our analysis was not multivariate repeated measures design, the lack of independence between observations might possibly remain. Fourth, the results should be interpreted cautiously in relation to other ethnicities because almost all of our subjects were Asian.

Conclusion

In a Japanese blood donor cohort, if syncopal-type reactions occurred after needle removal following WB donation, elderly donors and female donors had an increased risk of developing DR. In addition, elderly donors with syncopal-type reactions had a higher risk of falls.

Conflict of interest

The authors declare no conflict of interest.

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ORIGINAL PAPER

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Iron deficiency among French whole-blood donors: first assessment and identification of predictive factors

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Vox Sanguinis

Background The objectives of this study are to estimate the prevalence of iron deficiency (ID) among French whole-blood (WB) donors to identify factors associated with ID and to generate decision trees.

Study design and methods A prospective National multicentre study was performed on WB donors from March 11, to April 5th, 2019. Samples were selected randomly to perform serum ferritin. ID was defined as ferritin value under 26 ng/ml. All results were stratified by sex. Factors associated with ID were analysed using multivariate logistic regression model. CART algorithm was used for decision trees.

Results Eleven thousand two hundred fifty eight WB donors were included. ID was more frequent in women (39·5%) than in men (18·0%). Among 7200 repeated donors, women below 50 yo had a higher risk (OR = 2·37; [1·97–2·85] IC95) than those above 50 yo. Factors associated with ID were: haemoglobin level under the threshold at donation n-1 except for women and n-2 donation; a low mean corpuscular haemoglobin at n-1 and n-2 donations; a shorter interval since n-1 donation and between n-1 and n-2 donations except for women; and women who had given three or four times in the last year. CART algorithm defined high risk of ID subgroups within three populations of donors, new female donors, repeated male donors and repeated female donors. In these identified subgroups, prevalence of ID was up to 72·1%.

Conclusions Our study showed the high prevalence of ID among French WB donors, identified well-known and new factors associated with ID and defined algorithms predicting ID in three populations.

Key words: France, high-risk subgroups, iron deficiency, predictive factors, whole-blood donors.

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Introduction

Blood donation is a volunteer, anonymous and non-remunerated donation in France, as promoted by the WHO [1]. However, blood donation has its price which is 'iron' [2]. Indeed, each donation of a single whole-blood (WB) unit results in a 200–300 mg iron loss [3], and if the total body iron load is approximatively 2–3 g, daily intake of iron cannot exceed 2–4 mg due to negative feedback of serum iron level on digestive iron absorption [4]. The vast majority of iron is devoted to haemoglobin synthesis. Low haemoglobin level, frequently resulting from iron deficiency (ID), is well documented in blood donors and the management of anaemia relies on well-organized

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strategies to prevent anaemic donors from donating. To do so, in France, pre-donation haemoglobin testing is performed on new donors and on selected repeated donors (every 2 years, or when the previous haemoglobin level was close to or under the threshold, or when requested by physician), a number of 6 and 4 donations over a 12-month long period is authorized for men and women donors respectively. A 8-week inter-donation interval is mandatory between two WB donations [5]. Of note, haemoglobin thresholds in France are 0·5 g/dl under the European threshold (that is 13 g/dl for men donors and 12 g/dl for women donors). This adapted threshold was a specific request of France to take into account the donors of Afro-Caribbean origin from overseas departments and territories, who have an average haemoglobin level lower than metropolitan donors [6] and then to preserve self-sufficiency in those areas. Donor monitoring has been reinforced by performing a systematic blood count for all donations. At the present time, such an attention is not paid to ID and the question of iron stock assessment in blood donors is neither consensual nor mandatory; only few countries have implemented ferritin assay at the blood testing step, such as the Netherlands or Denmark [7]. Besides, ID without anaemia is also associated with symptoms. Iron is one of the key elements of the mitochondrial aerobic respiratory chain, thus ID is related to impaired physical endurance [8], asthenia, restless leg syndrome or Pica's disease [9]. This may not be such a concern if the prevalence of ID among non-anaemic donors was not so high as described in various studies [10–12]. Indeed, about 30% of overall donors suffer from ID with a wide range of differences: from 5% in male new donors to 70% of repeated women donors in various countries [10–12]. Nevertheless, no data were yet available for French donors. Some differences could be expected as far as dietary habits, advice to donors regarding iron loss, healthcare system or haemoglobin-related deferral thresholds are different from our country to others. We conducted a prospective study among French donors to estimate the prevalence of ID among WB donors. Our secondary aim was to identify ID-associated factors. Altogether our data will allow us to address strategies in order to manage this issue.

Material and methods

Study design and donors

This is a non-interventional cross-sectional multicentre study, performed in WB donors of the two French blood centres, the *Etablissement français du Sang* (EFS, the French blood transfusion establishment) and the *Centre de Transfusion des Armées* (CTSA, the French Military Blood Institute).

Whole-blood donors who met French criteria for WB [5] were potentially included. Donors were excluded when they came for bloodletting, for non-therapeutic donation or did not agree to participate to the study (refusal rate: 0·2–0·4%). Donors were informed about the ongoing study in the mandatory pre-donation form and that ferritin testing might be performed and that they could refuse to participate. Among participants, samples were selected randomly within all geographical areas and all mobile blood drives (MBD) and blood centres. No additional blood sampling was taken, serum ferritin was tested after blood donation screening on surplus amount. Donors either with low ferritin or high ferritin as described *infra*, were informed by letter advising them to consult their general practitioner and to postpone their next donation for at least six months for those which ferritin level was too low.

Study data

All the data were recorded in the medic-technic softwares used for medical interview. No additional question was asked and no additional data, except ferritin level, was registered as usual blood donation.

The following data were collected for each donation with ferritin testing: (1) Some are related to the donor: sex, age, birth department including continental and overseas ones and abroad, height and weight to calculate body mass index (BMI), donor status (new or repeated), number of donations in the previous 12 months; (2) Some are related to the nature of the blood drive (mobile drive or blood centres), the type of MBD (in towns, in teaching places, in companies), the regional transfusion establishment for EFS (11 in continental France, 3 overseas) and the military blood centre (CTSA); (3) Some are related to the current donation and to the two previous WB donations (called n-1 and n-2): haemoglobin level, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), the interval between the index and the n-1 donation, the interval between the n-1 and n-2 donation.

Anaemia was defined as haemoglobin level <12 g/dl for women and <13 g/dl for men.

The study n°2018-A02568-47 was approved by the French Ethical Research Committee.

Laboratory methods

Samples of peripheral blood were redirected at EFS and collected from the diversion pouch at CTSA. Serum ferritin level was assessed from a dry tube. Serum samples

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were sent for laboratory measurements to blood screening laboratories. Manufacture guidelines regarding the temperature storage between transportation and handling and the time-points from blood draw to analysis were strictly followed. Ferritin values were assessed using an assay linear from 0·2 to 1500 ng/ml ARCHITECT-Ferritin® on Architect analyser (Abbott, Longford, Ireland) at EFS and Access Immunoassay®-FERRITIN on DxI6000 analyser (Beckman-Coulter, CA, USA) at CTSA. A correlation of 0·99 between the two assays previously performed on 35 samples was observed; however, the values on DxI6000 analyser tended to be lower than on Architect analyser. Nonetheless, it has been chosen not to perform an adjustment on ferritin levels. The thresholds were defined under 26 ng/ml as a reduced iron store (ID) [13] and under 15 ng/ml as an absent iron store [14].

Statistical analysis and sample sizing

The first aim of the study was to estimate the prevalence of ID among WB donors thus sample sizing was based on the confidence interval of ID prevalence. As the prevalence was unknown, an estimation of 50% was used to maximize the sample size, and the precision of the confidence interval was fixed at ±1%. Therefore, the sample size needed was at least 9604 donors.

To be representative of the blood donor population of 2018, weights were calculated using marginal calibration. First, marginal calibration on sex -and- age group, geographical region (ETS) and donor status was applied separately for EFS and CTSA. Then, a second marginal calibration on the blood centres (ETS or CTSA) was performed. Weights from these two marginal calibrations were multiplied to obtain the final weights. Weights were used in all analyses.

Mean corpuscular volume and MCH were highly correlated. Subsequently, only MCH was included in the multivariate analysis.

All analyses were stratified on sex. Markers for ID were analysed by using multivariate logistic regression models. All independent variables were included in the models except for withdrawn volume and birth department.

Furthermore, Classification And Regression Trees (CART) algorithm was used to define classification trees in four subgroups: new female donors, new male donors, repeated female donors and repeated male donors. As previous analyses, the trees were stratified by sex but also by donor status because the set of independent variables available is dependent on donor status (history of donations). The predicted outcome was ID. For new donors, the input variables were characteristics at current donation. However, for repeated donors, the input variables also included information on the two previous donations: haemoglobin level kinetics and MCH kinetics. Haemoglobin level and MCH kinetics were defined as $(X_{n-1}-X_{n-2})/(T_{n-1}-T_{n-2})$ with X the measure of Hb or MCH, T the date of donation, n-1 the previous donation and n-2 the antepenultimate donation. The number of splits was determined by minimizing the cross-validation error.

Results

From 03/11 to 03/29/19 at EFS and from 03/11 to 04/05/19 at CTSA, 11 258 WB donors, 9711 from EFS (9,285 in continental France, 220 from French West-Indies and 206 from Réunion Island) and 1547 from CTSA (all in continental France) were included.

Donor characteristics

As expected, there were some weak discrepancies between study sample and donors population of 2018 according to geographical region, sex, age and donor status (Appendix S1). Therefore, all results were weighted to be representative of donor population of 2018. Table 1 shows that age, donor status, collection site of 11 258 donors included were significantly different between women and men. Women were younger and give blood more frequently in blood centres than in BMD, except in BMD teaching places, men were more frequently repeated donor and give blood more frequently in BMD than women, mainly in BMD that took place in companies. Differences in mean Hb, ferritin and MCH were also observed between men and women with lower values among women.

There were some discrepancies between the populations of donors from EFS and from CTSA. Blood donors from EFS were: (1) more frequently women (48·2% vs. 31·4% in CTSA, $P < 0·0001$) than men; (2) older than from CTSA (median 39·0 yo vs. 32·0 yo, respectively, $P < 0·0001$); (3) BMI distribution was different, EFS donors were more obese (BMI $\geq$ 30) (11·2%) and less normal weight (18·5 $\leq$ BMI < 25) (56·8%) compared to CTSA donors (6·4% obese, 61·9% normal weight) ($P < 0·0001$); (4) the distribution regarding the donor status was different, EFS donors being more frequently repeated donor than CTSA donors (86·3% vs. 74·1%, respectively, $P < 0·0001$) and (5) finally, EFS donors were more frequently anaemic than CTSA donors (2·3% vs. 0·8%, $P = 0·0403$).

Haemoglobin and ferritin levels in donors

Overall, prevalence of ID in French WB donors and of absent iron store were 29·3% and 13·2%, respectively and were more frequent in women (39·5% for ID and 19·3% for absent iron store) than in men (18·0% for ID and 6·4% for absent iron store; Table 1).

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Table 1 Characteristics of donors

Donor data	Women N	Women %	Men N	Men %	P
Age					
Less than 50 yo	4 363	73 7	3 508	65 7	<0 0001
More than 50 yo	1 559	26 3	1 828	34 3	
Donor status					
Repeated donor	4 740	80 0	4 544	85 2	<0 0001
New donor	1 182	20 0	792	14 8	
Collection site					
Blood centres	1 396	23 6	1 138	21 3	<0 0001
BMD in towns	3 729	63 0	3 404	63 8	
BMD in teaching place	619	10 5	535	10 0	
BMD in companies	145	2 4	241	4 5	
Unknown BMD	33	0 6	18	0 3	
Haemoglobin levels					
<Threshold[a]	219	3 7	48	0 9	<0 0001
≥Threshold	5 702	96 3	5 286	99 1	
Haemoglobin levels at N-1 donation					
<Threshold[a]	157	3 6	34	0 8	<0 0001
≥Threshold	4 234	96 4	4 319	99 2	
Haemoglobin levels at N-2 donation					
<Threshold[a]	85	2 4	28	0 7	<0 0001
≥Threshold	3 470	97 6	3 788	99 3	
Ferritin					
<15 ng/ml	1 143	19 3	344	6 4	<0 0001
≥15 ng/ml	4 777	80 7	4 991	93 6	
Ferritin					
<26 ng/ml	2 340	39 5	959	18 0	<0 0001
≥26 ng/ml	3 580	60 5	4 376	82 0	
Haemoglobin (g/dl)					
Mean (SD)	13 5 (1 0)		15 1 (1 0)		<0 0001
Median (IQR)	13 4 (12 8–14 0)		15 1 (14 4–15 7)		
Ferritin (ng/ml)					
Mean (SD)	44 6 (54 2)		84 5 (90 1)		<0 0001
Median (IQR)	32 6 (17 9–56 9)		60 9 (32 9–106 5)		
MCH (pg)					
Mean (SD)	29 2 (1 9)		29 6 (1 6)		<0 0001
Median (IQR)	29 3 (28 2–30 2)		29 7 (28 7–30 6)		
Interval between N-1 and index donation (months)					
N	4 724		4 526		<0 0001
Mean (SD)	18 0 (42 1)		12 5 (32 1)		
Median (IQR)	6 4 (3 3–13 8)		3 8 (2 1–8 8)		
MCH at N-1 donation (pg)					
N	4 591		4 449		<0 0001
Mean (SD)	29 2 (1 8)		29 7 (1 6)		
Median (IQR)	29 3 (28 3–30 2)		29 7 (28 8–30 7)		
Interval N-2 and index donation (months)					
N	4 171		4 149		<0 0001
Mean (SD)	32 2 (54 1)		21 5 (39 0)		
Median (IQR)	152 (88–30 9)		97 (57–19 0)		
MCH at N-2 donation (pg)					
N	3 848		3 987		<0 0001
Mean (SD)	29 2 (1 8)		29 7 (1 6)		
Median (IQR)	29 3 (28 2–30 2)		29 7 (28 7–30 6)		

BMD, blood mobile drive.
[a]12 g/dl in women, 13 g/dl in men.

Prevalence of ID was higher in donors from EFS than from CTSA (29·4% vs. 24·3%, respectively, $P < 0·001$). However, these difference could fully be explained by differences in sex distribution as there were more women in EFS population than in CTSA population.

As summarized in Table 2, the prevalence of ID was related to haemoglobin level. However, whatever the haemoglobin threshold was (French or European threshold), the prevalence of ID still remained high, always higher in women than in men.

Markers/Factors associated with ID

Markers related to the donor characteristics at the time of current donation were determined in a multivariate analysis among all donors (N = 11 244, Table 3). As expected, women below 50 years old had a higher risk (OR = 1·72; [1·47–2·02] IC95) than those above 50 years old while men below 50 years old had a lower risk (OR = 0·64; [0·54–0·76] IC95), except 18-19 years old men, than men above 50 years old. BMI was a risk factor, there was a gradient among men and women with underweight (BMI < 18·5) donors at higher risk and obese donors at lowest risk. The following variables were markers both in women and men: (1) repeated donor had a higher risk than new one; (2) the type of collection site where donor gave blood was associated with different risk of ID among men - BMD in teaching places being at lower risk than blood centres; (3) as expected, a haemoglobin level under the threshold at the time of donation was a marker and (4) a lower level of MCH.

Interestingly, place of birth, either continental France, French overseas territories or abroad, was not associated with ID (29·5%, 26·8%, 27·1% respectively, $P = 0·37$).

Factors associated with ID related to the donor characteristics and the history of donation were determined in multivariate analysis among repeated donors (N = 7200,

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Table 2 Prevalence of absence of iron store (AIS, ferritin < 15 ng/ml) and iron deficiency (ID, ferritin < 26 ng/ml) according to sex and haemoglobin level

	Women			Men		
Haemoglobin levels (g/dl)	***N***	**AIS (%)**	**ID (%)**	***N***	**AIS (%)**	**ID (%)**
<12·0	220	61·8	72·3	6	50·0	66·7
12·0–12·4	478	43·9	62·1	4	25·0	50·0
12·5–12·9	987	27·5	51·7	40	47·5	60·0
13·0–13·4	1300	19·6	41·4	176	32·4	47·7
13·5–13·9	1291	12·2	33·9	370	19·7	37·6
14·0–14·4	876	8·2	25·3	731	11·1	25·4
14·5–14·9	512	6·8	23·8	1 014	5·0	19·1
≥15·0	257	2·3	21·0			
15·0–15·4				1095	2·7	15·0
15·5–15·9				889	2·0	11·1
≥16·0				1010	1·0	6·3
Total	5921	19·3	39·5	5335	6·4	18·0

Table 3 Factors associated with iron deficiency related to the donor at the time of donation

	Women (*N* = 5161)			Men (*N* = 6083)		
Donor data at the time of donation	**OR**	**IC95**	***P***	**OR**	**IC95**	***P***
Age (vs. ≥ 50 yo)						
18–19 yo	2·54	[1·89; 3·41]	<0·0001	0·95	[0·65; 1·40]	<0·0001
20–24 yo	2·24	[1·79; 2·81]		0·62	[0·46; 0·83]	
25–49 yo	1·57	[1·33; 1·85]		0·64	[0·53; 0·76]	
BMI[a](vs. obesity)						
Underweight	3·43	[1·97; 5·97]	<0·0001	3·25	[1·00; 10·51]	<0·0001
Normal	2·30	[1·82; 2·91]		2·98	[2·21; 4·00]	
Overweight	1·41	[1·09; 1·82]		1·98	[1·46; 2·67]	
Donor status (vs. new donor)						
Repeated donor	3·04	[2·42; 3·82]	<0·0001	36·37	[17·93; 73·77]	<0·0001
Collection site (vs. blood centres)						
BMD[b] in towns	0·92	[0·76; 1·11]	0·0660	1·02	[0·81; 1·29]	0·0018
BMD in teaching places	1·05	[0·80; 1·39]		0·46	[0·31; 0·69]	
BMD in companies	0·46	[0·26; 0·80]		0·81	[0·45; 1·49]	
Unknown BMD	0·69	[0·30; 1·60]		1·09	[0·36; 3·32]	
Haemoglobin levels (vs. ≥ threshold)						
<Threshold[c]	1·63	[1·10; 2·42]	0·0152	4·01	[1·87; 8·62]	0·0004
MCH (pg)[d]	0·59	[0·56; 0·62]	<0·0001	0·54	[0·51; 0·57]	<0·0001

Adjusted for geographical region.
[a]Body mass index.
[b]Blood mobile drive.
[c]12 g/dl in women, 13 g/dl in men.
[d]Mean corpuscular haemoglobin.

Table 4). In agreement with result among all donors (Table 3), women below 50 years old had a higher risk (OR = 2·37; [1·97–2·85] IC95) than those above 50 years old while the age was not a risk factor for men. BMI was still associated with ID for both sexes. The haemoglobin level under the threshold at *n*-1, except for women, and *n*-2 donations was associated with ID. A low level of MCH at *n*-1 and *n*-2 donations was always associated with ID. A shorter interval between *n*-1 and index donation and a shorter interval between the *n*-1 and *n*-2 donations (only for men), were risk factors. Women who had given three or four times in the previous 12 months

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were at increased risk and men with increasing number of donations reaching five times had a tendency of increased risk.

Algorithm to predict ID based on classification tree

As the prevalence of ID was different between women and men (Table 1) and the donor status was a major risk factor (Table 3), algorithm to predict ID based on classification tree was defined in three subgroups: new female blood donors, repeated female donors and repeated male donors (Figs. 1, 2 and 3). New male blood donors were not modelized due to very low prevalence of ID (1%, n = 18). Indeed, the availability of data during the medical interview is specific to donor status.

The number of donors in each subgroup in a year was forecasted both from study data and from actual number of donors in 2018.

In the subgroup of new female donors (N = 160 304, actual number of donors in a year), those who were more likely to have ID (45·6%) were those under 29·5 years old and with haemoglobin level below 13·7 g/dl (Fig. 1).

In the subgroup of repeated female donors (N = 655 430 actual number of donors in a year), those who were more likely to have ID (72·1%) were those who had given a previous donation in an interval shorter than 4·5 months and with MCH at n-1 below 30 pg (Fig. 2). In addition, another sub-population was identified among female who had given in an interval shorter than 4·5 months, among those with MCH at n-1 above 30 pg, 71·1% of those under 31·5 years of age had ID (Fig. 2).

In the subgroup of repeated male donors (N = 596 879 actual number of donors in a year), those who were more likely to have ID (61·6%) were those who had given at least twice in the previous year and with MCH at n-1 below 29·5 pg and who had given a previous donation in an interval shorter than 3·5 months. In addition, another

Table 4 Factors associated with iron deficiency related to the donor and the history of donation

	Women (N = 3066)			Men (N = 4134)		
Donor and history of donation data	**OR**	**IC95**	***P***	**OR**	**IC95**	***P***
Age (vs. ≥ 50 yo)						
18–19 yo	3·06	[1·81; 5·16]	<0·0001	1·12	[0·68; 1·85]	0·5723
20–24 yo	3·39	[2·59; 4·44]		0·97	[0·70; 1·36]	
25–49 yo	2·12	[1·74; 2·58]		0·89	[0·73; 1·08]	
BMI[a] (vs. obesity)						
Underweight	2·63	[0·98; 7·11]	<0·0001	8·02	[1·57; 40·84]	<0·0001
Normal	2·39	[1·76; 3·25]		4·37	[3·15; 6·05]	
Overweight	1·62	[1·16; 2·24]		2·56	[1·85; 3·55]	
Interval between n-1 & index donation (months)	0·92	[0·89; 0·95]	<0·0001	0·80	[0·73; 0·87]	<0·0001
Hb[b] levels at n-1 donation (≥ threshold)						
<Threshold[c]	1·38	[0·83; 2·29]	0·2166	6·52	[2·00; 21·28]	0·0019
MCH[d] (pg) at n-1 donation	0·75	[0·68; 0·84]	<0·0001	0·69	[0·60; 0·80]	<0·0001
Interval between n-1 & N-2 donations (months)	0·99	[0·97; 1·00]	0·0833	0·95	[0·92; 0·98]	0·0010
Hb levels at n-2 donation (≥ threshold)						
<Threshold[c]	3·59	[1·87; 6·91]	0·0001	3·34	[1·25; 8·94]	0·0162
MCH (pg) at n-2 donation	0·88	[0·79; 0·97]	0·0148	0·84	[0·73; 0·97]	0·0186
Number of donations with RBC[e] (vs. 1)						
2	1·24	[0·85; 1·81]	<0·0001	0·36	[0·19; 0·68]	<0·0001
3	2·10	[1·32; 3·33]		0·53	[0·24; 1·16]	
4	3·31	[1·89; 5·77]		0·84	[0·35; 2·03]	
5				1·68	[0·64; 4·40]	
6				0·90	[0·27; 3·03]	

Adjusted for geographical region.

[a]Body mass index.

[b]Haemoglobin.

[c]12 g/dl in women, 13 g/dl in men.

[d]Mean corpuscular haemoglobin.

[e]Red blood cell.

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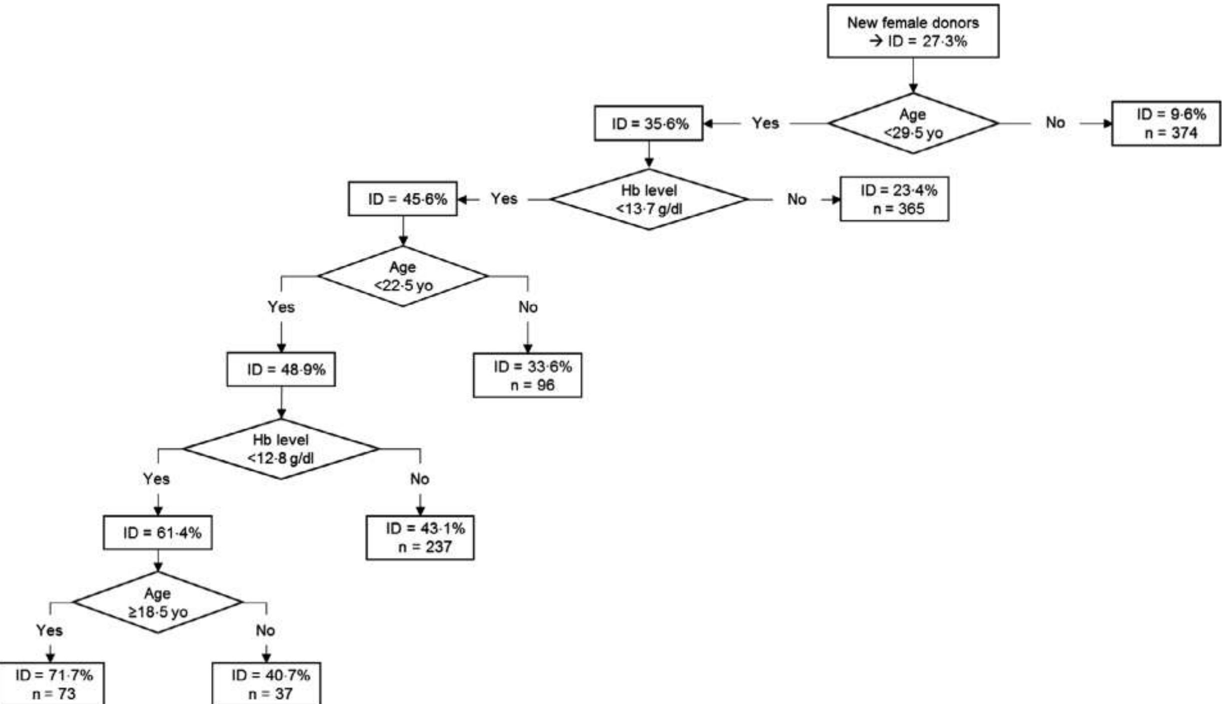

Fig. 1 Classification tree of new female donor. Hb, haemoglobin; ID, iron deficiency.

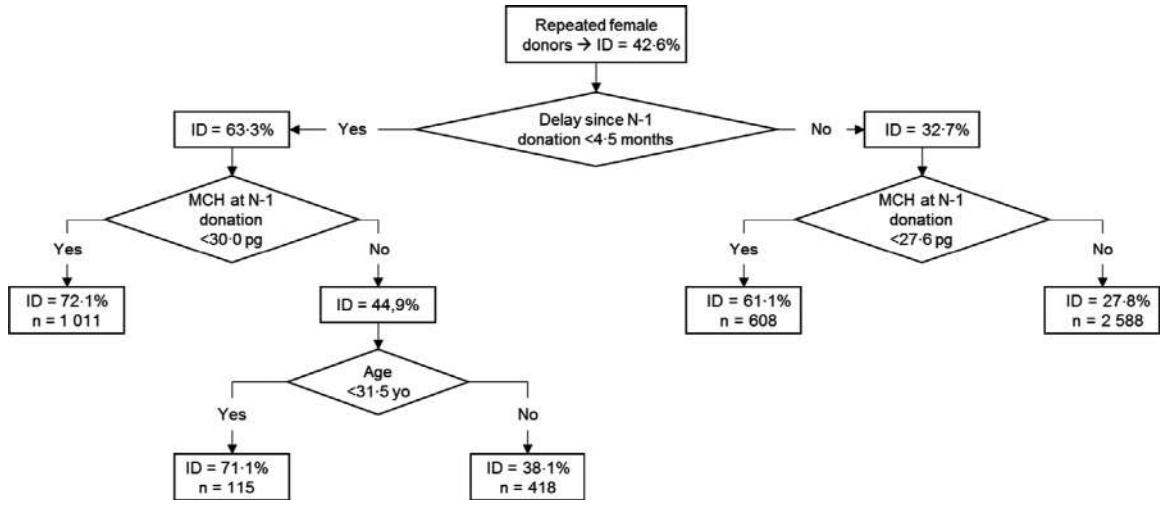

Fig. 2 Classification tree of repeated female donor. ID, iron deficiency; MCH, mean corpuscular haemoglobin.

sub-population was identified among male whose interval of previous donation was above 3·5 months and with MCH at n-1 donation below 27·4 pg, 66·3% had ID (Fig. 3).

Discussion

In this study, we estimated for the first time at the National level the prevalence of ID in France which was 39·5% among women and 18·0% among men blood donors. These results are in accordance with previous studies performed in different countries [10,13,15]. The prevalence of ID in France differed according to sex and to donor status, reaching 42·6% of women and 20·9% of men repeated donors while it was 27·3% of women and 1·0% of men new donors, in accordance with previous data [16].

Of note, ID is also a frequent issue in general population, as recently reported in France: 25·9% of women and

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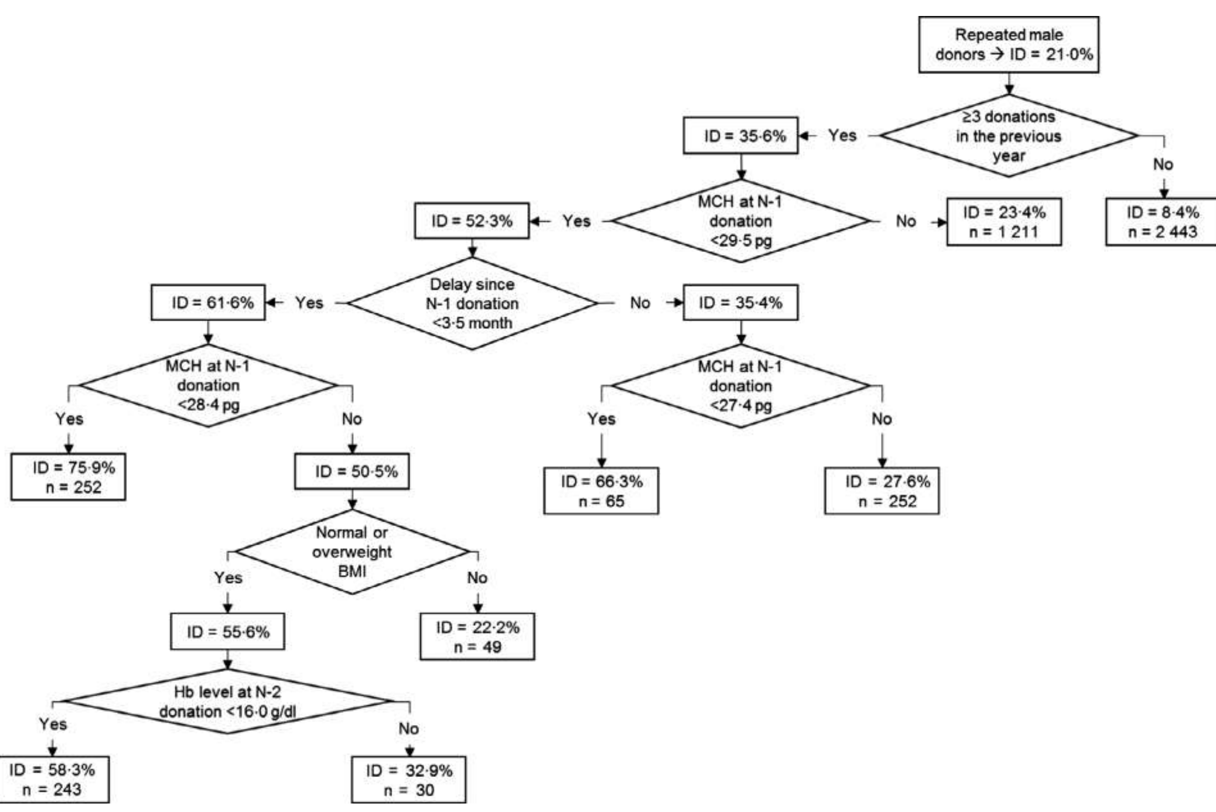

Fig. 3 Classification tree of repeated male donor. Hb, haemoglobin; ID, iron deficiency.

5·4% of men had ID [17]. They account for a part of ID of French blood donors.

Our study has some limitations. First, two different assays were used to measure ferritin level: preliminary evaluation showed that results were strongly correlated without relevant discrepancy (see supra). Secondly, the short duration of the study could be considered as a limitation. However, to be representative of 2018-blood donors, the results have been weighted on following criteria, sex and age, donor status and transfusion centre. Thirdly, different thresholds are reported in the literature and in agreement with recent studies [18,19], we chose the threshold of 26 ng/dl: threshold of 20 ng/dl or of 30 ng/dl could lead to overestimation or underestimation of ID prevalence. However, factors associated with ID were analysed with a threshold of 30 ng/dl and provided overall the same conclusions (data not shown). Finally, the relationship between Hb at n-1 and n-2 donations and risk for ID at index donation is subject to selection bias as many people who were below the Hb threshold at n-1 donation did not participate to this study because they were not qualified.

Conversely, the strengths of this study included analysis of a high number of donors, as high as 11 240, from continental France and overseas territories. There was no difference of ID prevalence between continental France (29·3%) and Martinique, Guadeloupe and Réunion Island (32·7%, 28·3% and 26·7%, respectively). These results of French West-Indies can be linked with a recent study which identified differences in ethnically diverse US blood donor population, 42·6% of African-American women and 17·6% of African-American men had ID while prevalence was 56·6% of female and 38% of male Caucasian-non-Hispanic donors [19]. This non-increasing risk of ID for donors of Afro-Caribbean origin, either from our study in French West-Indies or from African-American donors, in comparison with Caucasian-non-Hispanic donors, is a very interesting new information and could be analysed with recent REDS-III RBC-Omics study results showing that African–American background was associated with resistance to osmotic haemolysis compared with other ethnic groups [20]. Indeed, the mean level of haemoglobin is lower in donors of Afro-Caribbean origin, either from our previous study of WB donors in 2015 in French West-Indies (12·9 g/dl for women, 14·7 g/dl for men) [6] or from US American African donors (12·9 g/dl for women and 14·4 g/dl for men), than in Caucasian-non-Hispanic donors (13·4 g/dl for women, 15·1 g/dl for men) [21]. In donors of Afro-Caribbean origin, the mean low level of haemoglobin is very probably not linked with prevalence of ID which is usual.

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Additionally, the number and the relevance of data analysed, not only for current blood donation but also for the two previous donations, are of great interest. It allowed us to confirm some well-known factors associated with ID such as the young age of women and the donor status (repeated), the haemoglobin level under the threshold and a lower MCH level at *n*-1 donation which is correlated to the MCV, microcytosis being well-known as predictive of ID anaemia – and the shorter interval between the *n*-1 and index donation [10,22].

More interestingly, we found new factors associated with ID in multivariate analysis. At the time of donation among WB donors, one type of collection site, BMD in companies, had a lower risk than blood centres. We also confirmed that shorter is the interval between the *n*-1 and index donation, higher is the risk of ID. Regarding blood count information, a lower MCH level for both women and men, either at *n*-1 donation or at *n*-2 donation, was associated with a higher risk of ID which may allow us to predict its occurrence. Similarly, haemoglobin level below the threshold, except for women at *n*-1 donation, was predictive of occurrence of ID (Table 4).

Finally, an innovative approach generating decision trees was used and will allow us to set up news strategies whose impact can be estimated before their implementation. These strategies are still debated in France, as well as in other countries where no consensus still emerge to face the issue of ID in blood donors.

One of the less debated option is the implementation of a ferritin assay to screen ID among WB donors. Thanks to the decision trees, some algorithm could be generated (Figs. 1, 2 and 3) to focus the screening among the most exposed blood donors: new or repeated female donor and repeated male donor. This will help us to set up a cost-efficient strategy. In order to avoid ferritin assay which is expensive and as systematic blood count is performed for all donations and predictive value of haemoglobin and MCH levels have been demonstrated, other markers of blood numeration will be further analysed to be used as surrogate markers for ferritin. However, inherited haemoglobin disorders are not unusual in France and may interfere with MCH: this parameter should be taken into account in further analysis.

To manage the ID among blood donors, several measures are available. A prolonged deferral of 6 months might allow an iron stores recovery in the vast majority of new and repeated donors, whatever blood donor iron was deficient or not [23]. In line with this measure (increasing deferral), an increase donation interval [24] or a decrease number of donation per year in the donors most at-risk of ID, as highlighted in our study, could be implemented. Another option could be to propose systematically an iron supplementation whose duration and dose would depend on ID depth [10]. The question of an iron supplementation either by the general practitioner during a medical consultation after the donor has been advised of a low level of ferritin [25] or directly by the blood transfusion centre [26] has to be discussed [27]. Finally, we showed that ID prevalence was still high even if haemoglobin threshold is increased to European threshold, rending the measure of an increased haemoglobin threshold unlikely to be effective [28].

The impact of the identification of ID blood donors on deferral and the return to donation rate has to be carefully assessed [24]. First, the amount of blood products available can be drastically impacted and secondly, one should not forget the qualitative aspect of the blood supply that should be investigated. These measures should be further deeply considered taking into account the impacts, at short but also long term. Finally, healthier donors will be benefit both for the donor and for the whole transfusion chain.

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Equipment for ferritin testing is included for EFS in Biological Blood Qualification platform and for CTSA in Laboratory Medicine Department.

Conflict of interests

No conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Distribution of EFS sample and EFS donors population of 2018 according to geographical region, sex, age group and donor status.

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Antioxidant power measurement in platelet concentrates treated by two pathogen inactivation systems in different blood centres

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Vox Sanguinis

Background and objectives The antioxidant power measurement can be useful to validate the execution of the pathogen inactivation treatment of platelet concentrates. The aim of this study is to evaluate the technology on different blood preparations including INTERCEPT and Mirasol treatments that are in routine use in Belgium and Luxemburg.

Materials and methods The antioxidant power measurement was tested on 78 apheresis platelet concentrates and 54 pools of buffy-coats-derived platelet concentrates before and after INTERCEPT treatment. In addition, 100 Reveos platelet pools were tested before and after Mirasol treatment. The antioxidant power was quantified electrochemically using disposable devices and was expressed as equivalent ascorbic acid concentration.

Results Mean results for apheresis platelet concentrates were of 90 ± 14 and 35 ± 10 µmol/l eq. ascorbic acid before and after INTERCEPT treatment, respectively. The mean results for pools of buffy-coats-derived platelet concentrates were of 81 ± 10 and 29 ± 4 eq. µmol/l ascorbic acid before and after INTERCEPT treatment, respectively. For buffy-coats-derived platelet concentrates treated by Mirasol technology, the mean results were of 98 ± 11 and 32 ± 10 µmol/l eq. ascorbic acid before and after illumination, respectively.

Conclusion The antioxidant power significantly decreases with pathogen inactivation treatments for platelet concentrates treated by INTERCEPT or Mirasol technologies.

Key words: antioxidant power, platelet concentrates, pathogen inactivation.

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Introduction

Treatment of cellular blood products with pathogen inactivation technologies (PITs) is widely implemented in blood establishments to diminish the risk of bacterial contamination and to face the presence of new emerging pathogens in blood components [1–3]. The INTERCEPT Blood System (IBS, Cerus Corp.) has been developed for platelet concentrates (PCs) and plasma units [4]. This technology uses a photosensitizer, amotosalen HCl and ultraviolet A (UVA) illumination to photochemically inactivate most of contaminants, as well as contaminating white blood cells [3, 5]. Another PIT exists, called Mirasol Pathogen Reduction Technology (Terumo BCT), which uses UV light and riboflavin to inactivate pathogens and white blood cells in donor PCs or plasma units for transfusion [5, 6]. Since 2015 in Belgium, all PC units have been pathogen-reduced by INTERCEPT System. The

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Luxemburgish Red Cross uses the Mirasol technology for its PCs since 2014, and it is fully implemented since July 2019.

A quality control test is recommended by the Council of Europe to assess the efficacy of pathogen inactivation in blood components [7]. Different tools exist to be assured that a unit has been treated like a label applied to the illumination container after treatment to provide visual evidence that the unit received a complete treatment in the illuminator and should not be reilluminated, or a UV indicator label changing from light blue to dark blue after the UVA exposure [8]. After illumination, a report is automatically printed to provide the proof of completeness. The illuminator may also be connected to a data management system to block the release of non-illuminated or doubly illuminated products [3]. These checkpoints are necessary during the treatment processing to avoid errors but it is not a control on the product itself [3]. Finally, even more complex approaches based on the inhibition of mitochondrial DNA replication have been developed [9, 10].

A new technology based on the measuring antioxidant power (AOP) can ensure this QC assay on blood products. [3, 11, 12]. Oxygen metabolism naturally produces reactive oxygen species (ROS) that exist in all cells in steady state with antioxidants [3]. These antioxidants can directly participate in the scavenging of ROS or indirectly by intercepting the chain carrying radicals during the oxidative process [3, 13]. Oxidative stress appears when the ROS/antioxidant balance is disturbed because of ROS excess, antioxidant depletion or both [3]. Throughout the scavenging of ROS, AOP is an important determinant of platelet (PLT) function [14, 15]. During PLT activation, ROS are generated and participate in the regulation of PLT signalling [16, 17]. Abonnenc et al. in 2016 showed that, consecutively with INTERCEPT Blood System known to generate ROS [18], the resulting excess of ROS led to a decrease of the AOP of PCs or plasma units [3]. It was showed recently an equivalent impact using riboflavin and UVB treatment on PCs [19]. These ROS and the consecutive oxidative stress participate in accelerating storage lesions and oxidative damages [18–24].

The technology is an electrochemical device commercialized to measure AOP in a variety of samples, including biologic fluids [11, 25]. It is based on an electrical current recording using a linear sweep voltammetry. The recorded current corresponds to the ability of the sample to donate electrons for neutralizing free radicals [11].

This study has the goal of confirming the results from Abonnenc et al. in assessing the AOP of PC units treated by INTERCEPT technology. A collaboration with the Luxemburgish Red Cross allows to compare also our results with them obtained in PCs treated by Mirasol® technology. The perspective is to use such a test as a QC assay for documenting the execution of PITs during the preparation of PCs.

Materials and methods

Blood collection

Blood and blood-derived products were collected from healthy volunteer donors who gave their consent for the use of their blood components in research. The collection day was defined as Day 0.

At the Belgian Red Cross, buffy coat (BC) was obtained from whole-blood donations (CompoSelect, Fresenius Kabi, Bad Homburg, Germany) with a mean volume of 465 ml and was collected in accordance with local standards. Whole blood was cooled to 18-24°C within 18 h of donation and centrifuged at 4500 ***g*** before being separated on MacoPress (MacoPharma, Tourcoing, France). After 4 to 12 h resting time at room temperature, six ABO-compatible BCs (target volume: 44 ml) were pooled with 280 ml PAS-E (SSP + solution, MacoPharma, Tourcoing, France) at a target ratio of 35% plasma (acceptable range 32%–47%). The mixture was centrifuged with TACSI PL (Terumo BCT, Zaventem, Belgium) [26], and the supernatant PC was collected by expression through a leucoreduction filter (TACSITM PL set, Terumo BCT, Zaventem, Belgium).

At the Luxemburgish Red Cross, 475 ml of whole blood was collected into Reveos collection bag containing 63 ml of CPD (4FG456S0, Terumo BCT) from healthy donors and stored on eutectic plates (Compocool, Fresenius Kabi) for at least 5 h. Depending on the collection time, the blood was centrifuged and separated by using Reveos Automate either on the same day (Day 0) or after overnight hold at room temperature (Day 1). The main difference between both separation programs is the centrifugation time which is longer in overnight conditions (7 instead of 4·5 min). For each whole-blood unit, one red blood cell concentrate, one plasma unit, one interim platelet unit (IPU) and a residual leucocytes bag were obtained. After at least four hours of resting and agitation time, 4 or 5 ABO-compatible IPUs were pooled respectively in 200 or 250 ml of T-PAS+ (Terumo BCT) at a target ratio of 38% and filtered through a leucoreduction filter (LRF-XL Filter, Haemonetics). In order to optimize and to standardize the final PLT content, a software application that sorts and groups IPUs based on the estimated PLT yield by the Reveos system was used (IPU select, Terumo).

Apheresis PCs (APCs) were collected on Amicus (Fresenius Kabi, Warrendale, PA, USA) version 3.21 (double-needle apheresis procedure) or on Trima Accel Cell

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Separator (Terumo BCT, Lakewood, CO, USA) version 6.06 in accordance with the manufacturer's instructions with automatic addition of 62% PAS-E. PCs were stored overnight at 22 ± 2°C under agitation.

Pathogen inactivation treatment

PCs from Belgian Red Cross were treated at Day 1 with the IBS illuminator (INT100, Cerus Europe BV, Amersfoort, Netherlands) in accordance with the manufacturer's instructions. Each component was treated with a nominal of 150 μM amotosalen and 3·0 J/cm^2 UVA light (320 to 400 nm) followed by 6 h of incubation with a Compound Adsorption Device. During this step, PLT units were stored on a flatbed PLT agitator set at approximately 60 cycles per minute at a temperature of 22 ± 2°C. PCs from Luxemburgish Red Cross were treated at Day 1, straight ahead after the PLT leucofiltration with the Mirasol® technology (Terumo, Lakewood), using riboflavin and ultraviolet A/B illumination. Thirty-five millilitre of Riboflavin (500 mμ) was added to the PC which was exposed to UVA/B light (280–400 nm) for a few minutes, depending on the PLT volume. After the illumination, PCs were stored on a flatbed PLT agitator set at approximately 60 cycles per minute at a temperature of 22 ± 2°C (Helmer).

Study design

The AOP measurement was tested on 78 APCs and 54 buffy-coat platelet concentrates (BCPCs) before and after INTERCEPT treatment. The pretreatment measure was immediately realized after sampling. The post-treatment measure was realized in average 18 h after the end of treatment.

The AOP measurement was tested on 100 Reveos platelet pools before and after Mirasol treatment, right after sampling. The pretreatment measure was performed before adding the riboflavin solution. The post-treatment sample was taken after the illumination in order to measure the AOP. Furthermore, we performed an extra test on 20 PCs in order to assess the potential riboflavin effect alone on the AOP. For each illuminated product, a PLT sample was taken at different stages of the illumination process: before addition of the riboflavin (sample 1), before the illumination (but after the addition of Vitamin B2, i.e. riboflavin) (sample 2) and after the illumination (sample 3). Furthermore, for the sample 2, 10 of these were protected from light and the 10 others were not.

AOP measurements

Antioxidant power measurements were performed using a potentiostat electrochemical analyser and three electrode-based sensors (Edel-for-life SA, Lausanne, Switzerland) [11]. A 3 μl volume of sample is deposited over the electrodes on a single-use microchip. The measurement is based on a pseudo-titration of a linear sweep voltammogram (recorded from 0 to 1·2 V with a scan rate of 100 mV/sec, under ambient conditions) for rapidly measuring the water-soluble AOP in a sample. The AOP is expressed in μmol/l equivalent ascorbic acid and reflects the redox status of the extracellular low-molecular-weight antioxidants present in the plasma fraction of the PCs or in the total product of the plasma units. The measure is immediately realized after the sampling and takes only 1 min. The sensitivity of the assay is of 19 μmol/l eq. ascorbic acid, and the standard deviation (SD) is of ±6 μmol/l eq. ascorbic acid (data not shown).

Statistical analysis

Data are expressed as mean ± SD. For two-group comparisons, a *t*-test was used while for more than two groups, a repeated-measures one-way analysis of variance (ANOVA) was realized. To determine the threshold and sensitivity–specificity, a ROC curve (GraphPad Prism, GraphPad software, Inc.) was drawn. A *P*-value of less than 0·05 was considered as significantly differences.

Results

PC results – INTERCEPT treatment

The mean results for APC were of 90 ± 14 μmol/l eq. ascorbic acid before INTERCEPT and of 35 ± 10 μmol/l eq. ascorbic acid after INTERCEPT. The mean results for BCPCs were of 81 ± 10 μmol/l eq. ascorbic acid before INTERCEPT and of 29 ± 4 μmol/l eq. ascorbic acid after INTERCEPT (Fig. 1). These differences are statistically significant between before and after treatment. There is a significant difference between APC and BCPC results before treatment and also between these two groups after treatment. Concerning APC, the analysis was split on the donor sex. Twenty-eight women and 50 men were included in the study. Male donors exhibited a significantly higher AOP (94 ± 14 μmol/l eq. ascorbic acid before INTERCEPT and 37 ± 10 μmol/l eq. ascorbic acid after INTERCEPT) compared to female donors (83 ± 11 μmol/l eq. ascorbic acid before INTERCEPT and 32 ± 8 μmol/l eq. ascorbic acid after INTERCEPT) (Figs 2 and 3). In each gender group, the difference was statistically significant between before and after treatment.

The AOP threshold that discriminates PCs before and after IBS treatment was determined by a ROC curve [3]. This threshold is defined as the crossing point between the sensitivity and the specificity curve. For male APC

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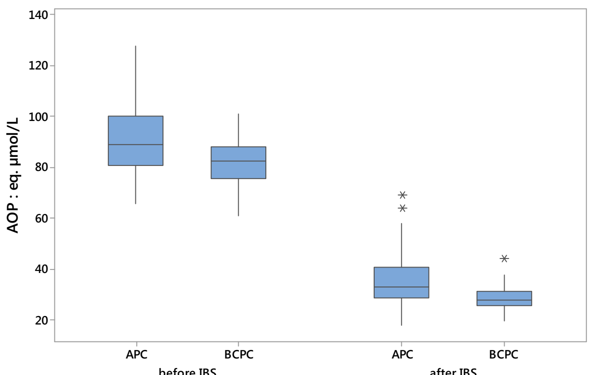

Fig. 1 Box plots representing antioxidant level expressed in µmol/L equivalent ascorbic acid in apheresis platelet concentrates (APC) and buffy-coat platelet concentrates (BCPC) before and after INTERCEPT Blood System (IBS). * are outliers.

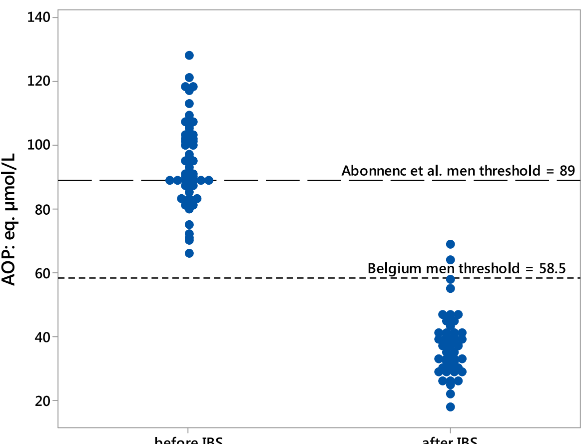

Fig. 2 Antioxidant level in function of donor sex expressed in µmol/l equivalent ascorbic acid: Men apheresis platelet concentrates (APC) before and after INTERCEPT Blood System. Each point represents a result. Dashed red lines stand for the calculated threshold based on the ROC curves, from the present studies and a reference study.

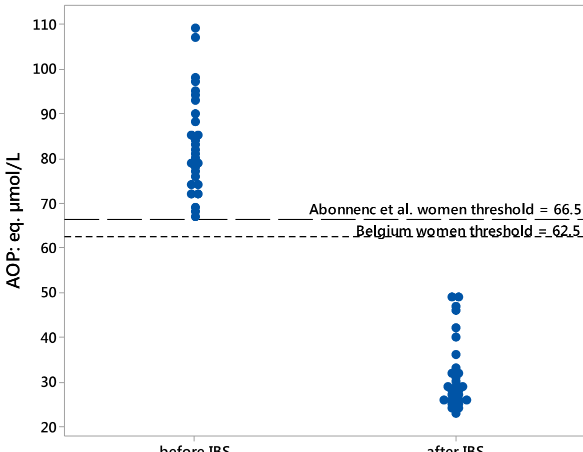

Fig. 3 Antioxidant level in function of donor sex expressed in µmol/l equivalent ascorbic acid: Women apheresis platelet concentrates (APC) before and after INTERCEPT Blood System. Each point represents a result. Dashed red lines stand for the calculated threshold based on the ROC curves, from the present studies and a reference study.

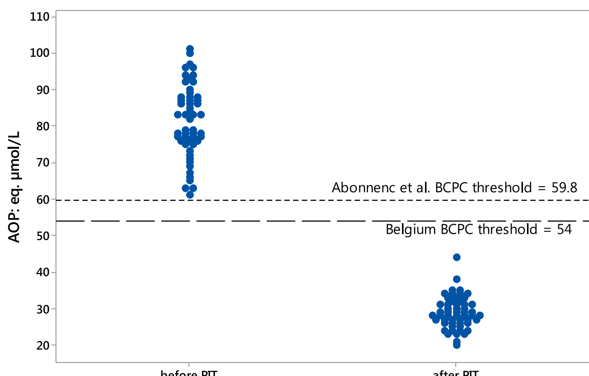

Fig. 4 Antioxidant level in buffy-coat platelet concentrates (BCPC) before and after INTERCEPT Blood System in Belgium expressed in µmol/l equivalent ascorbic acid. Dashed red lines stand for the calculated thresholds based on the ROC curves, from the present study and a reference study.

donors, the threshold was of 58·5 µmol/l eq. ascorbic acid (Fig. 2) compared to female APC donors with 62·5 µmol/l eq. ascorbic acid (Fig. 3). The threshold for BCPC was of 54 µmol/l eq. ascorbic acid (Fig. 4). All of these results had 100% of specificity. Below these thresholds, INTERCEPT treatment is considered to be executed. For men group, the threshold found by Abonnenc et al. was of 89 µmol/l eq. ascorbic acid. Our study showed a threshold at 58·5 µmol/l eq. ascorbic acid which is very low compared to the threshold of 89 µmol/l eq. ascorbic acid. (Fig. 2) The high values before IBS do not correspond to the high values after IBS. For women, our threshold was found at 62·5 µmol/l eq. ascorbic acid compared to 66·5 µmol/l eq. ascorbic acid for Abonnenc et al. (Fig. 3) As shown in the Fig. 4 concerning BCPCs, the two populations (before and after IBS) are very distinguishable and our threshold (54 µmol/l eq. ascorbic acid) is lower than Abonnenc's threshold which is of 59·8 µmol/l eq. ascorbic acid.

PC results – Mirasol treatment

For PCs treated by Mirasol technology, the mean results (n = 100) for BCPCs were of 98 ± 11 µmol/l eq. ascorbic acid before illumination and of 32 ± 10 µmol/l eq. ascorbic acid after illumination. These results are statistically

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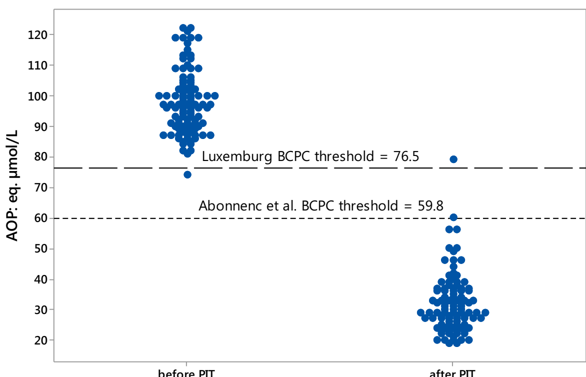

Fig. 5 Antioxidant level in buffy-coat platelet concentrates (BCPC) before and after Mirasol technology in Luxemburg expressed in µmol/l equivalent ascorbic acid. Dashed red lines stand for the calculated thresholds based on the ROC curves, from the present study and a reference study. [Colour figure can be viewed at wileyonlinelibrary.com]

different. The threshold for PCs from Mirasol was of 76·5 µmol/l eq. ascorbic acid (Fig. 5). One result after illumination was found aberrant, and over the threshold, no explanation was found. All of these results had 100% of specificity.

For the AOP measurements performed at different stages of the Mirasol process, the results showed a decrease of the AOP values. In the case where the samples were not protected from the light (n = 10), the mean results were of 105 ± 13 µmol/l eq. ascorbic acid before illumination and before riboflavin addition, of 45 ± 24 µmol/l eq. ascorbic acid after riboflavin addition and of 29 ± 13 µmol/l eq. ascorbic acid after illumination. For the second test (n = 10) (samples containing riboflavin protected from the light), the mean results were of 100 ± 12 µmol/l eq. ascorbic acid before illumination and before riboflavin addition, of 95 ± 11 µmol/l eq. ascorbic acid after riboflavin addition and of 31 ± 6 µmol/l eq. ascorbic acid after illumination.

Discussion

In this study, the obtained results prove that the AOP for PCs can be used to document the execution of the INTERCEPT and Mirasol treatment by a response of yes or no (no correlation with PI efficacy).

Abonnnec [3] has documented that the AOP decrease requires the presence of amotosalen HCl on Intercept treatment. The AOP decrease is mainly initiated by amotosalen HCl. UVA alone had a negligible effect on the AOP at a standard UVA dose of 3·9 J/cm^2.

To be used as a QC assay, an analysis has to be fast, straightforward, robust, reliable and with an affordable cost. The electrochemical-based EDEL technology meets these requirements, as it only requires the EDEL device that is plugged to a computer and single-use sensors. There is no sample preparation as only a few microlitres of the PC were dispensed into the sensor, and the analysis takes just a few seconds.

This study has the advantage to include several types of PCs treated by different PITs and provided from different blood centres, Mirasol treatment from Luxemburgish Red Blood Cross and INTERCEPT System for the PCs treatment from the Blood Service from Belgian Red Cross. These results were also compared with the results previously published by Abonnenc et al. on blood products produced in Switzerland and treated with INTERCEPT or Mirasol [3, 19]. Our strategy was to analyse paired samples pre- and post-treatment and then measure the difference of AOP between the two values. This difference was compared with a standard value determined thanks to previous assays and specific for each blood product and each blood establishment. Such analysis allows eliminating false-positive values and reducing false-negative rate (that could be present in APCs and not in BCPCs because of the pooling approach averaging the pretreatment AOP values). Another strategy can be performed which implies the measurement of post-treatment sample and compare the AOP to a determined threshold value with the same manner as the first strategy. Below the threshold value, the PIT is considered to be executed.

The prepared PCs for this study contain 35% of plasma and 65% of PAS. The AOP reflects the amount of different soluble antioxidants such as urate or ascorbic acid found in the plasma. Then, the initial AOP in untreated PCs depends on the percentage of plasma contained in the PC and it will be different from one blood centre to another. As Abonnenc et al. underlined, PCs produced in 100% of plasma would exhibit a higher AOP than those produced in PAS [3]. Therefore, each blood centre would have to define and validate its own AOP threshold for each tested blood product, as it is clearly pointed out here by threshold comparison in three different blood centres. The robustness of the assay can be impacted by the interdonor variability, especially for APC products, where male donors exhibited higher AOP values than females [3, 27]. Indeed, Demirbag et al. showed in their study that sex hormones have antioxidant activity and allow to decrease the oxidants production in different cells [28]. The timing of sampling and measurements can affect strongly the result, as it is important to realize the test immediately after sampling. In general for IBS treatment, thresholds obtained in this study are lower than determined thresholds by Abonnenc et al. As BCPCs are prepared from several donors, the biologic variability is not expressed in this case. However, the Luxemburgish

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threshold is much higher than those from Belgium and Switzerland. This difference could be explained by several reasons: plasma carry-over which is 3% higher in Luxemburgish platelets, difference in the PIT (other photosensitizer and wavelength), etc. In the extra-tests, we noticed a difference between samples protected from the light and those not protected before illumination. The protection against light of treated Mirasol blood products before illumination is known to prevent the decrease of AOP [29–31]. The mean before treatment is significantly lower in BCPCs than APCs and also after treatment, except for Mirasol results. The pooling of BCs clearly contributes to reduce the variability and favours the discrimination. The populations before and after treatment are by consequence well distinguished. The statistical analysis shows that the fall of results between before and after treatment is statistically significant.

Conclusion

The present data showed that the AOP is a reliable information to assess the completeness of PITs for quality control purposes on PCs. It also demonstrates the centre-to-centre differences in producing blood products and the needs in on-site validation procedures. All of these results must be confirmed by extended other studies to qualify and validate the AOP method as potential quality control assay. The dose kinetics for each pathogen inactivation method needs to be shown whether and how the decrease of AOP values after PIT correlates with the UV dose applied. Moreover, it is important to study how the PIT induces AOP reduction at the upper and lower limits of PC specifications (mainly plasma content, platelet concentration, PC volume and type of preparation method) to determine the ability of the AOP method to reliably discriminate between treated and untreated PCs. Finally, the influence of storage time and storage conditions on AOP results needs to be further investigated, particularly for the quality of MIRASOL-treated platelets.

Conflict of interest

The authors declare no conflict of interest. This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

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Non-phthalate plasticizer DEHT preserves adequate blood component quality during storage in PVC blood bags

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Vox Sanguinis

Background and objectives Commercial blood bags are predominantly made of polyvinyl chloride (PVC) plasticized with di(2-ethylhexyl) phthalate (DEHP). DEHP is favourable for storage of red blood cells (RBC). Historically, removal of DEHP from blood bags has been linked to unacceptable haemolysis levels. Oncoming regulatory restrictions for DEHP due to toxicity concerns increase the urgency to replace DEHP without compromising RBC quality. Di(2-ethylhexyl) terephthalate (DEHT) is one suggested substitute. The aim of this study was to compare PVC-DEHT to PVC-DEHP blood bags using additive solutions saline–adenine–glucose–mannitol (SAGM) and phosphate–adenine–glucose–guanosine–saline–mannitol (PAGGSM), to determine whether DEHT can maintain acceptable component quality.

Materials and methods RBC concentrates (N = 64), platelet concentrates (N = 16) and fresh frozen plasma (N = 32) were produced from whole blood collected into either DEHT or DEHP plasticized systems. Using a pool-and-split study design, pairs of identical RBC content were created within each plasticizer arm and assigned either SAGM or PAGGSM. Storage effects were assessed weekly for 49 days (RBC), 7 days (platelets) and before/after freezing (plasma).

Results Though haemolysis was slightly higher in DEHT, all study arms remained below half of the European limit 0·8%. K^+ was lower in DEHT than in DEHP independent of additive solution. The metabolic parameters were not influenced by choice of plasticizer. Platelet activation/metabolism and plasma content were similarly preserved.

Conclusion Our study demonstrates that the plasticizer DEHT provides adequate blood component quality. We propose DEHT as a strong future candidate for replacement of DEHP in blood bags.

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Introduction

Polyvinyl chloride (PVC) plasticized with di(2-ethylhexyl) phthalate (DEHP) has been the material of choice for

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commercial blood containers since the mid-20th century. Plasticizers are essential for material flexibility; facilitating centrifugation, sealing, transport and general handling of blood bags without risk of breakage and product loss [1]. DEHP is a dipolar, lipophilic molecule non-covalently bound to the PVC polymer, that leaches from the plastics when in contact with the stored blood component. By incorporation into the red blood cell (RBC) bilayer membrane, DEHP helps preserve membrane integrity [2–4].

However, concerns have been raised about DEHP-linked endocrine-disrupting toxicity observed in animal models [5]. Though actual evidence of toxic impact in humans is ambiguous, it cannot be excluded that DEHP and/or its metabolites may be harmful during prolonged exposure. Therefore, replacement of DEHP in blood bags with a corresponding non-toxic plasticizer has long been desired, but not at the expense of blood component quality [6, 7]. The lack of an acceptable plasticizer substitute for RBCs has slowed down the conversion process considerably, but recently, the demand became urgently motivated due to the updated European Commission regulation (EC) 2017/745 allowing a maximum concentration of 0·1% weight/weight of DEHP in medical devices [8]. Though exceptions are possible, finding a substitute is nonetheless a high priority.

Several plasticizers have been suggested as substitutes to DEHP in blood bags. Di(2-ethylhexyl) terephthalate (DEHT) and di-isononyl cyclohexane-1,2-dicarboxylic acid (DINCH) have shown the greatest potential so far, despite providing inferior RBC quality in previous studies [9–13].

DEHT is a structural isomer of DEHP with no reported resemblance of the DEHP toxicity pattern [14, 15]. A recent study assessing RBCs stored in PVC-DEHT bags with phosphate–adenine–glucose–guanosine–saline–mannitol (PAGGSM) and AS-1 as additive solution (AS) showed promising results for RBC storage [9]. To our knowledge, there are presently no published studies where the collection bag is plasticized with DEHT, nor are there published assessments of the AS saline–adenine–glucose–mannitol (SAGM) paired with PVC-DEHT. As SAGM is presently the most widely used AS in Europe, this would be a valuable contribution to the collective gathering of DEHT *in vitro* data.

Plasma, mostly prepared as fresh frozen plasma (FFP), is widely used, predominantly at emergency and critical care units as part of the treatment for critical bleeding [16]. Coagulation factors, except factor VIII, and to some extent factor V, are generally well preserved in FFP [17]. Although it is not expected that plasticizers influence the levels of coagulation factors, it is important to confirm this by comparing the concentrations of coagulation factors/inhibitors preserved in FFP during PVC-DEHT and PVC-DEHP storage.

Platelets are generally stored with plasticizers that provide superior gas permeability to DEHP, for instance n-butyryl-tri-n-hexyl citrate (BTHC) [18] or tri-(2-ethylhexyl) trimellitate (TOTM) [19]. However, when prepared from whole blood (WB), platelets are in contact with the processing set plasticizer until preparation and may thus be affected.

In this study, we focused on assessing the quality of RBCs produced and stored in PVC-DEHT blood bag systems paired with SAGM or PAGGSM, during a 49-day storage period. The effects were compared to corresponding storage in PVC-DEHP. Secondary, we compared plasma and platelet components stored in DEHT or DEHP, to ensure their compatibility in a future WB set.

Materials and methods

Blood collection and component production

Prototype blood bags made entirely of PVC-DEHT or PVC-DEHP, respectively (including tubes, ports and filters), were manufactured for the study (Macopharma, Mouvaux, France), mimicking the commercially available quintuple bottom-and-top NPT reference (bag configuration: Supporting Information 1). Sixty-four WB units (450 ml ± 10% in 63 ml citrate-phosphate-dextrose) from consenting donors were collected in the prototypes (N = 32; blood type A = 20, O = 12 of each plasticizer). Within each plasticizer arm, the WB units were pairwise ABO matched, pooled and split into new pairs of identical content. Each pair was assigned two additional sets of plasticizer-matched prototype bags for further WB processing into leucoreduced RBCs, leucoreduced plasma and buffy coat (BC). One of the sets contained 100 ml SAGM, the other 100 ml PAGGSM (Fig. 1). WB processing was performed according to Karolinska standard operating protocol (SOP): centrifugation at 3130 *g*, 11 minutes, MacoSpin, and separation by MacoPress Smart Revo (both MacoPharma).

Four study arms were generated for RBCs: DEHT/SAGM, DEHT/PAGGSM, DEHP/SAGM and DEHP/PAGGSM (N = 16 per arm). All units were leucoreduced by filtration and stored at 2–6°C within 8 hours of donation.

One plasma unit was randomly chosen from each processing pair, generating two plasma study arms: DEHT and DEHP (N = 16; blood type A = 10, O = 6 of each). They were leucoreduced by filtration, frozen in Lundair freeze LF Maxi (Ingenjörsfirman P-O Persson AB, Helsingborg, Sweden) and stored in ≤–25°C within 18 hours of donation.

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Double-dose BC platelet concentrates (PC) were prepared from pools of 8 same-plasticizer BCs and Platelet Additive Solution-E (PAS-E) using I-Platelet Pooling (IPP) set (Kansuk, Istanbul, Turkey), centrifugation (421 *g*, 9 minutes, Macospin) and extraction (Macopress Smart Revo), according to Karolinska SOP. Immediately after production, one of the units from each double dose was transferred into a prototype platelet storage bag plasticized with BTHC (ports of TOTM) and tubes of either DEHT or DEHP and then stored in platelet agitators at 18–22°C. Additional WB was collected until each of the two platelet study arms had N = 8.

All RBC and plasma components followed assigned plasticizer (DEHT or DEHP) from collection throughout the entire process including sampling. Similar for PCs, except for briefly during production, as the IPP set is plasticized with TOTM with a hard housing filter of polycarbonate, but contains DEHP in tubes and forks. Potential cross-contamination was avoided through separated storage and handling, both during bag manufacturing and of the collected and produced components. Measurements of raw material, semi-finished bags and sterilized finished products confirmed DEHP content <0·1% weight/weight in the DEHT material at all measuring points.

TSCDII (TerumoBCT, Lakewood, CO, USA) was used for all sterile docking. Seals were made with Qseal-free, Qseal-opti and Qseal-air (Conroy Medical AB, Upplands Väsby, Sweden).

Sampling

Prototype sampling bags (nominal volume 40 ml) were used. The RBCs were sampled every 7 days from day (d) 1 to 49. Plasma was sampled on the day of collection (d0 pre-freezing). After thawing (Barkey plasmatherm, Barkey GmbH & Co. KG Leopoldshöhe, Germany), the bags were kept at 2–6°C and sampled on d0 (within 30 minutes of thawing), d7 and d14 (factor VIII only) post-thawing. Aliquots were refrozen at ≤–65°C until analysis. PCs were sampled on d2, d5 and d7.

Analysis of storage effects

Production process

To verify conformity of the production process, residual white blood cells (WBC) were counted on the first day of sampling in all components, by use of cell counter ADAM-rWBC (NanoEnTek, Seoul, South Korea) (RBCs and plasma) or Nageotte/microscopy (PCs). RBC concentrates (RCC) were analysed for haematocrit (Hct) and Hb_{RCC} (g/unit and g/l) using Swelab Alfa Plus Basic hematology analyzer (Boule Diagnostics AB, Spånga, Sweden). Residual RBCs in plasma were counted by flow cytometry (CytoFLEX, Beckman Coulter, Brea, CA, USA). Platelet count (/l and /unit) was obtained by CA 620 Cellguard (Boule Medical, Stockholm, Sweden). The PCs were visually checked for swirling and aggregates.

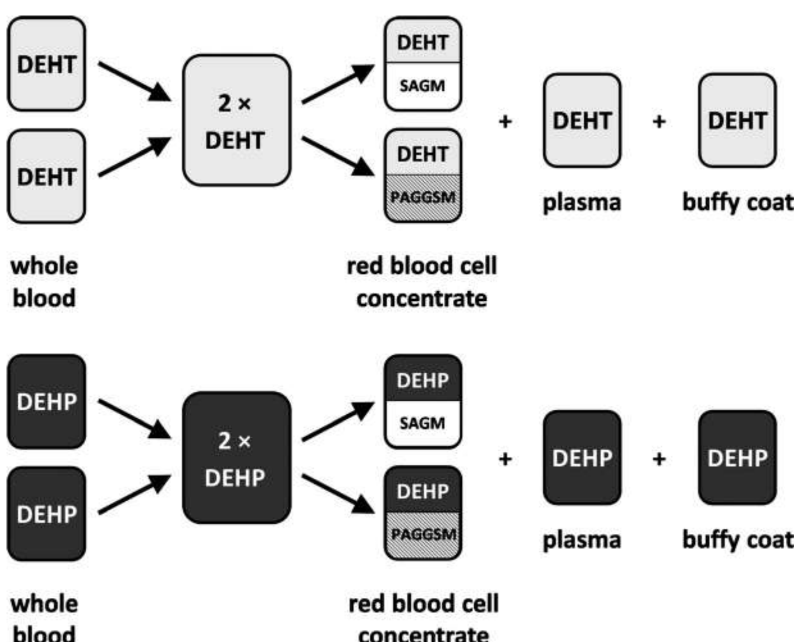

Figure 1 Pool-and-split study approach for PVC bags plasticized with DEHT (N = 32) or DEHP (N = 32), with additive solutions SAGM and PAGGSM for red blood cell storage.

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Serology tests were performed for all donors at donation. All units were subjected to bacterial screening after completed storage (Karolinska University Laboratory, Clinical chemistry and Clinical microbiology departments).

Red blood cells

The storage effects on RBCs were assessed by analysis of haemolysis, mean corpuscular volume (MCV), RBC microvesicle (RMV) count, RMV phosphatidylserine translocation, pH and concentrations of potassium ions (K^+), adenosine triphosphate (ATP), glucose, lactate and 2,3-diphosphoglycerate (2,3-DPG).

Haemolysis analysis was performed using HemoCue plasma/low haemoglobin photometer (Radiometer Medical ApS) following previous protocol [20]. Haemolysis (%) = (100-Hct) × $Hb_{supernatant}$ (g/l)/Hb_{RCC} (g/l). MCV (fL) was determined by Swelab Alfa. ABL 800 Flex blood gas analyzer (Radiometer Medical ApS, Brønshøj, Denmark) assessed extracellular pH (37°C) and concentrations (mmol/l) of K^+, glucose and lactate. Count and phosphatidylserine externalization (annexin V) of RMV were investigated by flow cytometry (CytoFLEX): PE-Cy7 and BV421 mouse anti-human CD235a (BD Biosciences, San Jose, CA, USA) were used for glycophorin A (marker for RBC origin) detection; aside from this, protocol and reagents have been previously described [20]. The results were analysed with FlowJo v.10.6 (Ashland, OR, US). Concentration of ATP (μmol/g Hb) was assessed by luminometry (Orion Microplate Luminometer, Berthold, Pforzheim, Germany) using ATP Kit SL (BioThema, Handen, Sweden), while 2,3-DPG (μmol/g Hb) was analysed through spectrophotometry (Jenway 6500 Spectrophotometer, Barloworld Scientific Ltd., Dunmow, Essex, UK) with 2,3-DPG test kit 10148334001 (Roche Diagnostics, Mannheim, Germany).

Plasma

Plasma was analysed for coagulation factors V, VII, VIII, X, XI (coagulation, turbidimetry), XIII, protein C (enzymatic activity, chromogenic), protein S-free (immunological, turbidimetry) and von Willebrand factor (photometry) (all IU/100 ml) on haemostasis analyser BCS-XP (Siemens, Munich, Germany). Triglycerides (enzymatic photometry, mmol/l) and α-1-antitrypsin (immunological, turbidimetry, g/l) were assessed using the Cobas 8000 platform (Roche Diagnostic, Basel, Switzerland). Fibrinogen (Clauss coagulation photometry, g/l) was measured with Sysmex CS 5100 (Kobe, Japan).

Platelets

Mean platelet volume (MPV, fL) was measured using CA 620 Cellguard. Cell disintegration was determined spectrophotometrically (Jenway 6500, test kit 063K6003 Sigma-Aldrich, St Louis, MO, USA) through determination of extracellular lactate dehydrogenase (LDH, %). Parameters of extracellular metabolism, pH (37°C, corrected to 22°C through Rosenthal's factor), glucose (mmol/l) and lactate (mmol/l), were measured, and bicarbonate (mmol/l) calculated, by ABL 800. Intracellular ATP content (μmol/10^{11} platelets) was assessed through luminometry (same equipment/reagents as RBCs). Mitochondrial membrane potential (determined through JC-1, %), activation level (CD62P, %), expression of surface glycoprotein CD42b (MFI), expression of platelet endothelial cell adhesion molecule PECAM-1 (MFI), and response capacity to agonist stimulation through PAC-1 with collagen, ADP and thrombin (%) were all analysed as previously described [21, 22].

Statistical analysis

Gaussian distribution was verified (D'Agostino-Pearson normality test), and mean ± standard deviation was calculated. Two-way ANOVA was chosen for statistical significance testing of RBCs, and unpaired t-test for plasma and platelets (RBCs: N = 16, plasma: N = 16, platelets: N = 8 per study arm), with Holm-Sidak's correction for multiple comparisons. For contingency test of 2,3-DPG at d21, Fisher's exact test was used to compare AS and plasticizers, respectively.

GraphPad Prism v.8.2 for Windows (GraphPad Software Inc., La Jolla, CA, USA) was used for computation of all statistics.

Ethical approval

Ethical approval was applied for but considered not applicable according to the Stockholm Regional Ethical Review Board.

Results

Component acceptance criteria

After production, all components fulfilled European [23] and local quality criteria without non-conformities (Supporting Information 2). In addition, screening for infectious markers [23] was negative for all donors.

There were no visual or physical differences between the plasticizers in processing functionality or handling. There were no leakages during transport, processing, storage or freezing/thawing of plasma. 100% of the tube seals and sterile dockings held adequate quality and sterile connections were easily opened.

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Minor effects on RBC membrane and metabolism

Haemolysis, RMV and K^+ were analysed to assess the effects of plasticizer and AS on cell membrane degradation.

Haemolysis was higher in DEHT than in DEHP from d14 onwards ($P < 0{\cdot}001$). From day 35, AS also affected haemolysis, with lower haemolysis levels in PAGGSM for both DEHT and DEHP ($P < 0{\cdot}05$ within plasticizers, $P < 0{\cdot}001$ for all other combinations). However, at d49, DEHT/SAGM, highest of the study arms (0·39%), still barely reached half of the European limit 0·8% [23] (Fig. 2a and b, Table 1, Supporting Information 3)).

RMV count is an indicator of membrane integrity loss. Reflecting the haemolysis results, the count was higher in DEHT than in DEHP and in SAGM than in PAGGSM (Fig. 2c, Table 1). We also noticed a similar highest-to-lowest order of glycophorin A expression decrease throughout storage (data not shown).

Externalization of RMV phosphatidylserine, apoptosis marker, was also elevated in DEHT/SAGM storage. At d49, DEHT/SAGM was higher than both DEHP arms ($P < 0{\cdot}05$), whereas DEHT stored with PAGGSM remained below or did not differ from the DEHP arms throughout storage (Fig. 2d, Table 1).

Elevated extracellular K^+ indicates membrane damage or inhibited metabolism. The extracellular K^+ levels were lower ($P < 0{\cdot}001$) in DEHT from d28 onwards, independent of AS (Fig. 3a). End K^+ concentration in DEHT finished 5-10 % lower than in DEHP (Table 1).

2,3-DPG preservation, associated with oxygen unloading ability, was notably higher ($P < 0{\cdot}05$ or higher significance level) in DEHT/SAGM at d14. At d21, a significantly higher percentage of DEHT-stored RCCs had some 2,3-DPG left (DEHT 66%, DEHP 38%, $P < 0{\cdot}05$), while there was no difference when comparing AS (SAGM 47%, PAGGSM 56%, ns)(Fig. 3b).

The RBC metabolism was further studied through analysis of pH (Fig. 4a), glucose, lactate and ATP (Fig. 4b, all Table 1). For units with the same AS, there were no significant differences (ns), regardless of the plasticizer used. An overall increased metabolism rate in SAGM, predominantly visualized by higher lactate generation ($P < 0{\cdot}01$ or higher significance level at d7, significance level decreasing over time), resulted in better ATP preservation in PAGGSM storage from d28 onwards ($P < 0{\cdot}05$ or higher significance level).

Mean corpuscular volume, indicator of cell swelling, was not affected by the plasticizer (Fig. 4c, Table 1), but showed markedly higher values for SAGM storage from d14 onwards ($P < 0{\cdot}05$ or higher significance level).

Complementary details for RBCs are available in Supporting Information 3.

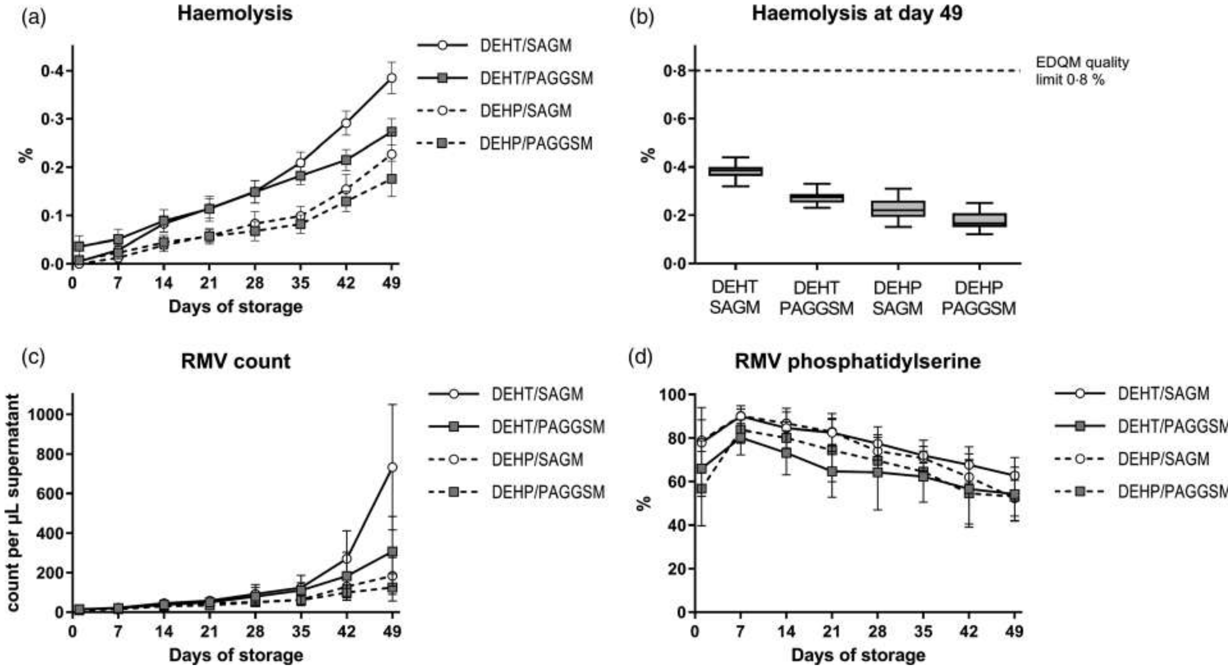

Figure 2 The RBC membrane was acceptably preserved in DEHT plasticized bags, visualized through (a) haemolysis over 49 days, (b) day 49 haemolysis in relation to European limit (0·8%, dashed line), (c) RBC microvesicle (RMV) count and (d) percentage of RMV phosphatidylserine externalization, all stored in DEHT or DEHP with additive solution SAGM or PAGGSM. Values are displayed as (a, c-d) mean ± standard deviation and (b) boxplot with Tukey whiskers. Significance was tested using two-way ANOVA with Holm-Sidak's correction for multiple comparisons (Table 1, Supporting Information 3).

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Table 1 Storage analysis results for red blood cells (N = 16 of each study arm) on day 1 and 49 (day 42: available in Supporting Information 3)

Analysis parameter, day 1	DEHT/SAGM Mean ± SD	DEHT/PAGGSM Mean ± SD	DEHP/SAGM Mean ± SD	DEHP/PAGGSM Mean ± SD
Haemolysis (%)	0·01 ± 0·01	0·04 ± 0·02	below detection limit	0·01 ± 0·01
	Significant differences: ***, ###, ¤¤¤			
Red blood cell microvesicles, count ($\times 10^3/\mu l$)	0·01 ± 0·01	0·02 ± 0·01	0·01 ± 0·01	0·01 ± 0
	Significant differences: ns			
Red blood cell microvesicles, phosphatidylserine positive (%)	77·9 ± 10·4	66·0 ± 12·9	78·9 ± 15·1	56·8 ± 17·1
	Significant differences: **, ‡‡‡, ##, ¤, §			
Extracellular K^+ (mmol/l)	2·9 ± 0·1	3·4 ± 0·2	3·0 ± 0·2	3·5 ± 0·2
	Significant differences: ns			
pH 37°C	6·991 ± 0·018	6·846 ± 0·017	6·988 ± 0·032	6·844 ± 0·025
	Significant differences: ***, ‡‡‡, ###, §§§			
Glucose (mmol/l)	29·8 ± 0·6	28·9 ± 0·6	29·7 ± 0·8	28·9 ± 0·8
	Significant differences: ns			
Lactate (mmol/l)	3·8 ± 0·3	3·5 ± 0·3	4·0 ± 0·5	3·6 ± 0·3
	Significant differences: ns			
Adenosine triphosphate (ATP) (μmol/g Hb)	5·8 ± 0·6	5·5 ± 0·4	5·7 ± 0·5	5·1 ± 0·6
	Significant differences: #			
Mean corpuscular volume (fl)	92·6 ± 2·5	92·7 ± 2·6	92·5 ± 2·7	92·6 ± 2·7
	Significant differences: ns			
2,3-diphosphoglycerate (2,3-DPG) (μmol/g Hb)	10·9 ± 1·8	11·6 ± 1·8	11·2 ± 1·7	10·8 ± 1·7
	Significant differences: ns			

Analysis parameter, day 49	DEHT/SAGM Mean ± SD	DEHT/PAGGSM Mean ± SD	DEHP/SAGM Mean ± SD	DEHP/PAGGSM Mean ± SD
Haemolysis (%)	0·39 ± 0·03	0·27 ± 0·03	0·23 ± 0·04	0·18 ± 0·04
	Significant differences: ***, †††, ‡‡‡, ###, ¤¤¤, §§§			
Red blood cell microvesicles, count ($\times 10^3/\mu l$)	0·73 ± 0·32	0·31 ± 0·18	0·18 ± 0·09	0·12 ± 0·07
	Significant differences: ***, †††, ‡‡‡, ###, ¤¤¤, §			
Red blood cell microvesicles, phosphatidylserine positive (%)	62·8 ± 8·3	54·2 ± 12·5	52·4 ± 8·2	53·0 ± 11·1
	Significant differences: †,‡			
Extracellular K^+ (mmol/l)	45·5 ± 1·4	44·7 ± 1·7	48·4 ± 1·7	48·7 ± 1·5
	Significant differences: †††, ‡‡‡, ###, ¤¤¤			
pH 37°C	6·275 ± 0·039	6·234 ± 0·040	6·291 ± 0·027	6·257 ± 0·029
	Significant differences: ***, ###, §§			
Glucose (mmol/l)	12·9 ± 1·5	11·5 ± 1·4	13·4 ± 1·1	12·3 ± 1·3
	Significant differences: **, ###, §			
Lactate (mmol/l)	33·4 ± 2·3	32·1 ± 2·2	34·0 ± 1·1	32·2 ± 1·3
	Significant differences: *, ‡, ##, §§			
Adenosine triphosphate (ATP) (μmol/g Hb)	2·9 ± 0·3	3·7 ± 0·2	3·0 ± 0·7	4·0 ± 0·8
	Significant differences: *, ‡‡‡, #, §§			
Mean corpuscular volume (fl)	102·4 ± 3·3	96·3 ± 3·0	100·7 ± 2·5	94·8 ± 2·4
	Significant differences: ***, ‡‡‡, ###, §§§			
2,3-diphosphoglycerate (2,3-DPG) (μmol/g Hb)	Not applicable			

Data is presented as mean ± standard deviation (SD). Significances are shown as *DEHT/SAGM vs. DEHT/PAGGSM, †DEHT/SAGM vs. DEHP/SAGM, ‡DEHT/SAGM vs. DEHP/PAGGSM, #DEHT/PAGGSM vs. DEHP/SAGM, ¤DEHT/PAGGSM vs. DEHP/PAGGSM, §DEHP/SAGM vs. DEHP/PAGGSM. Significance levels are shown as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns (no significance).

No differences in FFP coagulation factors/inhibitor preservation

The results of the plasma factors were consistent: similar levels were observed for both DEHT and DEHP units at all measured time points (Table 2).

The expected decrease of factor VIII was observed in both types of bags to a similar extent: DEHT: 72 ± 19 and DEHP: 68 ± 14 IU/100 ml directly after freezing/thawing, which was tolerable compared to the European limit (≥70 IU/100 ml [23]). At d14 post-thawing, both had decreased to 57 IU/100 ml (Fig. 5a). None of the

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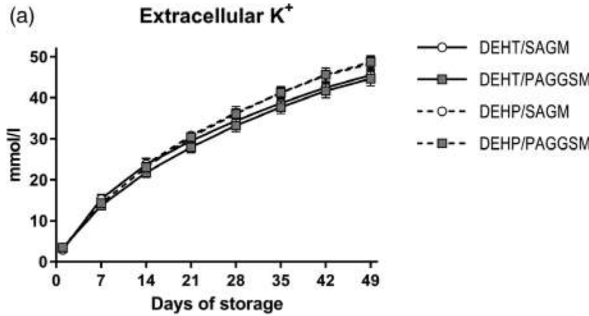

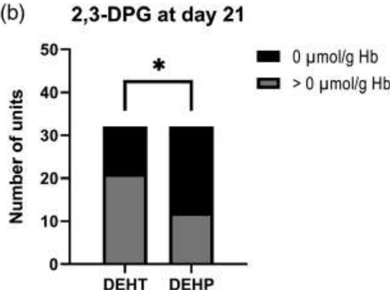

Figure 3 DEHT was slightly superior to DEHP for RBC storage independent of additive solution for the parameters (a) extracellular potassium (K^+) and (b) 2,3-DPG. Values are displayed as (a) mean ± standard deviation and (b) number of non-depleted (DEHT: 21, DEHP: 12) and depleted (DEHT: 11, DEHP: 20) units. Significance in (b) was tested with Fisher's exact test ($^*P < 0{\cdot}05$).

other parameters was noticeably affected over time with either plasticizer. Complementary details are available in Supporting Information 4.

Similar platelet reactivity, activation and metabolism

The platelets were exposed to DEHT or DEHP until preparation of the PCs and then again during sampling. There were no significant differences between the properties of platelets produced in DEHT or DEHP systems (Table 3). The collective assessment of the platelet parameters demonstrated an overall high quality, independent of plasticizer exposure.

No alterations in MPV were observed. Extracellular LDH levels implied normal platelet lysis rate in both plasticizers. Glucose consumption and lactate production correlated well, and a viable metabolism was confirmed by sufficient bicarbonate buffering capacity and ATP content [24, 25]. Mitochondrial membrane potential (JC-1) was well preserved in both study arms. Relative expression of surface receptor CD42b, containing vWF binding site, and PECAM-1, regulator of collagen stimulation, remained equally stable during storage for both study arms, with an expected moderate decrease of CD42b and increase in PECAM-1. Assessment of CD62P indicated no abnormal activation in either study arm (Fig. 5b). Response capacity to agonist stimulation was similarly reduced during storage (Fig. 5c). Complementary details are available in Supporting Information 5.

Discussion

This study presents a collection of *in vitro* evidence indicating that DEHT is a realistic candidate to replace DEHP in blood bags. The RBC quality markers were well within regulatory limits, and no adverse effects were seen for plasma or platelets.

In most aspects, DEHT-stored RCCs showed similar patterns to traditional DEHP storage [26, 27], likely attributed to a favourable WB processing strategy. Haemolysis levels of 0·29% (DEHT/SAGM) and 0·22% (DEHT/PAGGSM) at d42, common storage end time, are considered fully normal levels also in DEHP (Supporting Information 3). It is well established that DEHP migrates into and stabilizes the RBC membrane [3]. In a previous study conducted at our centre where RBCs were stored in polyolefin plastics [4], RBCs in PAGGSM exceeded 0·8% beyond 3 weeks storage. The PAGGSM results in our study may indicate that DEHT has a similar stabilizing mechanism to DEHP, although perhaps not as strong, since measurements of leached DEHT in RCCs yield markedly lower free levels than DEHP [9].

In two perspectives, DEHT may even be preferential to DEHP. Slightly lower K^+ levels in older DEHT-stored RCCs (Fig. 3a) and fewer 2,3-DPG depleted units at d21 during DEHT storage (Fig. 3b) were observed. K^+ has been associated with cardiac arrest [28], whereas 2,3-DPG facilitates oxygen unloading [29]. 2,3-DPG is dependent on pH, a metabolic factor that is influenced by AS composition [30–32]. Our results carefully suggest that plasticizer composition may be an additional variable impacting the 2,3-DPG depletion rate, though the possible mechanism, as well as clinical impact, requires further exploration.

The choice of AS clearly influenced the RBC storage lesion. Also with DEHT, PAGGSM offered a better membrane-protective storage environment than SAGM, visualized by reduced haemolysis, RMV count and percentage of RMV apoptosis marker (Fig. 2a–d), a pattern previously described in DEHP studies [30–32]. PAGGSM also implicated better ATP preservation (Fig. 4b) and less cell swelling (Fig. 4c). This indicates that PAGGSM would probably be the first-hand recommendation to be paired with DEHT, although, since overall differences were small, SAGM remains a satisfactory option.

We wanted to confirm that DEHP removal/DEHT addition did not detrimentally affect plasma and platelets, as has been previously investigated for DINCH [10]. For

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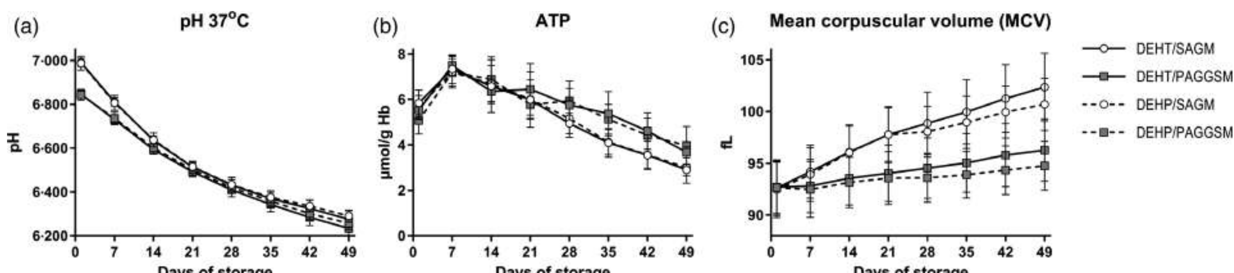

Figure 4 Plasticizer did not impact RBC metabolism parameters (a) pH, (b) ATP or (c) MCV. Graphs show storage over time in DEHT or DEHP combined with SAGM or PAGGSM. Values are displayed as mean ± standard deviation.

Table 2 Storage analysis results for plasma stored in DEHT or DEHP (N = 16 of each) on day 0 pre-freezing and day 7 post-thawing (day 0 post-thawing: available in Supporting Information 4).

	Day 0 (pre-freezing)		Day 7 (post-thawing)	
Analysis parameter	**DEHT Mean ± SD**	**DEHP Mean ± SD**	**DEHT Mean ± SD**	**DEHP Mean ± SD**
Protein S (IU/100 ml)	93 ± 10	92 ± 14	94 ± 09	94 ± 10
Protein C (IU/100 ml)	112 ± 13	109 ± 11	110 ± 12	106 ± 13
von Willebrand factor GP1bA (IU/100 ml)	104 ± 34	99 ± 31	101 ± 35	100 ± 34
Factor V (IU/100 ml)	104 ± 14	99 ± 12	90 ± 08	94 ± 09
Factor VII (IU/100 ml)	112 ± 14	103 ± 14	84 ± 18	81 ± 14
Factor VIII (IU/100 ml)	105 ± 31	96 ± 19	57 ± 16	55 ± 11
Factor X (IU/100 ml)	100 ± 13	95 ± 11	95 ± 13	88 ± 09
Factor XI (IU/100 ml)	97 ± 17	92 ± 09	84 ± 18	86 ± 09
Factor XIII (IU/100 ml)	125 ± 13	119 ± 15	128 ± 14	119 ± 14
Fibrinogen (g/l)	2·58 ± 0·34	2·52 ± 0·34	2·38 ± 0·33	2·39 ± 0·30
Triglycerides (mmol/l)	1·00 ± 0·29	1·08 ± 0·45	1·05 ± 0·30	1·10 ± 0·45
Alpha-1-antitrypsin (g/l)	0·95 ± 0·12	0·94 ± 0·13	0·92 ± 0·12	0·92 ± 0·11

Data are presented as mean ± standard deviation (SD). There were no significant differences (ns).

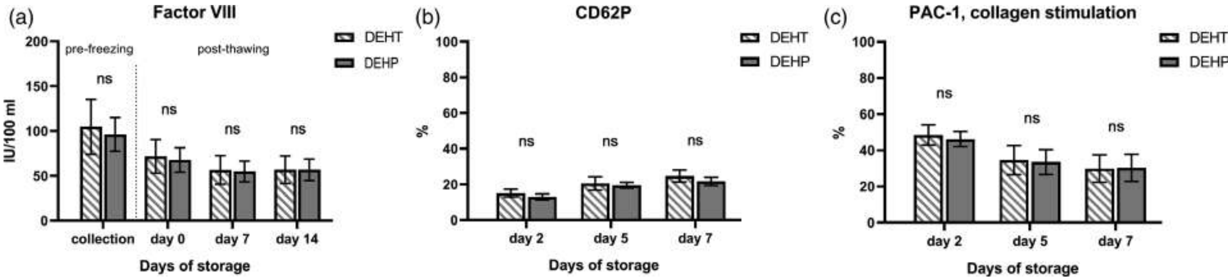

Figure 5 Effect of DEHT or DEHP plasticizer on fresh frozen plasma (FFP) through (a) coagulation factor VIII, and platelet concentrates through (b) activation marker CD62P and (c) response to agonists through PAC-1 collagen stimulation. Values are displayed as mean ± standard deviation. Significance was tested using unpaired t-test with Holm-Sidak's correction for multiple comparisons (ns: not significant).

plasma, we did not observe any difference in the levels of clotting factors/inhibitors in FFP stored in DEHT compared to DEHP at any of the investigated time points. Likewise, platelets did not show any inferior reactivity, activation or metabolism from DEHT processing and sampling.

The limitations of this study include the inability to completely exclude donor variability impacting comparisons as, to avoid plasticizer cross-contamination, a pool-and-split model was not possible between the two plasticizers. Another limitation is that all PCs were briefly in contact with DEHP when passing through the IPP system; we do not know how this affected the results. Complementing *in vivo* studies would also be valuable to obtain a complete picture. Furthermore, this is a single-centre study.

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Table 3 Storage analysis results for platelet concentrates on day 2 and 7 (day 5: available in Supporting Information 5), prepared from whole blood stored in DEHT or DEHP (N = 8 of each).

	Day 2		Day 7	
Analysis parameter	DEHT Mean ± SD	DEHP Mean ± SD	DEHT Mean ± SD	DEHP Mean ± SD
Mean platelet volume (fL)	9·3 ± 0·2	9·3 ± 0·6	9·5 ± 0·3	9·4 ± 0·5
Extracellular lactate dehydrogenase (%)	4·0 ± 1·6	3·6 ± 1·6	4·4 ± 1·3	4·5 ± 1·6
Glucose (mmol/l)	7·0 ± 0·4	7·1 ± 0·9	3·3 ± 0·6	3·9 ± 1·3
Lactate (mmol/l)	9·9 ± 1·1	9·7 ± 1·5	16·6 ± 1·8	15·6 ± 2·4
pH 22°C	7·293 ± 0·032	7·289 ± 0·060	7·403 ± 0·034	7·431 ± 0·077
Bicarbonate (mmol/l)	6·9 ± 0·4	7·0 ± 0·7	5·2 ± 0·5	5·5 ± 0·9
JC-1 (%)	97·4 ± 1·5	98·2 ± 0·5	96·1 ± 0·9	96·8 ± 0·5
CD62P (%)	15·2 ± 2·2	13·0 ± 1·7	24·7 ± 3·4	21·7 ± 2·3
ATP (μmol/10^{11} platelets)	7·0 ± 0·5	7·3 ± 0·8	6·6 ± 0·8	6·7 ± 0·8
CD42b (MFI)	69·1 ± 9·3	68·4 ± 11·2	59·8 ± 11·9	58·5 ± 11·7
PECAM-1 (MFI)	15·6 ± 1·6	14·7 ± 1·1	15·6 ± 0·6	15·0 ± 1·0
PAC-1 collagen stimulation (%)	48·5 ± 5·6	46·2 ± 4·2	30·0 ± 7·6	30·4 ± 7·5
PAC-1 ADP stimulation (%)	55·2 ± 7·6	55·0 ± 9·4	26·9 ± 7·3	26·6 ± 7·6
PAC-1 thrombin stimulation (%)	48·2 ± 7·0	46·6 ± 7·9	17·5 ± 5·4	16·6 ± 5·5

Data are presented as mean ± standard deviation (SD). There were no significant differences (ns).

It is well known that, for instance, haemolysis is influenced by multiple processing factors, for example WB holding time, RBC filtration, centrifugation force and sampling [30, 33–35], of which our centre fulfils numerous criteria favourable for low haemolysis with small standard deviation. Likely, differences in production processes will lead to different storage lesion results. In addition to the potential of alternative AS compositions [4, 10, 30–32], changes in processing may be another key factor to counteract disadvantageous RBC quality effects of DEHP removal [13]. As a future aspect, the individual contribution of different production parameters should be further explored.

In conclusion, this study demonstrates that PVC blood bags plasticized with DEHT provide adequate red blood cell quality during 49 days of storage. Furthermore, DEHT does not significantly alter the lesion profile of platelets or the degeneration of coagulation factors in plasma. PVC-DEHT bags hold as high physical and functional quality as bags plasticized with DEHP. Therefore, we consider PVC-DEHT blood bags a recommendable non-phthalate candidate for future blood component collection and storage.

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Conflict of interest

This study was conducted in collaboration with Macopharma; NF, SR and SC are Macopharma employees. All other authors declare no conflicts of interest.

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Supporting Information

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HIV incidence in South African blood donors from 2012 to 2016: a comparison of estimation methods

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Vox Sanguinis

Background Measuring incidence is important for monitoring and maintaining the safety of the blood supply. Blood collected from repeat-donors has provided the opportunity to follow blood donors over time and has been used to estimate the incidence of viral infections. These incidence estimates have been extrapolated to first-time donors using the ratio of NAT yield cases in first-time versus repeat-donors. We describe a model to estimate incidence in first-time donors using the limiting antigen (LAg) avidity assay and compare its results with those from established models.

Methods HIV-positive first-time donations were tested for recency using the LAg assay. Three models were compared; incidence estimated for (1) first-time donors using LAg avidity, (2) first-time and repeat-donors separately using the NAT yield window period (WP) model and (3) repeat-donors using the incidence/WP model.

Results HIV incidence in first-time donors was estimated at 3·32 (CI 3·11, 3·55) and 3·81 (CI 3·07, 4·73) per 1000 PY using the LAg assay and NAT yield WP models, respectively. Incidence in repeat-donors was between 2·0- and 2·5-fold lower than in first-time donors estimated at 1·56 (CI 1·37, 1·77) and 1·94 (CI 1·86–2·01) per 1000 PY using the NAT yield/WP and incidence/WP models, respectively.

Conclusion Testing HIV-positive donations using the LAg assay provides a reliable method to estimate incidence in first-time donors for countries that collect the majority of blood from first-time donors and do not screen with NAT.

Key words: transfusion - transmissible infections, residual risk estimation, blood safety, NAT testing.

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Introduction

Measuring the incidence of HIV is important for public health and maintaining the safety of the blood supply. Historically, incidence has been studied in expensive longitudinal cohort studies. Blood transfusion services that collect blood from mostly repeat-donors provide the opportunity to follow donors over time and have been used to estimate the incidence of viral infections in the blood supply [1]. Using the estimated incidence and an estimate of the mean duration of pre-detectable infectiousness (based on viral load growth during the ramp-up phase of infection and the HIV-1 minimum infectious dose), we can estimate the proportion of screen-negative blood donations which are potentially infectious, that is residual risk (RR) [2]. These repeat-donor-based incidence and consequent RR estimates have been extrapolated to

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first-time donors by multiplying the repeat-donor estimates with a factor such as the *first-time*-to-*repeat-donor* prevalence ratio or the *first-time*-to-*repeat-donor* NAT yield (HIV RNA positive, HIV antibody negative) ratio. The prevalence ratio is expected to overestimate the incidence and subsequent RR in first-time donors, especially in high endemic countries, as repeat-donors are prescreened for infection [3]. The NAT yield ratio can only be used in contexts that perform NAT testing in parallel with serology, and in most countries, the numbers of NAT yield cases are relatively small resulting in poor precision of the NAT yield ratio. Many blood services in developing countries collect blood mostly from first-time donors.

With the use of more sensitive NAT in parallel with serological assays a 'NAT yield window period (WP) model' can estimate first-time donor incidence and RR [4]. Many blood services that use NAT to screen blood use this NAT yield WP model to estimate RR. However, the small number of NAT yield donations, typically detected, limits the precision of risk estimates and the cost of NAT limits its application. Newer 'incidence assays' with lower sensitivity to HIV antibody allow the definition of a tunable (but longer) 'recent infection' WP compared to NAT. Unlike NAT testing on serological negative specimens, incidence assays need only be performed on the HIV antibody-positive donations and can yield a much larger sample of recent infections providing more precision for incidence estimations in first-time donors [5]. However, these more recent serologic approaches for incidence estimation have not been extensively validated in the blood bank setting.

As part of a five-year study, all HIV-seropositive donations were tested with an incidence assay to determine recent HIV infections [6–8]. We compared incidence estimates calculated from three approaches: (1) the cross-sectional LAg incidence assay method using first-time donors [7]; (2) the NAT yield WP model [4] in both repeat-donors and first-time donors, and (3) the incidence/WP (I/WP) classic method using repeat-donors [2]. In addition, we compared the incidence in first-time donors with that reported in the general population [9] and examined differences in incidence over the study period in different geographical regions and in donors of different ethnicities, gender and ages.

Methods

Setting

SANBS collects approximately 900 000 blood donations per annum in a high HIV endemic context and screens blood in parallel for NAT and anti-HIV leading to approximately 1600 confirmed HIV seropositive and 60 HIV NAT yield donations per year.

Laboratory testing algorithms

All blood donations were screened by the Prism (Abbott, Delkenheim, Germany) 3rd-generation anti-HIV, HBsAg and anti-HCV chemiluminescent immunoassays (ChLIA) in parallel with the Procleix Ultrio (Plus) NAT assay (Grifols, Barcelona, Spain) for HIV RNA, HCV RNA and HBV DNA. An extensive confirmatory and follow-up algorithm (previously described) [10] were in place for HIV, which classifies donations into four categories: (1) HIV negative, (2) HIV-concordant positive (HIV RNA by NAT and anti-HIV by ChIA), (3) HIV 'NAT yield' and (4) HIV 'serology yield' (RNA negative, anti-HIV ChIA repeat reactive and Western blot positive). The NAT yield cases are confirmed through seroconversion to anti-HIV positive at recall (approximately 70% return) or, for non-returning donors, replicate testing by Procleix Ultrio (Plus) discriminatory HIV NAT and quantitative PCR (Cobas Ampliprep/Cobas TaqMan HIV-1/2, Roche, Basel, Switzerland) or Real-time HIV-1 m2000rt (Abbott, Delkenheim, Germany), using stored fresh frozen plasma from the index donation.

HIV-concordant-positive donations were tested for recency of infection using the limiting antigen (LAg) avidity assay (Sedia Biosciences Corporation, Portland, Oregon). The single-well LAg avidity EIA is responsive to the avidity of HIV-1-specific IgG, as it quantifies antibody binding to a multi-subtype recombinant HIV-1 antigen coated onto assay plates at low density (hence the term 'limiting antigen', typically offering just a single binding site to multivalent IgG antibodies, and hence demonstrating a progressive increase in signal intensity over 3–6 months following seroconversion that can be used to infer duration of infection [11]. A normalized optical density (ODn) of <1·5 for the recent/long-standing threshold has been demonstrated to define a state of 'recent infection' with a mean duration of approximately 180 days for Clade C HIV infections [12]. Five recency or incidence assays, including the LAg avidity assay, have been robustly evaluated and validated by the Consortium for the Evaluation and Performance of HIV Incidence Assays. A false recency rate (FRR) of 1·3% (95% CI 0·3–3·2) was estimated for the LAg assay [5].

Incidence models

Only allogeneic whole blood donations were included in the analysis. The definition of a repeat-donor is different for models 2 and 3 (see Appendix 1). Age categories were collapsed for reporting based on incidence into three groups: younger than 20 years, 20–30 years and older

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than 30 years. Confidence limits for incidence estimates were derived from Poisson regression.

Model 1: The LAg first-time donor model
First-time donors were categorized into three groups: HIV negative, recent HIV infection and long-standing HIV infection. Recent HIV infection was defined as (1) HIV-concordant-positive and LAg avidity recent (ODn < 1·5) or (2) NAT RNA positive, anti-HIV negative. There were 123 (2%) confirmed HIV-positive donors with missing LAg results and were imputed as recent or long-standing HIV using a fully conditional specification multiple imputation logistic regression method (see Appendix 1). Donors with long-standing infection (ODn ≥ 1·5) were classified as prevalent and excluded from the analysis. Incidence was calculated as incident cases/1000 person-years (PY) using only cases classified as recent infections.

The denominator was defined as the total time for at-risk first-time donors with each uninfected first-time donor contributing the full mean duration of recent infection (MDRI) estimate and each recently infected donor contributing half the MDRI estimate. The MDRI of 195 (96% CI: 168–222) days estimated by Grebe and colleagues [12] was used which includes the length of the period that anti-HIV is positive and LAg with an ODn < 1·5 (179 days) and the length of the period that NAT is positive and anti-HIV is negative (16 days) [12].

Model 2: NAT yield WP model
The NAT yield WP model previously described [4] classifies incident infections as NAT positive, anti-HIV negative. Incidence was calculated as NAT yield cases/1000 PY. Each uninfected donor contributed the full NAT yield detection period, and each NAT yield donor contributed half the NAT yield period to the denominator. The length of the NAT yield detection period of 15·4 days was used [13, 14]. As this model assesses new infections cross-sectionally based on a brief NAT yield detection period, we used it for estimation in first-time and repeat-donors. For this model, a repeat-donor is classified as a donor who donated previously within or outside of the study period.

Model 3: the classic incidence/WP model
This model originally described by Schreiber et al. [2] only estimates incidence in repeat-donors. Donors are classified as repeating donors if they contributed 2 or more donations during the study period, the term 'repeating' is used to differentiate these donors from 'repeat' donors in model 2. (see Appendix 1) A donor is classified as having an incident HIV infection if they have two or more donations during the study period of which the first donation tested HIV negative and any subsequent donation tested HIV positive. Incidence was calculated as cases/1000 PY. The denominator was defined as the total follow-up time for the at-risk repeating donors. For those who remain free of infection throughout the study period, time at risk is the time from first to last donation in the study period. For those found to be infected at the second or subsequent donations, time at risk is time from the first donation in the study period to halfway between the last donation at which the donor was uninfected and the donation at which infection was detected.

Incidence in the general population

We compared the incidence derived in first-time donors with the incidence reported in the general population from the UNAIDS data 2019 report [9].

Results

SANBS collected 4 019 985 whole blood donations from January 2012 until December 2016. Of these 3 504 481 (87%) were collected from 723 166 repeat-donors and 515 504 (13%) were collected from first-time donors (used in models 1 and 2). There were 3 661 669 (91%) donations classified as coming from repeating donors for model 3. Table 1 provides the number of donations used to calculate person-years (PY) for each model. Table 2 provides the HIV positives used as incident cases in each model by year, province, ethnicity, gender and age. HIV prevalence in all donations, in first-time donations and in repeat donations was 0·23%, 1·11% and 0·1%, respectively, giving a *first-time*-to-*repeat-donor* HIV prevalence ratio of 11:1. The NAT yield rate in all donations, first-time donations and repeat donations was 0·0078%, 0·016% and 0·0066%, respectively, giving a *first-time*-to-*repeat-donor* NAT yield ratio of 2·42:1.

Incidence

Model 1: Among the 515 504 donations by first-time donors, 5540 (1·07%) confirmed anti-HIV positive. Of these, 879 (16%) were classified as recent infections. The denominator consisted of PY contributed by 510 862 at-risk donors [excluding 4642 HIV-positive donors with long-standing infections]. The overall incidence using model 1 and the LAg assay to determine recency in first-time donors was 3·32 (CI 3·11, 3·55) per 1000 PY.

Table 3 shows incidence by year, province, gender, ethnicity and age using model 1. Incidence varied slightly by year, declining from 4·08 in 2013 to 2·90 per 1000 PY in 2016. The highest incidence was in the Mpumalanga and KwaZulu-Natal provinces, and the lowest incidence was in the North West and Northern Cape provinces (Fig. 1).

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Table 1 Donations for each model.

	Model 1	Model 2			Model 3
	LAg first-time donors*	All	First-time	Repeat	Repeating
Total	513 334				
Total used in denominator	508 796	4 019 985	515 504	3 504 481	3 661 669
Year					
2012	98 690	780 826	100 229	680 597	697 581
2013	108 055	796 638	109 755	686 883	728 479
2014	103 000	803 641	104 323	699 318	742 204
2015	102 033	828 449	103 291	725 158	767 212
2016	97 018	810 431	97 906	712 525	726 223
Province					
Gauteng	226 558	1 805 636	229 145	1 576 491	1 645 924
KwaZulu-Natal	103 638	686 877	105 339	581 538	612 057
Mpumalanga	46 014	395 308	46 962	348 346	361 922
Eastern Cape	47 967	373 306	48 543	324 763	342 237
Free State	26 895	269 849	27 249	242 600	250 974
North West	23 940	227 523	24 140	203 383	210 101
Limpopo	20 842	138 395	21 074	117 321	124 689
Northern Cape	12 936	123 090	13 052	110 038	113 748
Ethnicity					
White	157 523	2 296 050	158 340	2 137 710	2 160 998
Black	272 117	1 156 029	277 350	878 679	991 200
Asian	36 174	301 480	36 525	264 955	272 142
Coloured	27 927	215 940	28 120	187 820	197 415
Unknown	15 055	50 486	15 169	35 317	39 944
Gender					
Female	283 185	1 741 727	287 345	1 454 382	1 544 856
Male	225 603	2 278 249	228 151	2 050 098	2 116 843
Age					
16–19	235 252	720 898	238 229	482 669	605 952
20–30	142 808	991 680	144 647	847 033	872 909
>30	130 736	2 309 771	132 450	2 177 321	2 180 826

*The first-time donations classified as long-standing are subtracted from the total. Long-standing donations are excluded from the denominator

Model 2: As the NAT yield method can be used to estimate incidence in all donations, first-time donations and repeat donations, we used it as a bridge to allow comparison to both models 1 and 3. In addition, we used this model to compare incidence in first-time and repeat-donors. Incidence, based on 312 NAT yields in all donations, was estimated at 1·84 (CI 1·65, 2·06) per 1000 PY. Using the 82 and 230 NAT yields from first-time and repeat-donors, incidence was estimated to be 2·44-fold higher in first-time donors at 3·81 (CI 3·07, 4·73) compared to 1·56 (CI 1·37, 1·77) per 1000 PY in repeat-donors (Table 3).

Model 3: The classic model to determine incidence included only donations from donors with at least two donations during the study period.

Among 3 661 699 whole blood donations by 602 329 repeating donors, 2425 tested confirmed HIV positive. Number of donations varied between 2 and 33 per donor. The median follow-up times, the amount of time during the study periods (used in the denominator) for HIV-positive and HIV-negative donors were 383·5 and 636 days, respectively. Incidence estimated using repeating donors was 1·94 (CI 1·86–2·01) per 1000 PY (Table 3).

Comparisons between Models

When comparing the models to determine incidence in first-time donors, model 2 yielded an estimate that was 1·15-fold higher than model 1 but this difference was not statistically significant. When we extrapolated the incidence derived from repeat-donors using model 3 to first-time donors using the NAT yield ratio of 1:2·42, the estimated incidence in first-time donors was 4·69 (CI 4·51,

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Table 2 classification of HIV positives for each model

	Model 1		Model 2									Model 3
	LAg recent		RNA+/Ab+			RNA–/Ab+/WB+			RNA+/Ab–			
	First-time	Missing	All	First-time	Repeat	All	First-time	Repeat	All	First-time	Repeat	New infections in Repeating donors
Long-standing	4538											
recent	879											
Total	879	123	8607	5462	3145	234	202	32	312	82	230	2425
Year												
2012	176	29	1548	1076	472	18	15	3	60	17	43	178
2013	228	33	1858	1239	619	36	34	2	82	26	56	448
2014	162	34	1795	1115	680	39	35	4	57	15	42	545
2015	165	7	1811	1082	729	63	54	9	54	14	40	644
2016	148	20	1595	950	645	78	64	14	59	10	49	610
Province												
Gauteng	312	22	3310	2154	1156	93	82	11	110	28	82	886
KwaZulu-Natal	235	44	1943	1165	778	57	50	7	84	32	52	573
Mpumalanga	142	23	1361	948	413	35	30	5	46	16	30	326
Eastern Cape	70	26	798	459	339	15	13	2	29	2	27	276
Free State	61	2	517	302	215	12	9	3	22	2	20	169
North West	18	5	211	141	70	6	5	1	8		8	58
Limpopo	29	1	311	203	108	12	10	2	8	1	7	93
Northern Cape	12	0	156	90	66	4	3	1	5	1	4	43
Ethnicity												
White	16	3	308	85	223	7	5	2	13	1	12	139
Black	831	114	7790	5126	2664	217	187	30	281	77	204	2113
Asian	2	1	85	31	54	3	3	0	5	1	4	35
Coloured	20	2	295	125	170	2	2	0	10	1	9	116
Unknown	10	3	129	95	34	5	5	0	3	2	1	22
Gender												
Female	651	83	5732	3763	1969	187	162	25	213	66	147	1563
Male	228	40	2875	1699	1176	47	40	7	99	16	83	862
Age												
16–19	268	34	1840	1371	469	28	26	2	59	22	37	426
20–30	418	42	3855	2263	1592	62	46	16	162	45	117	1211
>30	193	47	2913	1828	1085	144	130	14	91	15	76	788

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Table 3 Incidence determined by three models

	Incidence (Lag FT)/ 1000 person-years Model 1	Incidence (NAT yields)/1000 person-years Model 2			Incidence (Repeat, classic)/ 1000 person-years Model 3
		First-time	All	Repeat	
Overall			1·84 (1·65, 2·06)		
Donor type					
First-time	3·32 (3·11, 3·55)	3·81 (3·07, 4·73)			
Repeat				1·56 (1·37, 1·77)	1·94 (1·86–2·01)
Year					
2012	3·48 (3·01, 4·02)	4·06 (2·53, 6·54)	1·82 (1·42, 2·35)	1·50 (1·11, 2·02)	1·74 (1·50–2·01)
2013	4·08 (3·59, 4·64)	5·68 (3·87, 8·34)	2·45 (1·97, 3·04)	1·93 (1·49, 2·51)	1·79 (1·55–2·07)
2014	3·05 (2·62, 3·55)	3·45 (2·08, 5·72)	1·68 (1·30, 2·18)	1·42 (1·05, 1·93)	1·75 (1·52–2·02)
2015	3·05 (2·62, 3·55)	3·25 (1·92, 5·48)	1·55 (1·19, 2·02)	1·31 (0·96, 1·78)	2·00 (1·75–2·29)
2016	2·90 (2·47, 3·41)	2·45 (1·32, 4·55)	1·73 (1·34, 2·23)	1·63 (1·23, 2·16)	1·63 (1·40–1·89)
Gender					
Female	4·43 (4·11, 4·78)	5·52 (4·34, 7·03)	2·91 (2·54, 3·33)	2·40 (2·04, 2·82)	2·68 (2·55–2·82)
Male	1·94 (1·70, 2·20)	1·68 (1·03, 2·73)	0·86 (0·70, 1·07)	0·96 (0·77, 1·19)	1·29 (1·20–1·37)
Age					
16–19	2·21 (1·96, 2·48)	2·20 (1·45, 3·35)	1·95 (1·51, 2·51)	1·82 (1·32, 2·51)	2·27 (2·07–2·50)
20–30	5·58 (5·08, 6·14)	7·49 (5·59, 10·04)	3·89 (3·33, 4·53)	3·27 (2·73, 3·92)	3·57 (3·37–3·79)
>30	2·87 (2·49, 3·30)	2·72 (1·64, 4·52)	0·93 (0·76, 1·15)	0·83 (0·66, 1·04)	1·07 (0·99, 1·15)
Female					
16–19	3·51 (3·09, 3·99)	3·50 (2·23, 5·49)	3·29 (2·48, 4·36)	3·16 (2·20, 4·55)	3·51 (3·15–3·91)
20–30	6·94 (6·20, 7·77)	10·18 (7·31, 14·18)	5·68 (4·71, 6·84)	4·69 (3·74, 5·88)	4·61 (4·28–4·97)
>30	3·23 (2·71, 3·85)	3·88 (2·21, 6·84)	1·40 (1·07, 1·82)	1·19 (0·88, 1·60)	1·40 (1·28–1·55)
Male					
16–19	0·65 (0·47, 0·90)	0·66 (0·21, 2·04)	0·70 (0·39, 1·26)	0·72 (0·36, 1·43)	1·05 (0·86–1·28)
20–30	3·77 (3·15, 4·50)	3·89 (2·10, 7·24)	2·33 (1·78, 3·06)	2·13 (1·57, 2·88)	2·58 (2·34–2·85)
>30	2·40 (1·90, 3·02)	1·24 (0·40, 3·85)	0·62 (0·45, 0·86)	0·59 (0·42, 0·83)	0·80 (0·72–0·90)
Race					
White	0·19 (0·12, 0·32)	0·15 (0·02, 1·06)	0·13 (0·08, 0·23)	0·13 (0·08, 0·23)	0·20 (0·17–0·23)
Black	5·88 (5·50, 6·29)	6·71 (5·37, 8·39)	5·80 (5·16, 6·52)	5·52 (4·81, 6·33)	5·81 (5·57–6·06)
Asian	0·12 (0·03, 0·48)	0·65 (0·09, 4·61)	0·39 (0·16, 0·94)	0·36 (0·13, 0·95)	0·33 (0·24–0·46)
Coloured	1·38 (0·89, 2·13)	0·85 (0·12, 6·01)	1·10 (0·59, 2·04)	1·14 (0·59, 2·18)	1·67 (1·39–2·00)
Unknown	1·27 (0·68, 2·36)	3·15 (0·79, 12·58)	1·41 (0·46, 4·38)	0·67 (0·09, 4·77)	1·47 (0·96–2·23)
Province					
Gauteng	2·61 (2·34, 2·92)	2·92 (2·02, 4·24)	1·45 (1·20, 1·74)	1·23 (0·99, 1·53)	1·55 (1·45–1·66)
KwaZulu-Natal	4·42 (3·90, 5·02)	7·28 (5·15, 10·30)	2·91 (2·35, 3·60)	2·12 (1·62, 2·78)	2·47 (2·27–2·68)
Mpumalanga	5·92 (5·02, 6·97)	8·25 (5·05, 13·46)	2·77 (2·07, 3·70)	2·04 (1·43, 2·92)	2·81 (2·52–3·14)
Eastern Cape	2·98 (2·36, 3·75)	0·99 (0·25, 3·94)	1·85 (1·28, 2·66)	1·97 (1·26, 3·03)	2·34 (2·08–2·64)
Free State	4·29 (3·34, 5·51)	1·76 (0·44, 7·04)	1·94 (1·27, 2·94)	1·95 (1·26, 3·03)	2·00 (1·71–2·34)
North West	1·42 (0·90, 2·26)	0	0·83 (0·42, 1·67)	0·93 (0·47, 1·86)	0·84 (0·64–1·11)
Limpopo	2·61 (1·81, 3·75)	1·14 (0·16, 8·07)	1·37 (0·69, 2·75)	1·42 (0·67, 2·97)	2·28 (1·86–2·81)
Northern Cape	1·74 (0·99, 3·06)	1·83 (0·26, 12·98)	0·96 (0·40, 2·32)	0·86 (0·32, 2·30)	1·12 (0·83–1·52)

4·89) which was significantly higher (1·41-fold) than in model 1 but not statistically different from the results from Model 2.

Model 2 and Model 3 were used to estimate incidence in repeat-donors. Model 3 estimated incidence as 1·24-fold and significantly higher than in model 2. To investigate the reason for the higher estimates in model 3, we assessed the sensitivity of the incidence estimate on the inclusion of donors who donated twice only compared to donors who donated three or more times and found a 2-fold higher incidence in the former group. Thus, model 3 repeating donors included some donors, who were observed from their first to the second donation, when incidence is higher, whereas model 2 repeat donors only

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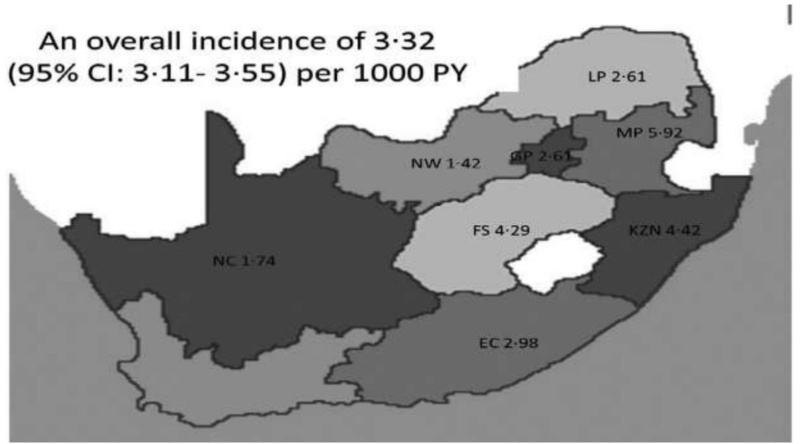

Fig. 1 Incidence in first-time donors determined using model 1.

included second and subsequent donations, when the incidence is lower.

Being female, aged 20–30 years old, of black ethnicity and living in KwaZulu-Natal and Mpumalanga were associated with a higher incidence for all models.

Comparison to HIV incidence in the general population

Incidence was approximately 3·5 times higher in the general population compared to first-time donors (Fig. 2). In the general population, incidence significantly decreased from 13·30 (CI 12·6, 13·9) in 2012 to 10·6 (CI 9·89, 11·3) per 1000 PY. A similar but not significant decrease, albeit lower, was seen in the first-time blood donors which declined from 3·48 (CI 3·01, 4·02) in 2012 to 2·90 (CI 2·47, 3·41) in 2016 (Table 3).

Discussion

The incidence of a viral infection in blood donors is used to estimate the RR of a transfusion-transmitted infection occurring as it establishes the number of new infections which may go undetected by the blood services screening strategy. South Africa has the largest burden of HIV in the world and although the prescreening donor interview and testing of first-time donors remove the majority of the prevalent infections, a substantial number of incident HIV infections are detected in our donor population, and hence, we project an elevated RR of transfusion-transmitted HIV infection relative to most other countries even with application of ID-NAT [3]. Accurate measurement of HIV incidence and consequent RR is essential to monitor risk reduction strategies. In this study, we were able to test HIV-positive samples from first-time donors to

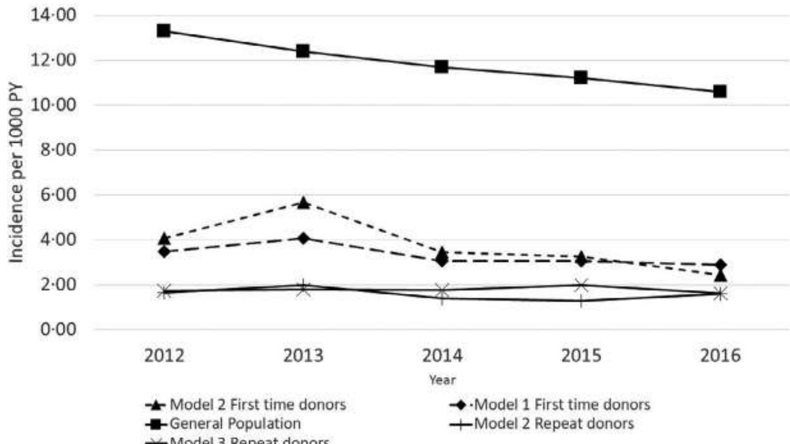

Fig. 2 Incidence in first-time donors using models 1 and 2 and incidence in repeat-donors using models 2 and 3 compared to the incidence in the general population [9].

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determine recent infection status using the LAg avidity assay and use the incident infections to compare the incidence derived by LAg with that derived by parallel testing using ID-NAT and the NAT yield WP model [4]. In addition, we compared incidence in repeat-donors derived by the I/WP model [2] with the NAT yield WP model [4]. Our high-level findings demonstrate that incidence rates determined by several methods were broadly consistent which is reassuring, although small discrepancies should spur re-examination of accuracy and implications of differences between the models.

Among first-time donors, we found similar overall HIV incidence using the LAg avidity and NAT yield models, adding confidence that these two cross-sectional incidence approaches are comparable and that the LAg avidity approach has promise in that it yields significantly larger numbers of recent infection cases and hence more precise incidence estimates than the NAT yield method. However, in repeat-donors we estimated a significantly higher incidence using the classic model compared to the NAT yield model. Furthermore, we found an extrapolated incidence from repeat-donors to first-time donors using the classic model that was one and a half-fold higher than the LAg avidity and NAT yield models, suggesting that previous corrections using NAT yield ratios for derivation of first-time incidence may be overestimated.

We speculate additional reasons for the higher incidence derived by the classic method: (1) in our study the median follow-up time (the time during the study period that contributes to the denominator) of HIV-positive repeating donors was nearly half the length of HIV-negative donors, as negative donors were able to give multiple donations that were counted for the entire study period, compared to HIV-positive donors who would have had truncated follow-up following detection of infection and deferral; (2) the inter-donation intervals for HIV-positive and HIV-negative donors were not random (data not shown), in part due to the preset periodic times of mobile clinics where 60% of the blood is collected; and (3) the classic method using repeating donors, included some first-time donors who donated a second donation during the study period. These newer donors may be at higher risk of having an incident infection compared to established repeat-donors. When we estimated incidence in donors who donated their second donation in the study and compared this to the incidence if they donated their 3rd or later donation, the former had a twofold higher incidence giving credibility to this assumption (data not shown).

The findings using the classic method may have relevance to the issue of self-deferral during the earlier stages of infection by repeat-donors, which consequently results in overestimation of RR. This is because a repeating donor may appropriately self-defer from donating blood for the mandatory 3 months following high-risk behaviour; therefore, although classified as an incident HIV infection using the classic model, such donors would not be contributing donations relevant to the RR as they self-deferred during the window period and would subsequently be detected using the current laboratory screening strategies. The lower incidence in repeat-donors based on the NAT yield WP approach is likely more accurate since detection of a NAT yield case is a direct measurement of a donor presenting to donate during the RNA-positive pre-seroconversion stage of infection that immediately follows the undetectable pre-NAT infectious WP.

Incidence in first-time donors was more than twice that of repeat-donors. This difference may be due to confounding or selection bias. First-time donors are enriched with young, female and black individuals, subgroups with higher HIV incidence. In addition, they have had no selection and education compared to repeat-donors who may self-defer from donating blood if their risk behaviour changed since their last negative donation. In contrast, first-time donors have not been educated on being a safe blood donor when they present for the first time and could donate blood for test seeking without the stigma of going to a testing clinic.

Like South Africa, many developed countries such as Canada, Australia, the USA and countries in Europe collect only a minority of their blood donations from first-time donors [1, 15–17]. For example, Canada collects 13% of their blood from first-time donors [15]. They calculated incidence using the I/WP model and repeat-donors and subsequently multiplied the proportion of first-time donors by 2 based on the hypothesis that incidence is double that in repeat-donors [16]. Australia collects 6% of their blood from first-time donors and includes only NAT yield first-time donors in their incidence and RR estimates [18].

Poorer countries, however, tend to have more first-time donors. In Tehran, 40% of the blood comes from first-time donors and these donations were excluded from their incidence calculations [19]. Zimbabwe similarly collects 44% of the blood from first-time donors [20]; however, they estimated RR in first-time donations by modifying the I/WP model; instead of using the inter-donation interval of a seroconverting repeat donor, they substituted the prevalence and duration of asymptomatic WHO stages 1 and 2 of 5 years [21]. Recently, a Brazilian study reported the prevalence, incidence and RR calculated using the I/WP model. They reported that 91% of donations came from first-time donors and were therefore excluded from the incidence calculations. It would be interesting to know whether the HIV positives in the first-time donors were long-standing infections (and therefore would have little impact on the RR) or recent infections (which would have an impact on the RR) [22].

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There are some limitations to this study. The LAg avidity assay is known to falsely classify as 'recent' approximately 60% of donors on anti-retroviral therapy (ART) and 13% of Elite controllers as recent infections [5, 7] due to a muted immune response especially if treatment is started early after acquisition [23, 24]. To mitigate this we excluded all donations that tested ID-NAT negative, anti-HIV positive – which is the test result pattern most commonly seen among HIV-positive donors taking surreptitious ART [25]. However, it is possible that some of our ID-NAT positive donors could be on ART with incomplete viral suppression and hence falsely classified as recent infections [25]. A second limitation is that we did not account for differences in inter-donation intervals of the seroconverting donors compared to the negative donors, non-random inter-donation intervals, and particularly longer inter-donation intervals in the HIV-positive donors in the immediate pre-seroconversion time-frame relative to their prior intervals which could bias the results of the I/WP model for repeat-donors and extrapolation of the results from that model to first-time donors using the NAT yield ratio [2]. Finally, although we performed LAg avidity testing on the HIV-concordant-positive donations from repeat-donors, an analysis to compare these data with the I/WP model results was beyond the scope of this analysis. Such an analysis would require adjustment to the contribution of person-time to the denominators for repeat-donors with inter-donation intervals that are shorter than the MDRI for LAg assay, which is common at SANBS.

Conclusion

The incidence model based upon LAg avidity testing of first-time donors provided similar incidence estimates when compared to the NAT yield WP model and substantially lower incidence compared to extrapolation of incidence in first-time donors from the classic method, supporting broader use of the LAg plus NAT yield-based approach in measuring HIV incidence. We believe testing HIV-positive donations from first-time donors using the LAg assay could provide blood establishments that do not perform NAT screening with a good tool to determine incidence in their first-time donor populations. By extrapolation, these findings also support the use of new incidence assays in cross-sectional HIV surveys of the general population.

Conflicts of interest

There are no conflicts of interest.

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Appendix 1

Definition of a repeat donor for model 2 and a repeating donor for model 3

A donation from a repeat donor in model 2 refers to all donations from non-first-time donors. For model 3, a repeating donor, irrespective of whether a donor donates for the first-time during the study period, or has donated before, as long as the donor donates again during the study period both within-study period donations will be classified as from a repeating donor. In contrast if a donor has donated once only during the study period irrespective of previously donating outside of the study period, the donation is not classified as from a repeating donor and is excluded.

Missing LAg data

The missing pattern was arbitrary based on comparison of demographics (data not shown), a fully conditional specification (FCS) multiple imputation logistic regression method was used to impute the missing LAg test results. The FCS method uses a multivariate imputation by chained equations method to impute values for a data set with an arbitrary missing pattern. The logistic model included the following covariates: outcome, age group, sex, race, fixed or mobile clinic, province and batch no. We used 5 multiple imputations, and the number of estimated recent HIV infections varied slightly between imputations y. Out of 123 donations missing Lag assay results, 23, 25, 21, 23 and 26 were imputed as recent HIV in the 5 multiple imputations, respectively.

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HIV incidence in South African blood donors from 2012 to 2016: a comparison of estimation methods

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Vox Sanguinis

Background Measuring incidence is important for monitoring and maintaining the safety of the blood supply. Blood collected from repeat-donors has provided the opportunity to follow blood donors over time and has been used to estimate the incidence of viral infections. These incidence estimates have been extrapolated to first-time donors using the ratio of NAT yield cases in first-time versus repeat-donors. We describe a model to estimate incidence in first-time donors using the limiting antigen (LAg) avidity assay and compare its results with those from established models.

Methods HIV-positive first-time donations were tested for recency using the LAg assay. Three models were compared; incidence estimated for (1) first-time donors using LAg avidity, (2) first-time and repeat-donors separately using the NAT yield window period (WP) model and (3) repeat-donors using the incidence/WP model.

Results HIV incidence in first-time donors was estimated at 3·32 (CI 3·11, 3·55) and 3·81 (CI 3·07, 4·73) per 1000 PY using the LAg assay and NAT yield WP models, respectively. Incidence in repeat-donors was between 2·0- and 2·5-fold lower than in first-time donors estimated at 1·56 (CI 1·37, 1·77) and 1·94 (CI 1·86–2·01) per 1000 PY using the NAT yield/WP and incidence/WP models, respectively.

Conclusion Testing HIV-positive donations using the LAg assay provides a reliable method to estimate incidence in first-time donors for countries that collect the majority of blood from first-time donors and do not screen with NAT.

Key words: transfusion - transmissible infections, residual risk estimation, blood safety, NAT testing.

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Introduction

Measuring the incidence of HIV is important for public health and maintaining the safety of the blood supply. Historically, incidence has been studied in expensive longitudinal cohort studies. Blood transfusion services that collect blood from mostly repeat-donors provide the opportunity to follow donors over time and have been used to estimate the incidence of viral infections in the blood supply [1]. Using the estimated incidence and an estimate of the mean duration of pre-detectable infectiousness (based on viral load growth during the ramp-up phase of infection and the HIV-1 minimum infectious dose), we can estimate the proportion of screen-negative blood donations which are potentially infectious, that is residual risk (RR) [2]. These repeat-donor-based incidence and consequent RR estimates have been extrapolated to

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first-time donors by multiplying the repeat-donor estimates with a factor such as the *first-time*-to-*repeat-donor* prevalence ratio or the *first-time*-to-*repeat-donor* NAT yield (HIV RNA positive, HIV antibody negative) ratio. The prevalence ratio is expected to overestimate the incidence and subsequent RR in first-time donors, especially in high endemic countries, as repeat-donors are prescreened for infection [3]. The NAT yield ratio can only be used in contexts that perform NAT testing in parallel with serology, and in most countries, the numbers of NAT yield cases are relatively small resulting in poor precision of the NAT yield ratio. Many blood services in developing countries collect blood mostly from first-time donors.

With the use of more sensitive NAT in parallel with serological assays a 'NAT yield window period (WP) model' can estimate first-time donor incidence and RR [4]. Many blood services that use NAT to screen blood use this NAT yield WP model to estimate RR. However, the small number of NAT yield donations, typically detected, limits the precision of risk estimates and the cost of NAT limits its application. Newer 'incidence assays' with lower sensitivity to HIV antibody allow the definition of a tunable (but longer) 'recent infection' WP compared to NAT. Unlike NAT testing on serological negative specimens, incidence assays need only be performed on the HIV antibody-positive donations and can yield a much larger sample of recent infections providing more precision for incidence estimations in first-time donors [5]. However, these more recent serologic approaches for incidence estimation have not been extensively validated in the blood bank setting.

As part of a five-year study, all HIV-seropositive donations were tested with an incidence assay to determine recent HIV infections [6–8]. We compared incidence estimates calculated from three approaches: (1) the cross-sectional LAg incidence assay method using first-time donors [7]; (2) the NAT yield WP model [4] in both repeat-donors and first-time donors, and (3) the incidence/WP (I/WP) classic method using repeat-donors [2]. In addition, we compared the incidence in first-time donors with that reported in the general population [9] and examined differences in incidence over the study period in different geographical regions and in donors of different ethnicities, gender and ages.

Methods

Setting

SANBS collects approximately 900 000 blood donations per annum in a high HIV endemic context and screens blood in parallel for NAT and anti-HIV leading to approximately 1600 confirmed HIV seropositive and 60 HIV NAT yield donations per year.

Laboratory testing algorithms

All blood donations were screened by the Prism (Abbott, Delkenheim, Germany) 3rd-generation anti-HIV, HBsAg and anti-HCV chemiluminescent immunoassays (ChLIA) in parallel with the Procleix Ultrio (Plus) NAT assay (Grifols, Barcelona, Spain) for HIV RNA, HCV RNA and HBV DNA. An extensive confirmatory and follow-up algorithm (previously described) [10] were in place for HIV, which classifies donations into four categories: (1) HIV negative, (2) HIV-concordant positive (HIV RNA by NAT and anti-HIV by ChIA), (3) HIV 'NAT yield' and (4) HIV 'serology yield' (RNA negative, anti-HIV ChIA repeat reactive and Western blot positive). The NAT yield cases are confirmed through seroconversion to anti-HIV positive at recall (approximately 70% return) or, for non-returning donors, replicate testing by Procleix Ultrio (Plus) discriminatory HIV NAT and quantitative PCR (Cobas Ampliprep/Cobas TaqMan HIV-1/2, Roche, Basel, Switzerland) or Real-time HIV-1 m2000rt (Abbott, Delkenheim, Germany), using stored fresh frozen plasma from the index donation.

HIV-concordant-positive donations were tested for recency of infection using the limiting antigen (LAg) avidity assay (Sedia Biosciences Corporation, Portland, Oregon). The single-well LAg avidity EIA is responsive to the avidity of HIV-1-specific IgG, as it quantifies antibody binding to a multi-subtype recombinant HIV-1 antigen coated onto assay plates at low density (hence the term 'limiting antigen', typically offering just a single binding site to multivalent IgG antibodies, and hence demonstrating a progressive increase in signal intensity over 3–6 months following seroconversion that can be used to infer duration of infection [11]. A normalized optical density (ODn) of <1·5 for the recent/long-standing threshold has been demonstrated to define a state of 're-cent infection' with a mean duration of approximately 180 days for Clade C HIV infections [12]. Five recency or incidence assays, including the LAg avidity assay, have been robustly evaluated and validated by the Consortium for the Evaluation and Performance of HIV Incidence Assays. A false recency rate (FRR) of 1·3% (95% CI 0·3–3·2) was estimated for the LAg assay [5].

Incidence models

Only allogeneic whole blood donations were included in the analysis. The definition of a repeat-donor is different for models 2 and 3 (see Appendix 1). Age categories were collapsed for reporting based on incidence into three groups: younger than 20 years, 20–30 years and older

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than 30 years. Confidence limits for incidence estimates were derived from Poisson regression.

Model 1: The LAg first-time donor model

First-time donors were categorized into three groups: HIV negative, recent HIV infection and long-standing HIV infection. Recent HIV infection was defined as (1) HIV-concordant-positive and LAg avidity recent (ODn < 1·5) or (2) NAT RNA positive, anti-HIV negative. There were 123 (2%) confirmed HIV-positive donors with missing LAg results and were imputed as recent or long-standing HIV using a fully conditional specification multiple imputation logistic regression method (see Appendix 1). Donors with long-standing infection (ODn ≥ 1·5) were classified as prevalent and excluded from the analysis. Incidence was calculated as incident cases/1000 person-years (PY) using only cases classified as recent infections.

The denominator was defined as the total time for at-risk first-time donors with each uninfected first-time donor contributing the full mean duration of recent infection (MDRI) estimate and each recently infected donor contributing half the MDRI estimate. The MDRI of 195 (96% CI: 168–222) days estimated by Grebe and colleagues [12] was used which includes the length of the period that anti-HIV is positive and LAg with an ODn < 1·5 (179 days) and the length of the period that NAT is positive and anti-HIV is negative (16 days) [12].

Model 2: NAT yield WP model

The NAT yield WP model previously described [4] classifies incident infections as NAT positive, anti-HIV negative. Incidence was calculated as NAT yield cases/1000 PY. Each uninfected donor contributed the full NAT yield detection period, and each NAT yield donor contributed half the NAT yield period to the denominator. The length of the NAT yield detection period of 15·4 days was used [13, 14]. As this model assesses new infections cross-sectionally based on a brief NAT yield detection period, we used it for estimation in first-time and repeat-donors. For this model, a repeat-donor is classified as a donor who donated previously within or outside of the study period.

Model 3: the classic incidence/WP model

This model originally described by Schreiber et al. [2] only estimates incidence in repeat-donors. Donors are classified as repeating donors if they contributed 2 or more donations during the study period, the term 'repeating' is used to differentiate these donors from 'repeat' donors in model 2. (see Appendix 1) A donor is classified as having an incident HIV infection if they have two or more donations during the study period of which the first donation tested HIV negative and any subsequent donation tested HIV positive. Incidence was calculated as cases/1000 PY. The denominator was defined as the total follow-up time for the at-risk repeating donors. For those who remain free of infection throughout the study period, time at risk is the time from first to last donation in the study period. For those found to be infected at the second or subsequent donations, time at risk is time from the first donation in the study period to halfway between the last donation at which the donor was uninfected and the donation at which infection was detected.

Incidence in the general population

We compared the incidence derived in first-time donors with the incidence reported in the general population from the UNAIDS data 2019 report [9].

Results

SANBS collected 4 019 985 whole blood donations from January 2012 until December 2016. Of these 3 504 481 (87%) were collected from 723 166 repeat-donors and 515 504 (13%) were collected from first-time donors (used in models 1 and 2). There were 3 661 669 (91%) donations classified as coming from repeating donors for model 3. Table 1 provides the number of donations used to calculate person-years (PY) for each model. Table 2 provides the HIV positives used as incident cases in each model by year, province, ethnicity, gender and age. HIV prevalence in all donations, in first-time donations and in repeat donations was 0·23%, 1·11% and 0·1%, respectively, giving a *first-time*-to-*repeat-donor* HIV prevalence ratio of 11:1. The NAT yield rate in all donations, first-time donations and repeat donations was 0·0078%, 0·016% and 0·0066%, respectively, giving a *first-time*-to-*repeat-donor* NAT yield ratio of 2·42:1.

Incidence

Model 1: Among the 515 504 donations by first-time donors, 5540 (1·07%) confirmed anti-HIV positive. Of these, 879 (16%) were classified as recent infections. The denominator consisted of PY contributed by 510 862 at-risk donors [excluding 4642 HIV-positive donors with long-standing infections]. The overall incidence using model 1 and the LAg assay to determine recency in first-time donors was 3·32 (CI 3·11, 3·55) per 1000 PY.

Table 3 shows incidence by year, province, gender, ethnicity and age using model 1. Incidence varied slightly by year, declining from 4·08 in 2013 to 2·90 per 1000 PY in 2016. The highest incidence was in the Mpumalanga and KwaZulu-Natal provinces, and the lowest incidence was in the North West and Northern Cape provinces (Fig. 1).

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Table 1 Donations for each model.

	Model 1	Model 2			Model 3
	LAg first-time donors*	All	First-time	Repeat	Repeating
Total	513 334				
Total used in denominator	508 796	4 019 985	515 504	3 504 481	3 661 669
Year					
2012	98 690	780 826	100 229	680 597	697 581
2013	108 055	796 638	109 755	686 883	728 479
2014	103 000	803 641	104 323	699 318	742 204
2015	102 033	828 449	103 291	725 158	767 212
2016	97 018	810 431	97 906	712 525	726 223
Province					
Gauteng	226 558	1 805 636	229 145	1 576 491	1 645 924
KwaZulu-Natal	103 638	686 877	105 339	581 538	612 057
Mpumalanga	46 014	395 308	46 962	348 346	361 922
Eastern Cape	47 967	373 306	48 543	324 763	342 237
Free State	26 895	269 849	27 249	242 600	250 974
North West	23 940	227 523	24 140	203 383	210 101
Limpopo	20 842	138 395	21 074	117 321	124 689
Northern Cape	12 936	123 090	13 052	110 038	113 748
Ethnicity					
White	157 523	2 296 050	158 340	2 137 710	2 160 998
Black	272 117	1 156 029	277 350	878 679	991 200
Asian	36 174	301 480	36 525	264 955	272 142
Coloured	27 927	215 940	28 120	187 820	197 415
Unknown	15 055	50 486	15 169	35 317	39 944
Gender					
Female	283 185	1 741 727	287 345	1 454 382	1 544 856
Male	225 603	2 278 249	228 151	2 050 098	2 116 843
Age					
16–19	235 252	720 898	238 229	482 669	605 952
20–30	142 808	991 680	144 647	847 033	872 909
>30	130 736	2 309 771	132 450	2 177 321	2 180 826

*The first-time donations classified as long-standing are subtracted from the total. Long-standing donations are excluded from the denominator

Model 2: As the NAT yield method can be used to estimate incidence in all donations, first-time donations and repeat donations, we used it as a bridge to allow comparison to both models 1 and 3. In addition, we used this model to compare incidence in first-time and repeat-donors. Incidence, based on 312 NAT yields in all donations, was estimated at 1·84 (CI 1·65, 2·06) per 1000 PY. Using the 82 and 230 NAT yields from first-time and repeat-donors, incidence was estimated to be 2·44-fold higher in first-time donors at 3·81 (CI 3·07, 4·73) compared to 1·56 (CI 1·37, 1·77) per 1000 PY in repeat-donors (Table 3).

Model 3: The classic model to determine incidence included only donations from donors with at least two donations during the study period.

Among 3 661 699 whole blood donations by 602 329 repeating donors, 2425 tested confirmed HIV positive. Number of donations varied between 2 and 33 per donor. The median follow-up times, the amount of time during the study periods (used in the denominator) for HIV-positive and HIV-negative donors were 383·5 and 636 days, respectively. Incidence estimated using repeating donors was 1·94 (CI 1·86–2·01) per 1000 PY (Table 3).

Comparisons between Models

When comparing the models to determine incidence in first-time donors, model 2 yielded an estimate that was 1·15-fold higher than model 1 but this difference was not statistically significant. When we extrapolated the incidence derived from repeat-donors using model 3 to first-time donors using the NAT yield ratio of 1:2·42, the estimated incidence in first-time donors was 4·69 (CI 4·51,

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Table 2 classification of HIV positives for each model

	Model 1		Model 2									Model 3
	LAg recent		RNA+/Ab+			RNA-/Ab+/WB+			RNA+/Ab–			
	First-time	Missing	All	First-time	Repeat	All	First-time	Repeat	All	First-time	Repeat	New infections in Repeating donors
Long-standing	4538											
recent	879											
Total	879	123	8607	5462	3145	234	202	32	312	82	230	2425
Year												
2012	176	29	1548	1076	472	18	15	3	60	17	43	178
2013	228	33	1858	1239	619	36	34	2	82	26	56	448
2014	162	34	1795	1115	680	39	35	4	57	15	42	545
2015	165	7	1811	1082	729	63	54	9	54	14	40	644
2016	148	20	1595	950	645	78	64	14	59	10	49	610
Province												
Gauteng	312	22	3310	2154	1156	93	82	11	110	28	82	886
KwaZulu-Natal	235	44	1943	1165	778	57	50	7	84	32	52	573
Mpumalanga	142	23	1361	948	413	35	30	5	46	16	30	326
Eastern Cape	70	26	798	459	339	15	13	2	29	2	27	276
Free State	61	2	517	302	215	12	9	3	22	2	20	169
North West	18	5	211	141	70	6	5	1	8		8	58
Limpopo	29	1	311	203	108	12	10	2	8	1	7	93
Northern Cape	12	0	156	90	66	4	3	1	5	1	4	43
Ethnicity												
White	16	3	308	85	223	7	5	2	13	1	12	139
Black	831	114	7790	5126	2664	217	187	30	281	77	204	2113
Asian	2	1	85	31	54	3	3	0	5	1	4	35
Coloured	20	2	295	125	170	2	2	0	10	1	9	116
Unknown	10	3	129	95	34	5	5	0	3	2	1	22
Gender												
Female	651	83	5732	3763	1969	187	162	25	213	66	147	1563
Male	228	40	2875	1699	1176	47	40	7	99	16	83	862
Age												
16–19	268	34	1840	1371	469	28	26	2	59	22	37	426
20–30	418	42	3855	2263	1592	62	46	16	162	45	117	1211
>30	193	47	2913	1828	1085	144	130	14	91	15	76	788

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Table 3 Incidence determined by three models

	Incidence (Lag FT)/ 1000 person-years Model 1	Incidence (NAT yields)/1000 person-years Model 2			Incidence (Repeat, classic)/ 1000 person-years Model 3
		First-time	All	Repeat	
Overall			1·84 (1·65, 2·06)		
Donor type					
First-time	3·32 (3·11, 3·55)	3·81 (3·07, 4·73)			
Repeat				1·56 (1·37, 1·77)	1·94 (1·86–2·01)
Year					
2012	3·48 (3·01, 4·02)	4·06 (2·53, 6·54)	1·82 (1·42, 2·35)	1·50 (1·11, 2·02)	1·74 (1·50–2·01)
2013	4·08 (3·59, 4·64)	5·68 (3·87, 8·34)	2·45 (1·97, 3·04)	1·93 (1·49, 2·51)	1·79 (1·55–2·07)
2014	3·05 (2·62, 3·55)	3·45 (2·08, 5·72)	1·68 (1·30, 2·18)	1·42 (1·05, 1·93)	1·75 (1·52–2·02)
2015	3·05 (2·62, 3·55)	3·25 (1·92, 5·48)	1·55 (1·19, 2·02)	1·31 (0·96, 1·78)	2·00 (1·75–2·29)
2016	2·90 (2·47, 3·41)	2·45 (1·32, 4·55)	1·73 (1·34, 2·23)	1·63 (1·23, 2·16)	1·63 (1·40–1·89)
Gender					
Female	4·43 (4·11, 4·78)	5·52 (4·34, 7·03)	2·91 (2·54, 3·33)	2·40 (2·04, 2·82)	2·68 (2·55–2·82)
Male	1·94 (1·70, 2·20)	1·68 (1·03, 2·73)	0·86 (0·70, 1·07)	0·96 (0·77, 1·19)	1·29 (1·20–1·37)
Age					
16–19	2·21 (1·96, 2·48)	2·20 (1·45, 3·35)	1·95 (1·51, 2·51)	1·82 (1·32, 2·51)	2·27 (2·07–2·50)
20–30	5·58 (5·08, 6·14)	7·49 (5·59, 10·04)	3·89 (3·33, 4·53)	3·27 (2·73, 3·92)	3·57 (3·37–3·79)
>30	2·87 (2·49, 3·30)	2·72 (1·64, 4·52)	0·93 (0·76, 1·15)	0·83 (0·66, 1·04)	1·07 (0·99, 1·15)
Female					
16–19	3·51 (3·09, 3·99)	3·50 (2·23, 5·49)	3·29 (2·48, 4·36)	3·16 (2·20, 4·55)	3·51 (3·15–3·91)
20–30	6·94 (6·20, 7·77)	10·18 (7·31, 14·18)	5·68 (4·71, 6·84)	4·69 (3·74, 5·88)	4·61 (4·28–4·97)
>30	3·23 (2·71, 3·85)	3·88 (2·21, 6·84)	1·40 (1·07, 1·82)	1·19 (0·88, 1·60)	1·40 (1·28–1·55)
Male					
16–19	0·65 (0·47, 0·90)	0·66 (0·21, 2·04)	0·70 (0·39, 1·26)	0·72 (0·36, 1·43)	1·05 (0·86–1·28)
20–30	3·77 (3·15, 4·50)	3·89 (2·10, 7·24)	2·33 (1·78, 3·06)	2·13 (1·57, 2·88)	2·58 (2·34–2·85)
>30	2·40 (1·90, 3·02)	1·24 (0·40, 3·85)	0·62 (0·45, 0·86)	0·59 (0·42, 0·83)	0·80 (0·72–0·90)
Race					
White	0·19 (0·12, 0·32)	0·15 (0·02, 1·06)	0·13 (0·08, 0·23)	0·13 (0·08, 0·23)	0·20 (0·17–0·23)
Black	5·88 (5·50, 6·29)	6·71 (5·37, 8·39)	5·80 (5·16, 6·52)	5·52 (4·81, 6·33)	5·81 (5·57–6·06)
Asian	0·12 (0·03, 0·48)	0·65 (0·09, 4·61)	0·39 (0·16, 0·94)	0·36 (0·13, 0·95)	0·33 (0·24–0·46)
Coloured	1·38 (0·89, 2·13)	0·85 (0·12, 6·01)	1·10 (0·59, 2·04)	1·14 (0·59, 2·18)	1·67 (1·39–2·00)
Unknown	1·27 (0·68, 2·36)	3·15 (0·79, 12·58)	1·41 (0·46, 4·38)	0·67 (0·09, 4·77)	1·47 (0·96–2·23)
Province					
Gauteng	2·61 (2·34, 2·92)	2·92 (2·02, 4·24)	1·45 (1·20, 1·74)	1·23 (0·99, 1·53)	1·55 (1·45–1·66)
KwaZulu-Natal	4·42 (3·90, 5·02)	7·28 (5·15, 10·30)	2·91 (2·35, 3·60)	2·12 (1·62, 2·78)	2·47 (2·27–2·68)
Mpumalanga	5·92 (5·02, 6·97)	8·25 (5·05, 13·46)	2·77 (2·07, 3·70)	2·04 (1·43, 2·92)	2·81 (2·52–3·14)
Eastern Cape	2·98 (2·36, 3·75)	0·99 (0·25, 3·94)	1·85 (1·28, 2·66)	1·97 (1·26, 3·03)	2·34 (2·08–2·64)
Free State	4·29 (3·34, 5·51)	1·76 (0·44, 7·04)	1·94 (1·27, 2·94)	1·95 (1·26, 3·03)	2·00 (1·71–2·34)
North West	1·42 (0·90, 2·26)	0	0·83 (0·42, 1·67)	0·93 (0·47, 1·86)	0·84 (0·64–1·11)
Limpopo	2·61 (1·81, 3·75)	1·14 (0·16, 8·07)	1·37 (0·69, 2·75)	1·42 (0·67, 2·97)	2·28 (1·86–2·81)
Northern Cape	1·74 (0·99, 3·06)	1·83 (0·26, 12·98)	0·96 (0·40, 2·32)	0·86 (0·32, 2·30)	1·12 (0·83–1·52)

4·89) which was significantly higher (1·41-fold) than in model 1 but not statistically different from the results from Model 2.

Model 2 and Model 3 were used to estimate incidence in repeat-donors. Model 3 estimated incidence as 1·24-fold and significantly higher than in model 2. To investigate the reason for the higher estimates in model 3, we assessed the sensitivity of the incidence estimate on the inclusion of donors who donated twice only compared to donors who donated three or more times and found a 2-fold higher incidence in the former group. Thus, model 3 repeating donors included some donors, who were observed from their first to the second donation, when incidence is higher, whereas model 2 repeat donors only

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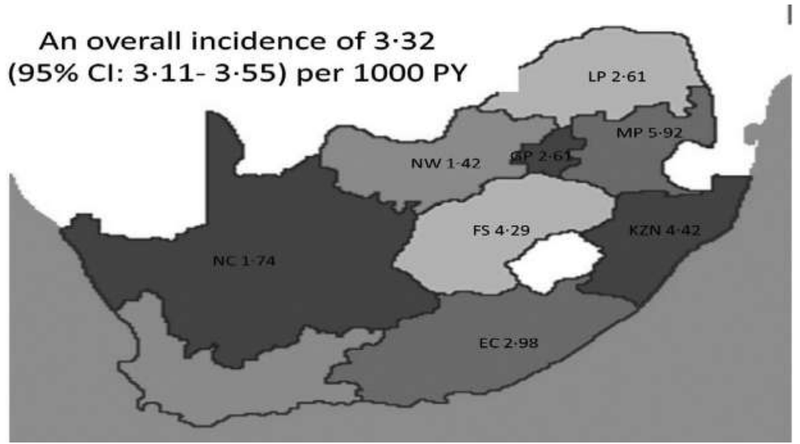

Fig. 1 Incidence in first-time donors determined using model 1.

included second and subsequent donations, when the incidence is lower.

Being female, aged 20–30 years old, of black ethnicity and living in KwaZulu-Natal and Mpumalanga were associated with a higher incidence for all models.

Comparison to HIV incidence in the general population

Incidence was approximately 3·5 times higher in the general population compared to first-time donors (Fig. 2). In the general population, incidence significantly decreased from 13·30 (CI 12·6, 13·9) in 2012 to 10·6 (CI 9·89, 11·3) per 1000 PY. A similar but not significant decrease, albeit lower, was seen in the first-time blood donors which declined from 3·48 (CI 3·01, 4·02) in 2012 to 2·90 (CI 2·47, 3·41) in 2016 (Table 3).

Discussion

The incidence of a viral infection in blood donors is used to estimate the RR of a transfusion-transmitted infection occurring as it establishes the number of new infections which may go undetected by the blood services screening strategy. South Africa has the largest burden of HIV in the world and although the prescreening donor interview and testing of first-time donors remove the majority of the prevalent infections, a substantial number of incident HIV infections are detected in our donor population, and hence, we project an elevated RR of transfusion-transmitted HIV infection relative to most other countries even with application of ID-NAT [3]. Accurate measurement of HIV incidence and consequent RR is essential to monitor risk reduction strategies. In this study, we were able to test HIV-positive samples from first-time donors to

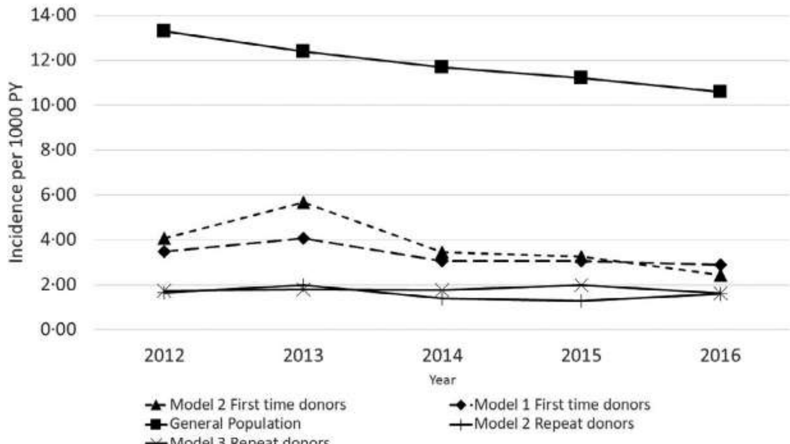

Fig. 2 Incidence in first-time donors using models 1 and 2 and incidence in repeat-donors using models 2 and 3 compared to the incidence in the general population [9].

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determine recent infection status using the LAg avidity assay and use the incident infections to compare the incidence derived by LAg with that derived by parallel testing using ID-NAT and the NAT yield WP model [4]. In addition, we compared incidence in repeat-donors derived by the I/WP model [2] with the NAT yield WP model [4]. Our high-level findings demonstrate that incidence rates determined by several methods were broadly consistent which is reassuring, although small discrepancies should spur re-examination of accuracy and implications of differences between the models.

Among first-time donors, we found similar overall HIV incidence using the LAg avidity and NAT yield models, adding confidence that these two cross-sectional incidence approaches are comparable and that the LAg avidity approach has promise in that it yields significantly larger numbers of recent infection cases and hence more precise incidence estimates than the NAT yield method. However, in repeat-donors we estimated a significantly higher incidence using the classic model compared to the NAT yield model. Furthermore, we found an extrapolated incidence from repeat-donors to first-time donors using the classic model that was one and a half-fold higher than the LAg avidity and NAT yield models, suggesting that previous corrections using NAT yield ratios for derivation of first-time incidence may be overestimated.

We speculate additional reasons for the higher incidence derived by the classic method: (1) in our study the median follow-up time (the time during the study period that contributes to the denominator) of HIV-positive repeating donors was nearly half the length of HIV-negative donors, as negative donors were able to give multiple donations that were counted for the entire study period, compared to HIV-positive donors who would have had truncated follow-up following detection of infection and deferral; (2) the inter-donation intervals for HIV-positive and HIV-negative donors were not random (data not shown), in part due to the preset periodic times of mobile clinics where 60% of the blood is collected; and (3) the classic method using repeating donors, included some first-time donors who donated a second donation during the study period. These newer donors may be at higher risk of having an incident infection compared to established repeat-donors. When we estimated incidence in donors who donated their second donation in the study and compared this to the incidence if they donated their 3rd or later donation, the former had a twofold higher incidence giving credibility to this assumption (data not shown).

The findings using the classic method may have relevance to the issue of self-deferral during the earlier stages of infection by repeat-donors, which consequently results in overestimation of RR. This is because a repeating donor may appropriately self-defer from donating blood for the mandatory 3 months following high-risk behaviour; therefore, although classified as an incident HIV infection using the classic model, such donors would not be contributing donations relevant to the RR as they self-deferred during the window period and would subsequently be detected using the current laboratory screening strategies. The lower incidence in repeat-donors based on the NAT yield WP approach is likely more accurate since detection of a NAT yield case is a direct measurement of a donor presenting to donate during the RNA-positive pre-seroconversion stage of infection that immediately follows the undetectable pre-NAT infectious WP.

Incidence in first-time donors was more than twice that of repeat-donors. This difference may be due to confounding or selection bias. First-time donors are enriched with young, female and black individuals, subgroups with higher HIV incidence. In addition, they have had no selection and education compared to repeat-donors who may self-defer from donating blood if their risk behaviour changed since their last negative donation. In contrast, first-time donors have not been educated on being a safe blood donor when they present for the first time and could donate blood for test seeking without the stigma of going to a testing clinic.

Like South Africa, many developed countries such as Canada, Australia, the USA and countries in Europe collect only a minority of their blood donations from first-time donors [1, 15–17]. For example, Canada collects 13% of their blood from first-time donors [15]. They calculated incidence using the I/WP model and repeat-donors and subsequently multiplied the proportion of first-time donors by 2 based on the hypothesis that incidence is double that in repeat-donors [16]. Australia collects 6% of their blood from first-time donors and includes only NAT yield first-time donors in their incidence and RR estimates [18].

Poorer countries, however, tend to have more first-time donors. In Tehran, 40% of the blood comes from first-time donors and these donations were excluded from their incidence calculations [19]. Zimbabwe similarly collects 44% of the blood from first-time donors [20]; however, they estimated RR in first-time donations by modifying the I/WP model; instead of using the inter-donation interval of a seroconverting repeat donor, they substituted the prevalence and duration of asymptomatic WHO stages 1 and 2 of 5 years [21]. Recently, a Brazilian study reported the prevalence, incidence and RR calculated using the I/WP model. They reported that 91% of donations came from first-time donors and were therefore excluded from the incidence calculations. It would be interesting to know whether the HIV positives in the first-time donors were long-standing infections (and therefore would have little impact on the RR) or recent infections (which would have an impact on the RR) [22].

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There are some limitations to this study. The LAg avidity assay is known to falsely classify as 'recent' approximately 60% of donors on anti-retroviral therapy (ART) and 13% of Elite controllers as recent infections [5, 7] due to a muted immune response especially if treatment is started early after acquisition [23, 24]. To mitigate this we excluded all donations that tested ID-NAT negative, anti-HIV positive – which is the test result pattern most commonly seen among HIV-positive donors taking surreptitious ART [25]. However, it is possible that some of our ID-NAT positive donors could be on ART with incomplete viral suppression and hence falsely classified as recent infections [25]. A second limitation is that we did not account for differences in inter-donation intervals of the seroconverting donors compared to the negative donors, non-random inter-donation intervals, and particularly longer inter-donation intervals in the HIV-positive donors in the immediate pre-seroconversion time-frame relative to their prior intervals which could bias the results of the I/WP model for repeat-donors and extrapolation of the results from that model to first-time donors using the NAT yield ratio [2]. Finally, although we performed LAg avidity testing on the HIV-concordant-positive donations from repeat-donors, an analysis to compare these data with the I/WP model results was beyond the scope of this analysis. Such an analysis would require adjustment to the contribution of person-time to the denominators for repeat-donors with inter-donation intervals that are shorter than the MDRI for LAg assay, which is common at SANBS.

Conclusion

The incidence model based upon LAg avidity testing of first-time donors provided similar incidence estimates when compared to the NAT yield WP model and substantially lower incidence compared to extrapolation of incidence in first-time donors from the classic method, supporting broader use of the LAg plus NAT yield-based approach in measuring HIV incidence. We believe testing HIV-positive donations from first-time donors using the LAg assay could provide blood establishments that do not perform NAT screening with a good tool to determine incidence in their first-time donor populations. By extrapolation, these findings also support the use of new incidence assays in cross-sectional HIV surveys of the general population.

Conflicts of interest

There are no conflicts of interest.

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Appendix 1

Definition of a repeat donor for model 2 and a repeating donor for model 3

A donation from a repeat donor in model 2 refers to all donations from non-first-time donors. For model 3, a repeating donor, irrespective of whether a donor donates for the first-time during the study period, or has donated before, as long as the donor donates again during the study period both within-study period donations will be classified as from a repeating donor. In contrast if a donor has donated once only during the study period irrespective of previously donating outside of the study period, the donation is not classified as from a repeating donor and is excluded.

Missing LAg data

The missing pattern was arbitrary based on comparison of demographics (data not shown), a fully conditional specification (FCS) multiple imputation logistic regression method was used to impute the missing LAg test results. The FCS method uses a multivariate imputation by chained equations method to impute values for a data set with an arbitrary missing pattern. The logistic model included the following covariates: outcome, age group, sex, race, fixed or mobile clinic, province and batch no. We used 5 multiple imputations, and the number of estimated recent HIV infections varied slightly between imputations y. Out of 123 donations missing Lag assay results, 23, 25, 21, 23 and 26 were imputed as recent HIV in the 5 multiple imputations, respectively.

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Perceptions on consumer information in transfusion. A qualitative study of consumers and prescribers

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Vox Sanguinis

Background and objectives Fresh blood product transfusion requires patient education for fully informed consent, and written consumer information is frequently used. Few studies have examined consumer preferences regarding written and verbal transfusion information provided. As a qualitative study, this research was designed to explore participant understanding and by analysing and integrating themes, generate a model to understand how transfusion information should be developed and used in practice.

Materials and methods Semi-structured interviews were conducted with healthcare consumers of transfusion information from various hospital clinical departments. Transcripts were coded to qualitatively compare nature/extent of content and opinions regarding transfusion information through thematic analysis.

Results Analysis identified themes relating to healthcare engagement, purpose of information, mode of delivery and content delivered. Differences were identified between perceived purpose of information provided to consumers between 13 transfusion prescribers and consumers. Prescribers viewed information as a tool for obtaining informed consent, whereas consumers desired reassurance and knowledge. Consumers described both the specialized nature and volume of information as limiting their ability to question professionals on whom they were dependent. Information provided should be tailored to consumers and utilize simple, succinct explanations.

Conclusion Both groups were satisfied with written information adjunctive to verbal information. These findings will be used to redesign transfusion information and may be employed at the bedside when discussing transfusion. They may have implications for consumer information in other settings.

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Key words: consent, consumer information, patient engagement, transfusion.

Introduction

Australian law and national healthcare standards mandate that patients are engaged in decisions regarding their care [1-3]. Except in emergencies, medical care requires informed consent. This is consistent with the ethical principle of respect for patient autonomy. Consumer information is frequently developed by organizations to communicate with and educate patients about therapies. It is often used in the informed consent process. In transfusion, information may include the nature of the blood products, the potential risks and benefits of transfusion, indications and alternative management approaches and advice on the transfusion process, but guidelines do not

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specifically state what is required within consumer information [4,5].

Information should ideally be developed with consumer involvement and conveyed in a manner that is meaningful to the intended recipient. In practice, information is often developed by healthcare professionals who hold the requisite content knowledge within the appropriate field. While this may ensure reliability of the medical content, healthcare professionals may have different perceptions on the optimal scope and purpose of information tools compared with consumers.

There have been various approaches to assessing quality of consumer information. The ability to recall has been used to measure the adequacy of information and the informed consent process more broadly, but it should be noted that subsequent recall is not a prerequisite to informed decision-making [6,7]. Process efficiency has also been assessed [8]. Readability and comprehension have also been used and are substantially better approaches if the purpose is to provide the information so that the recipient **can** integrate it into their decision-making [9]. Assessing the quality of the information should focus on how well it achieves its intent. This requires explicit identification of the purpose for providing information, and while this is often asserted, there is limited evidence on what recipients want from information in transfusion.

We sought to examine the role and composition of transfusion information from both the viewpoint of consumers and prescribers. To allow the emergence of new concepts and minimize framing the research within a particular ethical paradigm, a qualitative approach was adopted.

Materials and methods

Study setting and population

The research was conducted in a major public hospital servicing metropolitan and surrounding regional areas in Canberra, Australia. Participants were recruited opportunistically from various hospital departments selected purposefully based upon known department transfusion frequency, and access to prescribers and consumers. Sample size was not predefined but determined by the point at which no new unique themes emerged in the consumer and prescriber cohorts. Interviews were conducted on campus at the consumers' usual point of care.

Inclusion criteria selected consumers or their carers (where there was a legally recognized substitute decision-maker) who had been prescribed or considered at risk for fresh blood product transfusion. 'At risk' was defined as any one of the following:

- Haemoglobin < 80 g/l
- Platelet count < 20 x 10^9/l
- Having had a cross-match or group and screen requested within the last 14 days (other than for routine checking of blood group and red cell antibodies in pregnancy)
- Having a procedure where a cross-match or group and screen is indicated according to the hospital maximum surgical blood order schedule
- Undergoing chemotherapy where there is a high risk of transfusion requirement

Prescribers were chosen from the same departments as consumers. Recruitment purposefully included a nurse practitioner and junior medical officers who most frequently educate and seek consent for transfusion (although they may not be the primary decision-makers) and more experienced medical staff [10].

Study procedures

Approval for the study was obtained from the Australian Capital Territory Health Human Research Ethics Committee. After obtaining informed consent, semi-structured interviews with participants were conducted. Similar exploratory questions were used for prescribers and consumers, modified for their role delineation. While the questions formed the basis of the interviews, interviewers explored content and themes with participants as they arose.

The interviews were conducted in two phases. The first was an exploration of the expectations regarding transfusion information. The second presented the current hospital transfusion information to the participant, asking them to read it. The current information material had been in use for more than five years. It had been developed by the hospital's transfusion and blood products governance committee and included consultation with consumer focus groups. Participants were asked to place traffic light coloured stickers on areas of the information sheet they found notable, in a modification of a technique used by Hadden [11]. Green stickers represented areas they thought were useful to provide positive feedback; yellow for areas where they may have been some difficulty in interpretation, wording or content; and red for areas they felt required major changes, should be removed or had major omissions. The semi-structured interview question templates for consumers and prescribers are shown in Appendix A and Appendix B, respectively.

Demographic data including age, gender, highest level of education, transfusion history, diagnosis and occupation were elicited for consumers. Current work unit, role

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within the hospital and frequency of prescribing were obtained from prescribers.

Interviews were conducted by one of three authors. AL, external to the hospital, conducted all interviews with prescribers as PC, and MB had recognized transfusion governance roles to remove the potential for this to influence interviewee responses. Audio files of interviews were recorded and transcribed using a secure online automated transcription program (my.sonix.ai) and verified or corrected by the interviewers. The content was analysed by the authors for content and themes arising.

Data management and thematic analysis

Confidential participant information was secured per the NHMRC Statement of the Ethical Conduct of Human Research, relevant privacy legislation and hospital policies. After familiarization with the data, transcripts were coded inductively by a team member for ideas and themes. Thematic analysis and code development were conducted independently by two authors (AL, PC). The interview transcripts were segmented and annotated to identify key themes. This preliminary list of categories was refined and select exhibitory quotes noted. Themes identified were discussed by the team to minimalize possible interpretation bias attributable to coders' worldview.

Results

Of the 13 consumers interviewed, all were patients who had either received (9) or were at risk of receiving transfusion, recruited from haematology, orthopaedics, general surgery and obstetric departments. The consumers had a predominance of females (9) and median age of 64 years. They had diverse occupational backgrounds including homemakers, healthcare professionals and white- and blue-collar workers. Level of education attained included completing tertiary (5), secondary (5) and primary (2) studies. There were 11 prescribers recruited from the same departments. Prescribers included interns (postgraduate year 1, 4), postgraduate year 2, 3 residents (3), who rotate through various hospital departments, specialist registrars (2), a nurse practitioner and a haematology consultant.

Content and themes were identified relating to healthcare engagement, the purpose of transfusion information, the mode of delivery of information and the content. These were structured into a hierarchy, with higher order themes impacting on lower order themes and discrepancies in the role and function of information identified between the prescribers and consumers.

Healthcare engagement

This theme reflects how individuals perceived consumers need or capacity to be actively involved in healthcare decision-making in general, and transfusion decisions in particular. It formed an overarching distinction between consumers' and prescribers' use and interpretation of transfusion information.

Consumers very frequently reported being overwhelmed by their circumstances and that this impacted upon their ability to make decisions. This sentiment was epitomized by a consumer being treated for acute promyelocytic leukaemia, 'At the time I was a sick fella and I was just happy to put my faith in the doctors and the nurses and the people around me'. The volume of information presented to consumers in hospital was also an issue with consumers describing feeling 'bombarded' and 'debarred from getting on with the rest of my life', should it all be read. Likewise, the complexity of information was an issue, with consumers implicitly and explicitly stating reliance on the expertise of their treating doctors. An engineer cited his inability to comprehend medical details with the same level of understanding he applied to his own work, reflecting this back as an obligation to prescribers, 'when experts such as yourself are talking to people... you've then got to look at the ability... of that person to process it and understand it'. Other consumers without identifying the same sense of being overwhelmed simply accepted that the transfusion decision was best made by experts.

Prescribers' comments surrounding healthcare engagement were guided by the principle of patient autonomy. All perceived consumers as at least actively involved in decision-making, and in most cases, consumers were seen as primary decision-makers (n = 7). One registrar described the decision for transfusion as: 'purely by choice and will not take place without your prior consent'. Except in emergencies, prescribers largely saw themselves as facilitating consumers' choices. A few prescribers (n = 4) expressed concern for the consumers' ability to filter a large amount of information, which, if generalized in a standardized form, may not all be pertinent to the individual. In their own hospital, they felt that transfusions were usually only prescribed when necessary.

Purpose of transfusion information

Despite their expectation that transfusion decisions were to be made by prescribers, consumers still expressed a desire to understand transfusion. While some of this related to practicalities and what to expect, there was also curiosity about the origin of the product. Most, however,

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sought information to reassure. Generally, this was implicitly stated. For some, the purpose of information was overtly to 'do the job and to reassure me'. They were keen that risks be addressed, and although 'all' risks were frequently cited as important, there was a clear preference to acknowledge and demonstrate that notorious risks, particularly viral infections, were addressed. When prompted about new, more likely risks (such as pulmonary infiltrates), these were considered unnecessary by consumers.

For many prescribers, the main function of information was to facilitate the informed consent process. One prescriber suggested that the information sheet provided should be signed by the consumers as evidence of informed consent. The ability of consumers to be able to question their care was considered important. Some prescribers however acknowledged that information could increase fear, particularly if there is an emphasis on the potential risks.

Mode of delivery

A majority of consumers (n = 11) indicated a preference for verbal and written information. For consent, verbal information was universally preferred, consistent with this occurring within a relationship based on trust. As a rural contractor observed, 'you looked a man in the eye, or a woman, and shook their hand. That was your bond and that's why I still - I'd rather talk to someone than look at a bit of paper'. A substantial majority of consumers (n = 9) desired written information, but not as an aid to the consent process. It was there as a reference, to have the option to return to later, or as a tool for family members. While a paper-based brochure was preferred, one information technology professional anticipated this would be lost and suggested it be hosted on the hospital website. Most consumers were pleased that there were links to more detailed information online, but despite a number of consumers citing the need for information to include all risks, none said they would personally utilize the links provided.

Prescribers also mostly valued verbal provision of information and saw this in the context of obtaining consent. Written information was valued as an aid to consent with one medical officer suggesting the information sheet be modified so the consumers can sign it to acknowledge consent for transfusion. Accessing written materials for a diversity of conditions was seen as a frequent barrier to its use, and some medical officers (n = 2) were unaware that the hospital had information for consumers.

Consumers and prescribers were in agreement that the information, both verbal and written, should be simple, use common terminology and avoid jargon. A series of dot points were suggested by some, and one consumer favoured diagrams, acknowledging that this is his preferred method of conveying and receiving information and that this may not suit all (his occupation frequently involved describing processes).

Content of information

Both prescribers and consumers had the risks of transfusion featuring prominently in their preferred content. The types of risks to be covered varied: some requested all risks but did not find the current information inadequate; others felt that the risks should be moderated to include only the most significant. A retired law clerk observed that 'there are too many options that could go wrong', and not only would an extensive list not be adequately processed, but that it would also 'make people a bit iffy'. Many consumers picked up on the latter issue. Providing a list of risks did increase their level of anxiety. Numerous consumers opposed mentioning systemic issues and felt that we should not discuss the potential for process failures as a cause for adverse events, 'It's like the nurse's not going to do their job and match - you know the blood with you. I just, I just. That's the way it makes me feel'. The only value observed in mentioning these risks was to explain the reasons for repeated and detailed identification processes.

Consumers wanted to know the benefits of transfusion; 'that it will work'. Where consumers had been transfused, however, their understanding of the role of transfusion was often (in the authors' view) misguided. For example, one consumer with a large haematoma after a fall felt that it substituted for surgery and another consumer with acute lymphoblastic leukaemia, acknowledging that their understanding may be flawed, believed the red cell transfusion was necessary to 'bolster the good bits' in the bone marrow after induction.

Consumers sought to understand the difference between fresh products, blood collection and processing. All approved of background information on blood products and general indications, because it was 'just general knowledge'. Information had value for its own sake, rather than to meet some other purpose in the transfusion process. While there was some dissent, mostly consumers were not interested in transfusion alternatives being mentioned on the transfusion information sheet. If prescribed, consumers found no value in questioning this or discussing alternatives, 'if you're here and you're having a transfusion you obviously can't avoid it', or as a defence consultant observed, 'I mean we don't just start giving blood to people just for fun do we?' Possibility of inappropriate or unnecessary prescription was not considered. One consumer was very opposed to the concept of

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questioning the prescriber, equating it with parents who refuse to vaccinate their children: 'That's bullshit, you know. You're putting their kids and that in harm's way by not getting the injections and that type of thing'.

Prescribers likewise had an emphasis on risks. This was framed in the context of getting informed consent. Most prescribers (n = 9) acknowledged the need to pre-empt consumers' concerns and provide personalized information, however, time was a major barrier to this. The majority (n = 10) mentioned providing information catered to consumers' previous exposure, assuming consumers retained information previously provided. However, whereas other prescribers described providing 'briefer' information to consumers with previous transfusion history, a senior prescriber stated they provided additional, more specific information for this circumstance: 'there are other information I have to provide such as iron overload and chronic transfusion complications'. Some also mentioned tailoring risks to the clinical history, such as circulatory overload when there is a history of heart disease. In contrast, others attempt to provide 'standard' information to all consumers particularly regarding risks, for implied ethico-legal reasons.

Prescribers less frequently mentioned expected benefits of transfusion. One senior prescriber felt indications had no place in transfusion information as they vary between consumers and it would only serve to confuse consumers. Some prescribers framed this differently, pointing to the consequences they expected in the absence of transfusion.

Prescribers did not appear to value information for itself. Some did recognize the value of information to reassure consumers and its potential to create or increase anxiety. A minority mentioned the importance of consumer information in 'empowering them to aid in their own health journey'.

Discussion

While it is generally accepted that transfusion information be provided to consumers, the purpose and value to consumers is seldom enunciated and more rarely from the viewpoint of the recipients. This study explored the perceived role, function and content of transfusion information and found major differences between prescribers and consumers. Prescribers' views, to a large extent, reflected the ethical paradigm of patient autonomy and saw consumers as active participants in decision-making. By contrast, consumers felt overwhelmed, both by their health concerns, of which the potential need for transfusion was often seen as a minor aspect, and volume of information encountered. This translated into a reliance on the beneficence of healthcare systems and professionals, in which they placed their trust. These different perceptions form a framework upon which health information should be developed and utilized.

The dependency on prescribers to make decisions in transfusions was reported in a previous qualitative study [12]. In this study, as in ours, consumers did not question the transfusion decision, with the findings attributed both to medical paternalism and consumers being overwhelmed. Physicians were not interviewed. Our prescribers largely perceived their role as an educator and facilitator in the consumers' decision-making process, seldom recognizing the limitations that consumers felt within a complex healthcare environment. Consumers choose a more dependent posture than their medical attendants acknowledged, fostered by an understanding of their limitations in terms of knowledge, comprehension and stressful circumstances. The impact of data complexity on the ability to provide informed consent has been discussed in the context of the increasing scope of medical knowledge. Our study highlights its relevance to more routine decisions in everyday medicine [13].

The need for information to be delivered within a relationship of trust may explain previous data on transfusion consent. Chan *et al.* [6] reported on the success of a standardized video in improving consumers' own perceived knowledge of transfusion, but it did not significantly improve their levels of comfort in receiving a transfusion. However, Furumaki [14] showed improved sense of ease when an additional discussion about transfusion was provided by a transfusion technician. Our study identified human interaction to create trust as a factor that consumers felt created comfort in the transfusion process.

The nature of risks and benefits to be discussed was interesting. The focus on viral infection is consistent with the need to allay pre-existing anxieties. By contrast, more common severe reactions were not cited, a finding previously noted by Friedman and others [15]. Their quantitative assessment also found all prescribers and most consumers recalled information on benefits had been provided. This contrasts with our consumer cohort, where there was a presumption of benefit if a transfusion was required.

Implications for the application of transfusion information

Consumers reported utilizing transfusion information for a different purpose to that assumed by prescribers in creating written information as a tool for obtaining informed consent. By contrast, consumers felt consent was a personalized process which was best facilitated by a knowledgeable clinician guiding decisions. Consumers wanted

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knowledge as background to their condition and treatment, but more importantly reassurance. Perhaps this arises from a perception of written information as ancillary. As such, they are tolerant of shortcomings [16]. While they frequently wanted all risks discussed, they were content when not all were presented, and they did not want additional fears to be created. Information that lists risks needs to be tempered to prevent unnecessary fears, but risks need to be acknowledged to reassure consumers that the risks they are aware of, particularly viral infections, have been managed. Respecting patient autonomy and as an essential component of informed consent, all risks that may impact a consumer's decision should be discussed. This is complex, varies between circumstances and may change. Our study suggests that consumers prefer that this be addressed case-by-case and that consumer information may be more general.

One way to address this would be to use written information as a secondary tool. Not as a stand-alone or routine adjunct to the verbal process of informing and obtaining consent but offered and contextualized during that process. This would be consistent with the preferences identified in our consumer cohort, as they frequently felt too overwhelmed or exhausted to read it but were nevertheless reassured by its availability. Language should be simple and aim to reassure. The function and expected outcomes (indications) of transfusion should be addressed. Importantly, a decision needs to be made to address consumer rather than prescriber needs when developing information, as their perceptions were not the same.

Implications for quality improvement

The importance of consumer consultation in patient tool development is highlighted by differing perceptions between consumers and prescribers on information purpose. Informing consumers, seeking them to be actively engaged in their health care, questioning and advocating for treatment are important aspects of care. While probable person-centred care will enhance healthcare system quality, our results suggest that this approach should not be relied upon as a quality improvement strategy. Few actively question their care, more commonly consumers trusted and accepted doctors' decisions. One was openly hostile to the concept of questioning his own care, mirroring community attitudes towards those who do not follow medical advice (in vaccination); many remarked on their own limitations in making decisions beyond their areas of expertise, a sentiment independent of educational attainment; others displayed unquestioning acceptance of medical advice, perhaps due to socialization. Lack of active questioning from individual consumer means that consumer advocacy may not be an effective practice improvement tool. Quality improvement strategies should rely on robust system changes rather than vulnerable people to question the experts in whom they trust. This has potential applications beyond transfusion.

Consumers are often informed about potential systemic risks of transfusion, such as receiving the wrong blood [17]. Our consumer cohort felt vulnerable and preferred not to be encumbered with the threat of failure in the healthcare process, which created anxiety. Consumers expect that there are systems in place to ensure appropriate prescribing and prevent errors, which they felt was undermined by informing them of the chance of system failure. The onus is upon institutions to prevent errors rather than inform consumers of their inability to do so.

Limitations

As a qualitative study, this research was designed to explore participant understanding and, by analysing and integrating themes, generate a model to understand how transfusion information should be developed and used in practice. There will be uncertainty about how well these apply in other hospitals and with different populations. Whilst the patient population sampled approximately mirrors the departmental frequency of transfusion, the small sample size may have presented a limited range of perspectives. High median consumer age may additionally limit external validity; however, Australian Institute of Health and Welfare data found that most RBC and plasma transfusions occur in patients over the age of 65 [18]. Further, accessibility of information to non-English speakers remains problematic. Another potential limitation is relative homogeneity of prescriber experience level. While the general principles identified here may apply to other situations where information is provided and informed consent sought, this should be done with caution. Most participants saw transfusion as a component of a larger treatment plan, and responses may have been different where the treatment was the major intervention.

Conclusion

This study found differences between the underlying purpose of blood transfusion information as perceived by consumers and prescribers. Prescribers viewed information as a tool for obtaining informed consent, whereas consumers desired knowledge and understanding. The need for reassurance was high, and consumers felt that this could only be obtained within a relationship of trust. Unlike prescribers who described seeking to engage consumers in decision-making with transfusion information,

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consumers described their lack of understanding and excessive information as limiting their ability to question professionals on whom they were dependent.

Conflicts of interest

The authors declare no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:
Appendix A. Interview questions - consumers.
Appendix B. Interview questions - transfusion prescribers.

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Clinical use of Convalescent Plasma in the COVID-19 pandemic: a transfusion-focussed gap analysis with recommendations for future research priorities

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Background and objectives Use of convalescent plasma for coronavirus disease 2019 (COVID-19) treatment has gained interest worldwide. However, there is lack of evidence on its dosing, safety and effectiveness. Until data from clinical studies are available to provide solid evidence for worldwide applicable guidelines, there is a need to provide guidance to the transfusion community and researchers on this emergent therapeutic option. This paper aims to identify existing key gaps in current knowledge in the clinical application of COVID-19 convalescent plasma (CCP).

Materials and methods The International Society of Blood Transfusion (ISBT) initiated a multidisciplinary working group with worldwide representation from all six continents with the aim of reviewing existing practices on CCP use from

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donor, product and patient perspectives. A subgroup of clinical transfusion professionals was formed to draft a document for CCP clinical application to identify the gaps in knowledge in existing literature.

Results Gaps in knowledge were identified in the following main domains: study design, patient eligibility, CCP dose, frequency and timing of CCP administration, parameters to assess response to CCP treatment and long-term outcome, adverse events and CCP application in less-resourced countries as well as in paediatrics and neonates.

Conclusion This paper outlines a framework of gaps in the knowledge of clinical deployment of CPP that were identified as being most relevant. Studies to address the identified gaps are required to provide better evidence on the effectiveness and safety of CCP use.

Key words: convalescent plasma, COVID-19, gap analysis, patient outcome, SARS-CoV-2.

Introduction

Coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was declared a pandemic by the World Health Organization (WHO) on 11 March 2020 [1]. To date, there are no proven therapies for infected patients. Moreover, no vaccines are available, although many are in rapid development and some may be available soon. Based on the concept of passive immunization, human convalescent plasma (CP) from COVID-19-convalescent donors has emerged as an option for prevention and treatment of COVID-19 considering that it can rapidly be made available and, theoretically, could be used for providing immediate immunity to susceptible individuals through viral neutralization [2]. Other proposed mechanisms of action include antibody-dependent cellular cytotoxicity and/or phagocytosis [3]. Moreover, the use of CP may provide an immunomodulatory benefit via amelioration of macrophage activation and systemic hyper-inflammation or 'cytokine storm' [4].

The interest in the use of CP in managing COVID-19-infected patients is based on the historical use of CP in other viral outbreaks such as measles [5], mumps [6] and influenza [7,8]. CP was also used in viral epidemics such as Spanish influenza A (H1N1) [9], avian influenza A (H5N1), SARS [10], Middle East Respiratory Syndrome and Ebola disease [3,11,12]. A retrospective meta-analysis concluded that CP from survivors of these diseases may reduce mortality, but should be studied in the context of well-designed trials due to the lack of high-quality studies and paucity in the published literature [13]. The early case series published from China on the therapeutic use of COVID-19 convalescent plasma (CCP) showed a potential role in improving clinical symptoms, decreasing viral load and raising serum neutralizing antibody titres [14,15]. However, these studies featured important limitations, with implications for the conclusions that can be drawn. The WHO recommended scientific studies to explore the feasibility and medical effectiveness for CCP collection and use, and to establish appropriate regulatory conditions including monitoring and reporting patient outcomes [16].

To deploy CCP therapy, various donor, product and patient-related conditions should be addressed [3]. Guidance is needed to direct blood centres and transfusion services on collection and manufacture of CCP and to support clinicians developing evidence-based treatment strategies. Existing gaps in knowledge regarding CCP trials need to be identified to enable developing and defining recommendations on patient eligibility, administration, safety and monitoring of adverse events. The deliverables from this project will facilitate study design and analysis of clinical data to determine CCP efficacy and safety, and outcomes can be used to identify areas that need to be explored when facing similar viral pandemics in the future.

Materials and methods

The International Society of Blood Transfusion (ISBT) through the Clinical Transfusion Working Party (WP) reached out to the ISBT global network to establish a multidisciplinary working group (WG) to address existing practice and gaps in knowledge on the use of CCP. The WG is comprised of members from all WHO regions, with expertise in blood banking, clinical transfusion medicine, adult and paediatric haematology and virology.

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Altogether, representatives from the ISBT clinical transfusion WP, the ISBT WP on global blood safety, the ISBT WP on transfusion transmissible infectious diseases, the ISBT WP on Haemovigilance, the Asia-Pacific Blood Network and AABB (formerly American Association of Blood Banks) were included. During weekly teleconferences (April to May 2020) a series of questions pertaining to donor, product and patient domains were devised. The outcome of this project is published in two separate papers. Here, we summarize the knowledge gaps in content areas pertaining to clinical deployment of CCP, based on existing literature at the time of publication, and input from clinical transfusion professionals. Donor- and product-related issues are addressed in a separate paper.

Results

Trial and study design

Randomized controlled trials (RCTs) with careful study designs and appropriate control group(s) are preferable. The comparative arm may include standard care, or another intervention, such as non-convalescent plasma or crystalloid fluid. RCTs will provide the most robust data, but a range of practical considerations will influence study designs possible during the outbreak. Double-blinded studies may be difficult to conduct in a pandemic situation when it is important to test new treatments as rapidly as possible. To date, there is no obvious placebo comparator to CCP, and non-convalescent plasma (that does not contain specific anti-SARS-CoV-2 antibodies) could lead to known transfusion-associated adverse events such as transfusion-associated circulatory overload (TACO), transfusion-related acute lung injury (TRALI), allergies or transfusion-transmitted infections (TTIs). Instead of applying a placebo, a way to decrease bias within the trials is to perform large pragmatic trials that use objective measures of effectiveness, such as assessing a decrease in all-cause mortality. Platform trials with adaptive design where more than one 'domain' may be active concurrently and, where patients can be allocated quickly to promising therapies, may also be efficient ways to conduct high-quality studies.

Considering that setting up RCTs may not be feasible in all medical settings, other study designs can be employed including cohort studies, case-control studies and observational studies such as using registries [17]. These studies may provide information that could still be of great value to assess other perspectives, such as the feasibility of collecting, processing and administration of CCP at pre-defined doses and treatment time-points in different clinical settings. The choice of study design may be dictated by country-specific challenges such as feasibility of conducting RCTs and cultural acceptability of enrolment in clinical research in the setting of a pandemic threat.

Patient eligibility

COVID-19 convalescent plasma should only be offered as therapy to patients with a laboratory-confirmed COVID-19 diagnosis or as prophylaxis in well-monitored clinical trials exploring prevention of COVID-19 infection in high-risk populations. The patient cohorts studied in most of the current therapeutic clinical trials consist of adults with moderate or severe respiratory disease. Eligibility criteria vary substantially, ranging from patients who do not require hospital admission [18] to patients with severe COVID-19 disease requiring mechanical ventilation [19,20]. CCP use may have its greatest benefit in patients early in their disease and prior to being placed on mechanical ventilation; however, studies are lacking to make any definitive conclusions [10]. Multiple studies exclude pregnant or breast-feeding women or patients with co-morbidities such as renal or cardiac disease [20,21]. Compassionate use of CCP has been started in settings outside clinical trials for patients with serious or life-threatening COVID-19 disease, who are not eligible or who are unable to participate in RCTs [22]. Prophylaxis trials of non-infected, but at risk subjects including these with history of exposure, are also being designed and now open for inclusion [23,24].

Considering that the COVID-19 pandemic is globally dynamic, enrolling patients in clinical trials can be a challenge in countries that are at the acute stage of the pandemic. Obtaining ethical and/or regulatory approvals can delay initiation of the trials. In addition, it becomes a challenge to meet the enrolment target in late stages of the pandemic when the number of eligible cases is decreasing significantly. Collaborative international trials are underway and will be useful in such circumstances [25]. Decision to initiate a CCP trial should be made early in a pandemic after assessing feasibility, benefits and risks. Although blood products are now very safe in most countries with the existing testing for TTIs, recovered COVID-19-infected patients who have received CCP manufactured without pathogen reduction treatment (PRT) may be deferred from subsequent CCP collection. In addition, CCP donors may be eligible to receive allogeneic CCP in case of COVID-19 re-infection or reactivation.

COVID-19 convalescent plasma dose, frequency and timing of administration

COVID-19 convalescent plasma can be collected by apheresis or whole blood donation. Apheresis yields 200–

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800 ml of CCP that can be divided into 1–4 separate units before freezing. There is a wide variation in the CCP dose used [26] due to a lack of standardization in study design, variation in CCP collection methods, production and administration. In early studies from China, two consecutive transfusions of 200–250 ml of ABO-compatible CCP were used in one study [14], whereas a single 200 ml dose with anti-SARS-CoV-2 titre >1:640 was tested in another study [27]. A dose of 200 ml, followed by 1–2 doses of 200 ml according to disease severity and patient tolerance, has been recommended by some authors [28]. This was also the case for CP use in other viral outbreaks, including H5N1 [8].

At this stage, there is no evidence for which dose and timing is best to optimize patient outcome [26]. A variety of CCP doses is currently under evaluation in registered clinical trials ranging between 200 and 600 ml per adult patient, or defined according to the patient's body weight or by the number of units to be transfused [29]. The minimal effective dose, and whether that is related to a specific neutralizing antibody titre, is currently unknown. Because viraemia peaks in the first week of infection in most viral illnesses, and because the primary immune response typically develops by day 10–14, followed by viral clearance, administering CCP early in the disease course could theoretically be more effective [10]. Based on the experience in other viral infections, CP should be used early in the disease before the inflammatory syndrome starts and the peak of production of endogenous IgM and IgG antibodies [8,30]. In early studies of SARS patients, better outcomes were seen in those given CCP before day 14, as compared to later time-points [10]. This approach would be expected to be most effective in COVID-19-infected patients; however, it has not yet been shown in clinical trials. Early administration for COVID-19-infected patients is also believed to prevent innate immune cell migration and avoid lung damage [4].

Whether additional transfusions should be given, and when, is also currently unknown. Factors to be analysed include transfused volume, response to treatment, and the risk of adverse events. Patient clinical and laboratory criteria that may determine the need of an additional dose and the timing of its administration from the first dose are yet to be defined.

Parameters to assess response and outcome

The safety and efficacy of CCP transfusion are not yet established and new data on patient outcomes are emerging continuously [27,31–33]. Previous meta-analyses on CP use in other viral infections reported improvement in clinical signs and symptoms, hospital length of stay, viral load and mortality [8,13]. One meta-analysis identified the potential for CP to reduce mortality in severe acute respiratory infections of other aetiologies including SARS-CoV-1 and H1N1 influenza [13]. Outcomes included mortality, hospital length of stay, requirement for and duration of critical care support, viral antibody level, viral load and adverse events. However, the same meta-analysis reported methodological heterogeneity and moderate-high risk of bias in the studies conducted.

Limited early data on CCP use suggest clinical benefit with reductions in body temperature, improved Sequential Organ Failure Assessment (SOFA) score, less need of respiratory support, improved lymphocyte count and inflammatory markers, increases in IgG, IgM and neutralizing antibody titres, and reduction in viral load [14,27]. Another study reported reduced pulmonary lesions, by chest CT scans, after CCP transfusion [27]. Use of globally accepted objective disease severity definitions and mobility end-points when assessing response to CCP is preferred to enable comparison between studies [34]. The European Commission recommended that hospitals report various parameters, including clinical symptoms, laboratory results, a disease progression scale, length of hospitalization, and serious adverse events [35]. A publicly accessible database is set up to gather outcome data and to allow for meta-analyses to evaluate safety and efficacy on a regular basis [17]. It is paramount to report end-point results of such studies regardless of their study design to enable acquisition of data and information on the feasibility of CCP use, its effectiveness and safety.

The authors acknowledge the need for RCTs to evaluate CCP use objectively among different patient populations. It is important to assess feasibility and efficacy under standard regulatory conditions, particularly regarding ethical conduct, appropriate CCP collection, and the monitoring and reporting of patient responses and outcomes [16]. Assessing the impact of other confounders, such as patient co-morbidities, timing of administration and effects of other treatments (e.g., anti-viral drugs and cytokine inhibitors), necessitates stringent evaluation based on pre-defined clinical signs and symptoms and laboratory parameters (e.g., inflammatory markers, serum cytokine and viral antibody levels, viral load). Other outcomes include mortality, hospital length of stay, duration of critical care support (e.g., days on a ventilator and/or in an intensive care unit), severe adverse events and treatment complications.

Comparing clinical and laboratory patient responses to CCP antibody characteristics may identify patient and donor factors that predict clinical efficacy. Access to reliable COVID-19 antibody testing varies, and is rapidly evolving, affecting the ability to qualify CCP donors and characterize their donations. Prior to implementation of COVID-19 antibody assays with titre thresholds as release

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criterium for CCP, clinical trials should consider requesting retention samples from CCP units to be stored until assays are available. This will improve the ability for objectively measuring product characteristics and efficacy and outcomes of CCP infused prior to routine use of these assays.

Adverse events

No serious adverse events were reported in the systematic review of CP use in other viral infections [13]. In addition, no serious adverse effects were reported from the initially published studies regarding CCP use [27]. That said, from the first Cochrane review of reported case series, the adverse events rates were reported to be very low [26]. Moreover, a study reported a low rate of serious adverse events in the first four hours of transfusion (<1%) [36]. However, under reporting of adverse events cannot be excluded. Risks associated with CCP are likely to be the same as those with standard plasma, including TTIs, mild transfusion reactions (e.g., allergic and febrile) to potentially life-threatening transfusion reactions (e.g., TACO, TRALI and anaphylaxis/anaphylactoid reactions) [37]. TACO and TRALI are particularly concerning in severe COVID-19 given the underlying acute lung injury and potential priming of the pulmonary endothelium [38,39]; this highlights the importance of CCP donor selection to avoid high risk donors. Thus, the European Union program requires CCP donors without a history of blood transfusion and female donors who have never been pregnant, or are tested and found negative for anti-HLA/HPA/HNA antibodies using a validated assay [35]. Pretreatment to minimize transfusion reactions (e.g. acetaminophen and diphenhydramine) may be considered, as needed, or if the patient had previously needed pre-medication for blood transfusions. Whatever dose of CCP is used, patients at risk of TACO (small stature, low body weight, elderly, known or suspected renal or cardiac dysfunction) should be transfused slowly – at a rate as low as 1 ml/kg/h – and closely monitored throughout the infusion [40].

Reporting adverse events using internationally agreed haemovigilance definitions will assist in comparing results between studies [41–43]. Donor and patient adverse events need to be reported within institutional and national haemovigilance frameworks using internationally agreed definitions to gather more information on the safety of CCP collection and its use in adult and paediatric patients. Cooperation with international haemovigilance programs is preferable.

There is a theoretical risk of transmitting SARS-CoV-2 by transfusion, especially with the current lack of donor screening for common respiratory viruses [38]. In one recent study, four asymptomatic donors, out of 2430 screened platelet and whole blood donations, had detectable SARS-CoV-2 RNA in their blood [44]. However, detectable RNA does not necessarily imply infectivity. To the best of our knowledge, there has never been a report of respiratory virus transmission via blood transfusion; nonetheless, this needs to be assessed by ongoing surveillance. Another potential risk is antibody-dependent enhancement (ADE), whereby antibodies developed during past infection with a different viral serotype exacerbate clinical severity of the current illness. This was seen with dengue virus, among other viral infections [45,46]. It is hypothesized that the mechanism involves IgG antibody Fc-region binding to the Fc gamma receptor on an immune cell, such that the Fc gamma receptor functionally mimics the actual viral receptor and, thereby, mediates viral entry [47]. There have been no reports of this phenomenon occurring with the SARS-CoV-1 or MERS viruses as a result of CP transfusion. Nonetheless, specific studies to assess this potential risk are required, particularly regarding vaccine design, use of PRT and monoclonal antibody-based therapy. Finally, there is a theoretical risk that CCP could exacerbate underlying coagulopathy associated with severe COVID-19 [48]. This was not reported in any of the recently published studies [26,33,36]. These potential risks and the fact that, at this stage, there is no specific anti-SARS-CoV-2 treatment available, should be discussed with the patient at time of enrolment in CCP clinical trials.

Application in paediatric and neonatal medicine

Early data suggest that paediatric COVID-19 cases might experience different symptoms than adults with children overall showing less severe disease than adults [49]. Based on all reports so far, less than 10% of children had severe or critical disease and mortality was rare [49]. Children with chronic lung and/or cardiovascular disease or immunodeficiency or on immunosuppression may be at higher risk for worse outcomes [50–52]. There is a scarcity of data specifically on CP use in paediatric and neonatal populations. During the Spanish flu, paediatric single doses of 50 ml of CP were attempted but no clear paediatric-specific outcomes are listed [8]. Given the dearth of other treatment options, CP can potentially play a key role and be a safe and efficacious treatment modality in children and neonates, which if instituted in a timely manner may reduce progression from mild to more severe disease. But this will need extremely careful evaluation in well-planned clinical trials and prospective studies.

Very few trials on use of CPP currently include children or neonates; however, few trials are planning to

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include children [24,53,54]. In the United States, a study aims to evaluate the safety and pharmacokinetics of human CCP in high-risk children (1 month–18 years), either with confirmed infection or with high risk exposure. CCP, with anti-SARS-CoV-2 antibody titres ≥1:320, at a dose of 5 ml/kg, with a maximum volume of 500 ml, will be used [24]. In Canada, a randomised, multi-centred, open-label Phase 2 clinical trial of the safety and efficacy of CCP for treatment of COVID-19 disease in hospitalised children has been launched [53]. This protocol allows a 10 ml/kg dose up to a maximum dose of 500 ml vs. standard of care as the control arm. It is challenging to perform clinical trials in this age group due to lower patient numbers. Therefore, the importance of multi-institutional collaborative national and international efforts cannot be overemphasized. Multi-institute registries collating observational data on CCP use and outcomes can be very important in the interim, while trial results are awaited.

Kawasaki-like inflammatory syndromes (Multisystem Inflammatory Syndrome in Children; MIS-C) have been reported in children with COVID-19 [55]. Most reports, both anecdotal and published, described the MIS-C occurring after infection with SARS-CoV-2. However, some report that the virus can be detected concurrently with MIS-C [56]. Whether affected children may benefit from CCP remains unclear and no recommendation can be made with certainty. If the patient is actively infected with SARS-CoV-2 at time of MIS-C then treatment with CCP might be beneficial. More studies are required to establish safety and efficacy of CCP in this syndrome.

Less-resourced countries

Resource constraints at both micro- and macro-level impact the provision of healthcare infrastructure as well as the ability to access this limited healthcare. The net effect is late presentation of significantly ill patients who compete for limited resources with other patients, often displacing such patients and further depleting the available resources. In addition, poor socio-economic circumstances contribute to rapid spread of disease, high rates of co-morbidities, all of which may contribute to poor patient outcomes, especially given limited availability of critical care facilities. At high rates of community outbreaks, however, especially once healthcare resources are saturated, higher resourced countries see similar issues.

There are many challenges faced by medical systems in low- and middle-income countries (LMICs) that may limit enrolling patients in CCP treatment programmes. Performing robust clinical trials against this background is problematic, as is borne out by the work on CP use during the previous Ebola crisis [57,58] leading to the potential empirical or observational use of CP in these settings. All possible attempts should be made to confirm safety and efficacy prior to redirecting limited resource to large-scale collection and provision of CCP. It is important to avoid empirical use of CCP based on symptomatology; particularly because COVID-19 has a wide range of presenting symptoms with significant overlap with other communicable diseases. Where feasible, CCP use should ideally be part of clinical trials, even if limited in scope, with clear, preferably clinical end-points, which do not require sophisticated laboratory investigations. However, plasma infusions in these setting are not without risk; high levels of communicable diseases and limited access to robust testing systems, poses particular risks in these settings, necessitating detailed risk-benefit assessments. Supply from other countries across international borders is impeded by many regulatory, financial and logistic barriers and challenges.

Ethical consideration

The adoption of CCP for treatment of COVID-19 has introduced a number of ethical challenges. Foremost, it remains an unproven therapy, despite a growing literature suggesting that it may be beneficial. Indeed, CCP was adopted quickly and widely in the absence of strong evidence of benefit, instead relying on case reports and uncontrolled observational studies to support its use [14,15]. Those data suffer from serious methodologic limitations, not least of which is the potential for confounding in late-stage disease. As one example, most of the patients who have received CCP have also been subjected to a range of other therapies. Second, recent data have afforded insight into the safety of use, suggesting that the risk of CCP is comparable to that of standard plasma [36]. Nonetheless, early in the pandemic when treatment options were otherwise minimal, transfusion of CCP was undertaken before safety data was available to specifically address the theoretical risks of SARS-CoV-2 transmission, ADE and exacerbation of underlying coagulopathy [38].

The demand for CCP continues to increase as it gains media attention as a viable treatment due to its anecdotal successes, its relative ease to manufacture from recovered patients, and as other therapeutic modalities fail to show benefit in clinical trials. There is insufficient inventory to support all patients in the notable absence of robust clinical data. While dual inventories could avoid competition between the clinical trials and compassionate use of CCP, there is more likely to be a single source to draw from. Compassionate CCP use may also impede enrolment into clinical trials, particularly if the study design includes randomisation offering the potential of placebo rather than CCP.

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Table 1 Identified knowledge gaps and points for consideration in the use of CCP for treating COVID-19 patients

Objective	Identified gaps	Points to consider
Trial and study design	• What is the best way to assess effectives and safety of CCP? • What control arm treatment should be employed in designing randomized clinical trials? • What other treatment alternatives to CCP are available? • What supportive measures will be available in the inclusion arm?	• Wherever possible, use of CCP should be within the context of a clinical trial until or unless its efficacy and safety are established. If CCP is used outside a clinical trial, data should still be collected to gather experience and outcomes. • Effectiveness of CPP compared to other treatment alternatives should be assessed whenever possible. • All patients should receive the best available supportive care as soon as being appropriate and available.
Patient eligibility	• Who would benefit most from CCP treatment? • Who would benefit from CCP prophylaxis? • What is the role of compassionate use of CCP, if any, outside a clinical trial? • Could CCP be not beneficial or even harmful?	Clinical trials should: • Define the disease settings to assess which patients will benefit the most from therapeutic and prophylactic CCP and/or when CCP is not beneficial or even harmful. • Define patient eligibility criteria if any for compassionate use of CCP.
CCP dose, frequency and timing of administration	• What is the minimal acceptable CCP dose to be effective? • What is the optimal dose of CCP? • Does CCP dose vary between clinical settings (e.g. disease severity, different patient groups)? • When should CCP be administered in the course of the disease? • What clinical criteria define the need for a repeat dose(s)?	Clinical trials should: • Define minimum CCP dose needed for efficacious treatment. • Assess optimum CCP dose in a range of disease severities and clinical settings. • Define appropriate time-points for CCP administration in the course of the disease for efficacious treatment. • Define clinical criteria that allow (repeated) CCP administration.
Parameter to assess response and outcome	• What clinical and laboratory parameters should be used to monitor response? • What are the best clinical outcomes to measure and what morbidity end-points should be assessed? • How outcomes are related to antibody characteristics and titre levels? • What confounders could impact patients' outcomes?	• Use of routinely collected data as much as possible to reduce workload pressure on front-line staff caring for patients. • Use precisely defined and globally accepted objective disease severity definitions, morbidity and quality of life end-points to assess clinical impact of CCP transfusions and to allow comparison of studies. • Assess for CCP antibody characteristics and titre levels, and compare these with the laboratory and clinical response. • Assess and control for confounders that could impact patients' outcomes.
Adverse events	• Is CCP use safe? When is CCP uniquely unsafe? • Is CCP transfusion associated with higher risks of adverse events compared to standard plasma? • What hemovigilance definitions should be used to characterized adverse events in transfused patients? • Can SARS-CoV-2 be transmitted by blood transfusion? • Can CCP transfusion induce ADE or exacerbate underlying coagulopathy? • Is pathogen reduction technology warranted to reduce TTI risk? • Are there any novel adverse events that occur with CCP?	• Monitor safety of CCP and define settings in which it should not be used. • Monitor patients for adverse events while on treatment. • Use internationally agreed definitions to gather more information on the safety of CCP collection and its use. • Monitor whether SARS-CoV-2 can be transmitted through blood and blood products. • Monitor whether CCP transfusion can induce ADE or exacerbate underlying coagulopathy. • Determine if use of pathogen reduction technology is warranted. • Assess for any SURARs that may occur with CCP use.

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Table 1 (Continued)

Objective	Identified gaps	Points to consider
Application in paediatric and neonatal medicine	• What would be the eligibility criteria for use of CCP in paediatrics and neonates? • What CCP dose to be used and how frequent? • What clinical outcome should be assessed?	Clinical trials should: • Define eligibility criteria for use of CCP in paediatrics and neonates. • Define CCP dose and frequency of administration. • Define paediatric-specific clinical outcome measures to be assessed, especially outcome measures that can be objectively measured.
Less-resourced countries	• How to determine if use of CCP is feasible in settings of limited resources? • Are there any international programs to facilitate access to CCP for patients in medical systems with limited resources?	• Perform risk assessments for the use of CCP ideally including a review of the safety of the country's blood supply in general and within the context of individual facilities in particular. • Develop (international) programs to facilitate access to CCP for patients in medical systems with limited resources.
Ethical considerations	• How to prioritize CCP use if limited supply or if competition exists on the existing inventory between clinical trials and compassionate use? • When to consider a cross-over of patients from the control arm to the treatment arm in CCP clinical trials? • How to implement CCP in settings with challenges in providing sufficient blood supply in LMICs?	• Define a mechanism on how to meet demand with insufficient CCP supply. • In the absence of current effective treatment options, consideration should be given to patients in control arms crossing over to CCP treatment arms in case of disease progression. • Diversion of resources away from routine blood collections in LMICs need to be carefully assessed.

COVID-19, Coronavirus disease 2019; CCP, COVID-19 convalescent plasma; LMICs, Low- and middle-income countries; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; SURARs, Suspected unexpected serious adverse reactions; TACO, Transfusion-associated circulatory overload; TRALI; Transfusion-related acute lung injury; TTI, Transfusion-transmitted infection.

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From an ethical perspective, all patients – including those enrolled in RCTs – should receive the best available supportive care as soon as appropriate and available. Considering the lack of high-level evidence to support the use of CCP at this time, the option to allow patients to cross over to the 'treatment group' who were initially randomized into the control group, and who show progressive disease after the primary end-point of the trial has been reached, can be considered. The latter may not only be an ethical option but could also allow data acquisition on an additional CCP infusion time-point in a given study after assessment of the primary end-points.

A broader question relates to the ethics of implementation of an unproven therapy in LMICs, the majority of which are unable to meet transfusion demand given a myriad of systemic challenges [59,60], but which may also have extremely limited access to other therapies. CCP may further strain those transfusion services. Using whole blood collection techniques could decrease the overall safety of the local blood supply by collecting (largely) first time donors, who traditionally have higher rates of TTIs. Diverting limited healthcare and blood establishment resources to the collection and provision of products with limited evidence of efficacy within a particular setting may more broadly negatively affect public health delivery.

Conclusions

We identified key questions and gaps in knowledge pertaining to the clinical use of CCP, including in special settings for paediatric and neonatal patients, as well as in less-resourced countries, and we suggest points to consider for developing new trials (Table 1). Acknowledging the substantial heterogeneity of the clinical CCP landscape the medical field has to deal with, this gap paper could help to put some studies into context, and it could contribute to conduct more streamlined and coordinated future studies. Central questions to be clarified are whether CCP is safe and effective for adult and paediatric patients with COVID-19. A recent Cochrane Systematic Review has shown no evidence to support CCP use based on the very limited existing reports [26]. However, substantial amounts of data are being published every day. Thus, addressing gaps in knowledge identified in this document, together with emerging evidence, is expected to identify the benefits and risks of CCP, thereby providing a robust basis for defining its future therapeutic use.

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Conflict of interests

Evan M. Bloch reports personal fees and non-financial support from Terumo BCT, personal fees and non-financial support from Grifols Diagnostics Solutions, outside of the submitted work; EMB is a member of the United States Food and Drug Administration (FDA) Blood Products Advisory Committee. Any views or opinions that are expressed in this manuscript are that of the author's, based on his own scientific expertise and professional judgment; they do not necessarily represent the views of either the Blood Products Advisory Committee or the formal position of FDA and also do not bind or otherwise obligate or commit either Advisory Committee or the Agency to the views expressed. Dana Devine a member of the scientific advisory board of Macopharma and received research support from TerumoBCT, Hemanext and Macopharma.

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ORIGINAL PAPER

Development of an updated assay for prekallikrein activator in albumin and immunoglobulin therapeutics

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Background Prekallikrein activator (PKA) is a contaminating enzyme found in therapeutic albumin and immunoglobulin products. The level is commonly measured using methods such as that defined by the European Pharmacopoeia (Ph Eur) with traceability to the WHO International Standard for PKA. This method generally works well, but problems are sometimes observed.

Materials and methods A simplified one-step method has been developed to replace the existing Ph Eur two-step method which consists of kallikrein generation followed by kallikrein measurement using a chromogenic substrate. Analysis of data from the one-stage method is simplified by the use of a dedicated online app.

Results The one-stage method was validated against the current Ph Eur method using batches of albumin and immunoglobulins. Problem batches of immunoglobulins were investigated using the one-stage method. Improved methodology using true initial rate determinations and use of acid-treated prekallikrein substrate (PKS) helped understand and reduce artefactual results.

Conclusions The one-stage method and associated app streamline real-time determination of PKA and promote good principles of enzyme assays to limit substrate depletion, while also conserving expensive PKS. Blanking steps and reproducibility are simplified.

Key words: prekallikrein activator, kallikrein, albumin, immunoglobulin, method development, online apps, reproducibility.

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Introduction

Human albumin for therapeutic use is a long-established blood product with a good safety record [1], although there remain some questions around clinical benefit [2, 3]. Production and quality control includes determination of the level of contaminating prekallikrein activator (PKA), a fragment derived from Hageman factor, factor XII, responsible for the generation of kallikrein from prekallikrein [4]. Kallikrein catalyses the formation of the vasodilator bradykinin, leading to hypotensive reactions in animal models and patients [5, 6]. A widely used assay method for PKA is outlined in the European Pharmacopoeia (Ph Eur) [7]. The level of PKA must be below 35 IU/ml, traceable to the WHO International Standard (IS), which defines the international unit (IU) [8]. Similarly, immunoglobulins are tested for contaminating PKA, with activity traceable back to the same WHO IS. The WHO IS is a freeze-dried 20% albumin with a high level of PKA, and there is no specific IS for PKA in immunoglobulins. The Ph Eur test, (and other PKA pharmacopoeial methods, e.g. [9]), outline a relative potency determination against a standard in a two-step procedure: (i) an activation step where PK in prekallikrein substrate (PKS) made from plasma is activated for a fixed time to generate kallikrein; and ii) this solution or a subsample is mixed with chromogenic substrate to estimate kallikrein activity by measuring amidolytic activity. Variations

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within this basic method were investigated previously in the collaborative study that established the WHO 2nd International Standard (IS) for PKA in albumin [8]. This study identified some deviations from the Ph Eur monograph, for example in the ratio of PKS:sample volume, which is supposed to be 9:1 to avoid artefacts due to different salt concentrations between test samples or tests and standards. It was noted that this rule was not always adhered to, probably to conserve PKS, which is time-consuming to make in-house or costly to buy. The study [8] also showed variations in the way participants interpreted blanking in their protocols, which suggested that the general advice given in the Ph Eur method was not entirely clear.

Theoretically, it is possible to update the existing two-stage method as outlined in the Ph Eur [7] to a simplified one-stage method, as for instance used in the assay of plasminogen activators [10]. Recently, we have simplified this procedure by developing online apps that can be easily used in a computer browser with benefits for reproducibility and transparency [11, 12]. Such apps have been used successfully in a recent collaborative study to establish the WHO 4th IS for Streptokinase [13]. With this experience, we now propose a simplified one-stage method to determine PKA activity in albumin or immunoglobulin solutions which is facilitated by a dedicated online app to simplify rate calculations and associated blanking steps. The use of this new method also makes it easier to investigate a well-known problem in the measurement of PKA in some immunoglobulin solutions, where apparent negative values for PKA activity can be observed after blanking steps using the standard Ph Eur procedure [14]. Work is presented to compare the new one-stage method against the existing Ph Eur method, and an explanation and solution to problems associated with PKA measurements in immunoglobulins are offered.

Materials and methods

The Ph Eur method

The method used follows Ph Eur guidelines [7]. PKA potencies of test and internal control (IC) samples were determined relative to the WHO 2nd IS for PKA (02/168, NIBSC, UK), a freeze-dried 20% albumin preparation with an assigned potency of 29 IU/ml. The IC sample (NIBSC code 00/488) was a freeze-dried 5% albumin batch with a potency of 10 IU/ml. Test albumin batches were from routine testing and coded AL1-AL4. For the first stage of PKA activation, 10 µl of test solution was incubated with 90 µl of PKS (prepared in-house, see below) at 37°C for 10 min. The second step of kallikrein measurement was started with the addition of 100 µl of 0·4 mM S2302 chromogenic substrate (Pro-Phe-Arg-pNA, Chromogenix, Milan, Italy) to the reaction well. All reactions were carried out in a microtitre plate, and each reaction had a corresponding blank for subtraction on the plate consisting of 10 µl of test solution plus 90 µl of buffer to replace the PKS in the first step. Dose–response curves for standards and tests were performed in duplicate, and results analysed by parallel line bioassay methods as outlined in the Ph Eur and previously [7, 8].

PKS preparation

Preparation of PKS followed Ph Eur guidelines [7]. The dialysis and column buffer used was 0·05 M Tris–HCl, pH 8·0, containing 0·02 M NaCl and 50 µg/ml of polybrene (hexadimethrine bromide). Briefly, blood (approximately 45 ml) was collected fresh into 3·8 % w/v sodium citrate containing 1 mg/ml polybrene and PKS isolated using a DEAE-Sephacel column. The PKS fraction does not bind to the column. This fraction was pooled, divided into aliquots and flash-frozen.

One-stage method

The reaction mixture consisted of 10 µl of sample, 50 µl of 1 mM S2302 and 40 µl of PKS, all warmed to 37°C and added to the wells of a microtitre plate in that order, using multichannel pipettes. The plate was divided into + and – PKS sections as for the Ph Eur method. Absorbance at 405 nm was read as soon as possible for at least 60 min. Exported results as time versus absorbance were read into a dedicated app [15], which subtracts background absorbance changes from pre-existing kallikrein activity (–PKS wells) from rates of kallikrein generation (+PKS wells) and calculates PKA activity from slopes of absorbance due to pNA generation versus time squared. Rates were calculated over a change in absorbance value of 0·1 or a maximum of 100 points, for a read interval of 30 s. Detailed instructions and a link to the app are available online [16]. Relative potencies of PKA were calculated from rates of absorbance versus time squared using the same parallel line bioassay methods as for the Ph Eur method, above. Immunoglobulins coded IG1-IG4 were used to investigate PKA measurement in immunoglobulins and acid-treated PKS.

PKS acid treatment

Acid treatment of the PKS substrate described above was as outlined in the US Pharmacopoeia [9]. Briefly, 2 ml of PKS at 37 °C was added to 1 ml of 0·33 M HCl and the

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resulting solution was incubated at room temperature for 15 min, before addition of 1 ml of 0·33 M NaOH and 100 µl of 1M Tris, buffer pH 8·0. Any precipitate was removed by centrifugation at 3000 × *g* for 3 min.

Software and statistical analysis

Parallel line analysis to calculate relative potencies of PKA against a standard were performed using Combistats v 5 [17]. Software designed to automate the calculation of rates of absorbance vs. time squared and deal with blanking steps in PKA assays was developed using the programming language R [18]. A browser-based user interface (the app) was written in R using the Shiny package [19]. Statistical tests were performed using R or Minitab v 18 (Minitab LLC, PA, USA).

Results

One-stage PKA assay using a dedicated app

The starting point for method development was the current Ph Eur method for the determination of PKA in albumin and immunoglobulin solutions [7]. The main change in the new method is to use a one-stage process to measure the generation of kallikrein. Thus, test or standard is mixed with PKS and chromogenic substrate and the reaction is followed immediately, avoiding the two-step process. In line with other similar reactions [10, 13, 20] this means that the activity of PKA, the desired measurand, can be calculated from plots of absorbance versus time squared. To simplify this process, software has been developed using the programming language R [18] and data may be analysed using a browser by means of an app, developed using the Shiny package [19]. The app also handles blank subtraction of pre-existing kallikrein enzyme activity in samples from test wells. In contrast to the current Ph Eur method, subtraction of background kallikrein activity is performed in real time, point by point, rather than at the end as final calculated slopes. A working version of the app may be explored [15], and additional detailed help notes are available online [16].

The one-stage method was investigated over a number of assays that included a common sample, internal control code 00/488 and several other albumin products, coded AL1 to AL4. Results are shown in Fig. 1 as a box plot with pairs of results for each test sample, using the one-stage method and the current Ph Eur method.

The results shown in Fig. 1 suggest that the one-stage method gives equivalent results to the current Ph Eur method as performed at NIBSC.

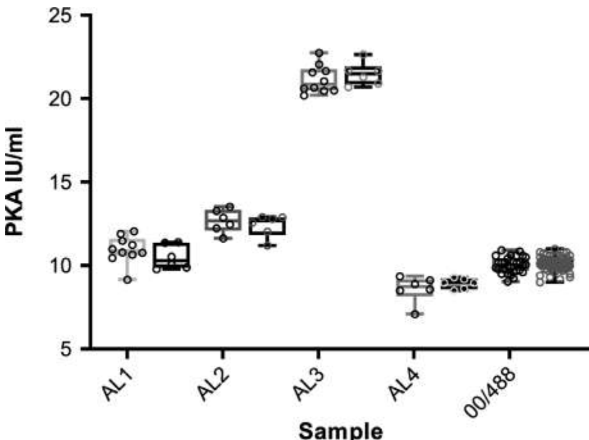

Fig. 1 Comparison of one-stage method and Ph Eur method for PKA determination in albumin samples. Each pair of boxes shows results from assays using the one-stage method (grey box/ black circles) or Ph Eur method (black box/grey circles). There was no significant difference in mean values between Ph Eur and one-stage methods using these samples (Student's *t*-test results were $P = 0{\cdot}28$, 0·52, 0·41, 0·38, 0·4, for AL1-AL4 and 00/488, respectively).

Acid-treated PK substrate

Some methods for PKA determination recommend an acid treatment step for the PKS [9], and acid treatment of the PKS was explored first of all with batches of albumin. In fact, the results shown in Fig. 1 include one-stage assay results using both normal PKS and acid-treated PKS. The results shown in Fig. 1 for 00/488 are elaborated in Fig. 2 to show more clearly the overlap between methods and differentiate normal and acid-treated PKS results. Statistical analysis applying a Shapiro–Wilk normality test to these results showed there was no significant deviation from a normal distribution for 00/488, for the two methods, Ph Eur and one-stage. A full breakdown of results by method and substrate is shown in Table 1 for the total of 168 determinations shown in Figs.1 and 2.

Acid treatment of PKS did not appear to affect assay results but has the disadvantage of adding time and extra manipulations to substrate preparation. Furthermore, there were problems caused by continued precipitation of proteins in the reaction wells after the acid treatment protocol, adding noise to the time courses of kallikrein generation. This developing precipitate may make the acid-treated PKS less suitable for the Ph Eur method which incorporates more PKS in the reaction mixes, especially the first activation step. Therefore, it is not recommended for routine PKA assays for albumin batches.

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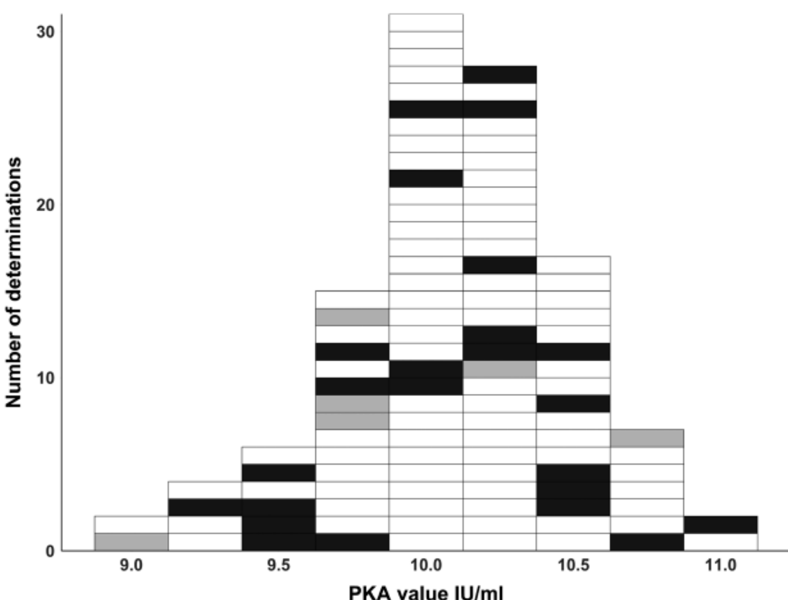

Fig. 2 Summary of PKA determinations in sample 00/488 in different assay formats. Each box in the stacked histogram represents the result from an individual determination of PKA value for the same sample, 00/488, using the Ph Eur method (open boxes), or the one-stage method (shaded boxes), where normal PK substrate (dark shading) or acid-treated PK substrate (light shading) was used. The vertical shading shows the distribution of results within each PKA value bin.

Table 1 Summary of PKA assay results for albumin samples using Ph Eur method or one-stage method with normal PK substrate or acid-treated PK substrate.

Sample	Method	Substrate	Mean PKA IU/ml	SD	N
AL1	Ph Eur	Normal	10·51	0·73	6
	One-stage	Normal	10·81	1·22	4
	One-stage	Acid-treated	11·05	0·55	6
AL2	Ph Eur	Normal	12·41	0·67	6
	One-stage	Normal	12·05	0·59	2
	One-stage	Acid-treated	12·98	0·56	4
AL3	Ph Eur	Normal	21·50	0·69	6
	One-stage	Normal	21·11	0·53	5
	One-stage	Acid-treated	21·20	1·11	5
AL4	Ph Eur	Normal	8·93	0·31	6
	One-stage	Normal	7·80	1·08	2
	One-stage	Acid-treated	8·99	0·33	4
00/488	Ph Eur	Normal	10·12	0·37	82
	One-stage	Normal	10·08	0·46	24
	One-stage	Acid-treated	9·85	0·55	6

PKA assays in immunoglobulins

While PKA assays are generally reliable and reproducible with good dose–responses with albumin products, this is not always the case with immunoglobulins. Complications are reported, for example [14], when using the Ph Eur assay where subtraction of the no-PKS blank wells can result in apparent negative values for PKA in some immunoglobulin batches. The reasons for this artefact are not fully understood but may be explained by observations laid out below. First of all, the problem is illustrated in Fig. 3a where several different preparations of therapeutic immunoglobulins were assayed alongside the IS for PKA (02/168) and the internal control sample (00/488), using the one-stage assay. Similar problems were seen using the Ph Eur assay. The standard and control albumin samples behaved as expected, but the immunoglobulin samples were characterized by apparent negative PKA values, lack of dose–response and variable results. Figure 3b shows clearly that the negative results were associated with high background in immunoglobulin samples, which may be termed 'kallikrein-like' activity as there may be other proteases present that cleave the chromogenic substrate S2302. It is likely that subtracting very high background rates, seen in Fig. 3b in the presence of low levels of PKA would contribute to errors and variability shown in Fig. 3a.

A theoretical contributing factor to apparent negative calculated values for PKA is the presence of an inhibitor of kallikrein-like activity in PKS, and this was explored using acid-treated PKS. Figure 4 shows a summary of results using one of the immunoglobulin samples also used in Fig. 3, IG4 (dark grey bars). Figure 4 presents results from the one-stage method over the 3-point

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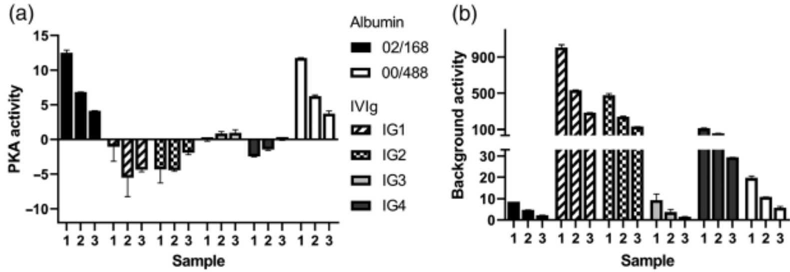

Fig. 3 Attempted determination of PKA in batches of immunoglobulin. Panel a shows results from a one-stage PKA assay using normal PKS, including results from the WHO 2nd IS (02/168, black bars) and internal control (00/488, white bars), where 3 doubling dilutions of samples are numbered 1–3. PKA activity is presented as rate of chromogenic substrate cleavage as absorbance change per $s^2 \times 10^9$, after subtraction of absorbance from no-PKS wells according to the normal protocol. The hatched and shaded bars are results from 4 different immunoglobulin batches, IG1 to IG4. Panel b shows the results for the same samples from no-PKS wells run in parallel for background kallikrein-like activity against chromogenic substrate as absorbance change per s $\times 10^6$. All results are means with SD error bars, n = 2.

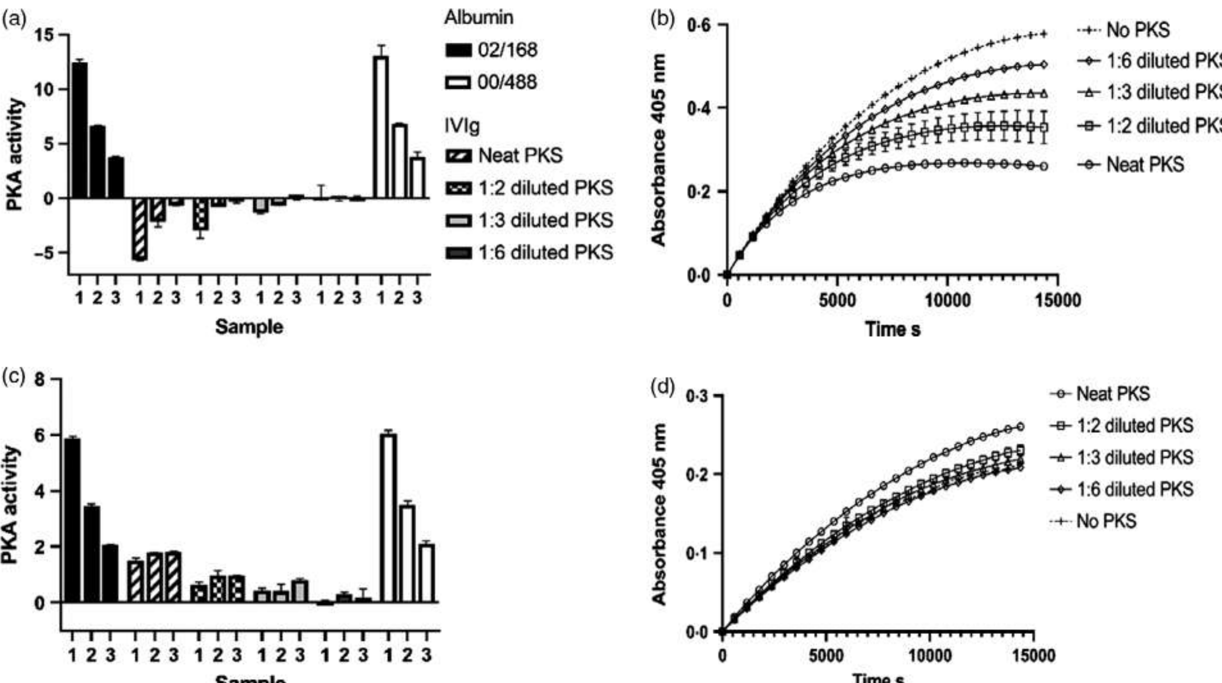

Fig. 4 Measurement of PKA in immunoglobulin and the effect of acid-treated PKS. Panel a shows results from a one-stage PKA assay using normal PKS, including results from the WHO 2nd IS (02/168, black bars) and internal control (00/488, white bars) alongside immunoglobulin sample IG4 using PKS neat (hatched bars), 2x dilution (checks), 3× dilution (light grey) and 6× dilution (dark grey). Each bar 1–3 is a 2-fold dilution of the albumin or immunoglobulin sample. PKA activity is presented as rate of chromogenic substrate cleavage in absorbance change per $s^2 \times 10^9$. Panel b shows the kinetics of kallikrein generation in a one-stage assay as absorbance at 405 nm versus time in s. A single concentration of IG4 is shown (5× dilution of stock 10% solution), over a range of PKS: neat, 2×, 3×, 6× dilutions and no PKS, as circles, squares, triangles diamonds and crosses, respectively. Panels c and d are identical to a and b but using acid-treated PKS. The bars and kinetic traces results are means with SD error bars, n = 2.

sample dilutions as shown in Fig. 3, but now also including a range of PKS concentrations so the influence of an enzyme inhibitor might be seen. Figure 4 shows both a summary of apparent final calculated values, after subtraction of background (panels a and c) and the raw kinetic data of all kallikrein-like activity before any subtraction of background rates (panels b and d) from a one-stage assay. Panels a and b are with normal PKS; c and d are with acid-treated PKS. Panel 4a again illustrates the issue of apparent negative values after background subtraction, which is dependent on the sample and PKS concentrations. Panel b explains the phenomenon. Without PKS (crosses), there is a high rate of kallikrein-like activity that rapidly hydrolyses

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chromogenic substrate (the decreasing rate over time is most likely due to chromogenic substrate depletion). However, as PKS is added in increasing concentrations, the rate of chromogenic substrate hydrolysis is depressed. The profiles seen in Fig. 4b are entirely consistent with a slow-binding inhibitor (e.g. a serpin) which gradually knocks out the activity of background amidolytic activity (e.g. see [21]). After acid treatment of the PKS, negative values of PKA are not calculated (Fig. 4c), but there is still no good dose–response for PKA determination with increasing immunoglobulin concentration, in each set sample dilutions 1–3, as there is for the albumin samples. The kinetics using the acid-treated PKS (Fig. 4d) do show a different pattern of amidolytic activity (the order of the curves is reversed in Fig. 4b and d), suggesting that the inhibitor proposed in 4b is much reduced after acid treatment of PKS. Increasing the concentration of acid-treated PKS does lead to a small increase PKA activity, as expected. It is apparent from Fig. 4b and d that under the conditions used in this assay, there would be minimal interference of the PKS inhibitor(s) for time-points below 45 min. Importantly, because more PKS is used in the first step in the Ph Eur assay, and this step has no competing chromogenic substrate present, there is more scope for inhibitor binding to existing or generated kallikrein, which could lead to an underestimate of PKA.

Discussion

The results presented above suggest that the newly developed one-stage assay for PKA works as well as the existing two-step assay to give the same results, but has a number of practical, economic and theoretical advantages, summarized below.

- Fewer manipulations and time critical steps
- Measures initial rates before significant substrate depletion (both PKS and S2302)
- Lower use of PKS: 40 μl per reaction in one-stage method, 90 μl in Ph Eur
- True rate of kallikrein generation is estimated using time squared transformation
- Reactions monitored throughout the measurement procedure (no blind first step)
- Large dilution of sample in well, by PKS and S2302 so efficient use of PKS
- True initial rates measured so less time for slow-binding inhibitors in the PKS
- Reaction wells and background wells can be observed in real time side by side
- The app performs time squared transformation and all rate calculations
- Plate layout can be varied to avoid plate edge or heating artefacts
- Blanking is clear and easy to understand and can be easily recorded
- The app performs a check on a set of data before each run
- Settings are recorded by the app and can be stored with data to aid reproducibility.

Testing, data analysis and results interpretation are usually straightforward for albumin, using existing methods or the one-stage variation, but problems have been noted when PKA is measured in some immunoglobulins [14]. Part of the problem may be due to a matrix effect because of the lack of a standard for PKA in immunoglobulins and the use of the albumin standard. However, the current work suggests that apparent negative results for PKA values after performing the expected blanking steps, are caused by high background levels of protease ('kallikrein-like' activity) and the presence of a corresponding protease inhibitor in the PKS. Albumin products rarely have high background rates, and the problem of apparent negative PKA activities, as seen in Figs 3 and 4 with immunoglobulins, is not observed. The subject of contaminating proteases in immunoglobulin preparations was a topic of major interest a few years ago when an increased rate of thromboembolic events (including strokes and venous or arterial thrombosis) was found to be associated with some batches of therapeutic immunoglobulins from some manufacturers. Subsequent research and collaborative efforts by manufacturers, regulators and researchers established that the procoagulant agent in the high-risk batches was activated factor eleven (FXIa) [14, 22, 23]. These studies also showed that the background 'kallikrein-like' activity routinely seen in PKA assays with albumin or immunoglobulins is dominated by kallikrein, but this is unlikely to contribute to prothrombotic activity. As none of the immunoglobulin samples we explored in the current work were implicated in thromboembolic events, it is likely that the 'kallikrein-like' activity we observed was in fact kallikrein. Nevertheless, it has been noted that high levels of contaminating kallikrein may be a cause for some concern and deserves more attention [13].

As mentioned above in Results, the suppression of amidolytic activity in Fig. 4 in the presence of PKS could be explained by the presence of a slow-binding inhibitor in the PKS, which can be largely eliminated by acid treatment. Currently, the identity of this inhibitor is not known. One of the advantages of the one-stage method is that the whole reaction showing the development of kallikrein and cleavage of chromogenic substrate is monitored, so problems can be observed. Furthermore, early

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initial rates are used, thus reducing the effect of remaining slow-binding inhibitors in the PKS. The traditional Ph Eur method includes the first, blind step of PK activation in the presence of a high concentration of PKS. Issues such as precipitate formation or interference by inhibitors in the PKS can occur during this step.

Overall, the updated one-stage method and associated app provides several theoretical, economical and practical advantages over the existing widely used two-stage Ph Eur method and should provide more reliable results when measuring PKA in immunoglobulin samples. If this approach is adopted more widely, we anticipate that variability of results obtained by different laboratories should be reduced.

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Slot blotting and flow cytometry: two efficient assays for platelet antibody screening among patients with platelet refractoriness

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Background and Objectives Frequent platelet transfusion may lead to the formation of alloantibodies and immune-mediated platelet destruction. Currently, identifying economic and effective screening methods is necessary for the management of platelet transfusion while different tests were recommended. The present study aims to challenge the performance of slot blotting (SB) and flow cytometry (FC) assays in detecting immune platelet refractoriness.

Materials and Methods Sera from 118 patients who received blood components and were clinically suspected of platelet refractoriness were enrolled. Platelet-reactive antibodies were explored in parallel by SB, FC and monoclonal antibody-specific immobilization of platelet antigens (MAIPA) techniques. In a further study, chloroquine-treated platelets were incubated with MAIPA-positive serum, and then, the results of the SB and FC techniques were compared.

Results Using MAIPA as a reference, antibodies were detected in 51 sera, with specificity for human leucocyte antigens (HLA), human platelet antigens (HPA) or both HLA/HPA, in 27, 18 and 6 patients, respectively. The sensitivity and specificity of SB and FC were 86·3%, 88·1%, 82·4% and 95·5%, respectively. The Spearman correlation revealed significant ($P < 0{\cdot}001$) correlations between FC ($r = 0{\cdot}763$) and SB ($r = 0{\cdot}738$) with MAIPA. In respect to HPA antibody detection, SB had 83·3% sensitivity and 92·6% specificity compared to 91·7% and 96·3% for FC while both approaches are acceptable ($P < 0{\cdot}001$, $r = 0{\cdot}69$; $P < 0{\cdot}001$, $r = 0{\cdot}773$) and can be recommended.

Conclusions The present study acknowledges that among the used methods, the flow cytometry's performance is the most appropriate, but slot blotting, with acceptable sensitivity, can be used as an acceptable and convenient procedure for platelet antibody screening.

Key words: flow cytometry, immune refractoriness, MAIPA assay, platelet, Slot blotting.

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Introduction

Platelets are small and non-nucleated cell elements that play important roles in haemostasis [1,2] and express various types of antigens, including ABO, class I human leucocyte (HLA) and platelet (HPA) antigens [3–6]. Thrombocytopenia due to platelet dysfunction, bone marrow suppression and chemotherapy increases haemorrhagic risk and may be associated with petechiae, purpura, ecchymosis, intracranial haemorrhage and even death [1]. Although platelet products are commonly used to attenuate bleeding disorders [3,7,8], frequent use may lead to platelet transfusion refractoriness (PR), which is defined as the failure to achieve an adequate increment in platelet count following consecutive transfusions [8,9]. This challenging and multifactorial scenario may be caused by immune and non-immune factors [10] the former of which involve HLA class I and HPA alloimmunization. The latter causes of PR are the consequences of splenomegaly, sepsis, antibiotic usage or consumption coagulopathy [10,11]. In an attempt to manage refractory patients, choosing platelets from antigen-compatible donors is pivotal [4]. Accordingly, testing for antibodies is technically demanding, and several common approaches such as enzyme-linked immunosorbent assay (ELISA) and monoclonal antibody-specific immobilization of platelet antigen (MAIPA) assay have been proposed for platelet cross-matching [12–16]. The former has low sensitivity, and the latter possesses some drawbacks such as being extremely costly, being very laborious and time-consuming [12]. Although the MAIPA assay is taken as a gold standard and permits the qualification and platelet antibodies' quantification [10], recognition of a rapid, effective, and economical antibody-screening technique is valuable for PR. Another important issue is discriminating between HLA class I and HPA alloantibodies. Previous experiments introduced chloroquine treatment of platelets to dissociate HLA class I antigens without affecting HPA antigens [17,18]. Flow cytometry (FC) is already known to be a reliable technique that has been widely used to detect antigens/antibodies on the cell surface through measurement of the fluorescence intensity emitted from an excited fluorochrome-conjugated antibody [19,20]. However, the analysis conducted using this method is subjective and needs skilled personnel and expensive instrumentation. Another used method, slot blotting (SB), is a molecular technique applied to detect proteins/biomolecules and represents a simplification of the Western blot method with the advantages of rapidity, low cost, simplicity and availability that allows multiple samples to be evaluated simultaneously [21,22]. In this method, the sample is loaded directly on a nitrocellulose membrane, and the blotting procedure is performed. It seems that this method, with some modifications, could be possibly used to evaluate the expression of cell surface antigens and serum antibodies. Based on our literature review, there is no experiment on the efficiency of this method for PR screening. Given the above concerns, a modified approach of SB was applied in the present study and the performance of SB and FC methods for antibody screening, before and after chloroquine treatment, was evaluated.

Methods

Patients

The current study was carried out during the period from October 2015 to January 2018 and was approved by the ethical review committee affiliated with Kerman University of Medical Sciences, Kerman, Iran. After receiving the informed consent, 118 patients (57 female and 61 male) suffering from acute leukaemias, lymphomas, myelodysplastic syndromes (MDS) and idiopathic thrombocytopenic purpura (ITP) with a median age of 36·4 years were enrolled (Table 1). Most of these patients were being treated for their haematologic or oncologic diseases and had received several leucocyte-contaminated blood components. To allow comparison, pooled platelets from 10 O+ healthy male donors, with no history of previous sensitization, were used as the platelet source in all assay. For platelet-rich plasma (PRP) preparation, ten millilitres of acid–citrate–dextrose (ACD) whole blood was centrifuged (300 g/10 min) and obtained PRPs were pooled. Next, stored serum samples (–80°C) from patients and serum control reagents were incubated with pooled PRP from the healthy donors and tested in parallel by SB, FC and MAIPA assays. These assays were performed in triplicate for each serum.

MAIPA Assay

The MAIPA assay was performed as a gold standard test to detect and discriminate platelet antibodies [23]. The assay was done as described by the manufacturer (apDia). Briefly, 50 µl of pooled platelets suspension (3 × 105 platelets/µl) was incubated with 50µl of patient's serum (60 min at 37°C). Washed platelets were treated with 50 µl of mouse monoclonal antibodies against platelet glycoproteins GPIb/IX, GPIIb/IIIa, GPIa/IIa and HLA/β2-microglobulin (apDia, 900004) in different wells (30 min at 37°C) and subsequently were washed 3 times with phosphate-buffered saline (PBS). Sensitized platelets were lysed with PBS containing 1% Triton-X-100 (15 min at 4°C); the lysates were then cleared by centrifugation (15 000 g/30 min); and 100 µl of it was placed in the wells of a microplate pre-coated with goat

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Table 1 Clinical and demographic characteristics of the enrolled patients

Age (mean)	Gender		Disease					
			Oncologic			Non-oncologic		
	Female	Male	AML	ALL	L	MDS	ITP	BSS
36.4	61 (51·7%)	57 (48·3%)	37	30	22	10	17	2

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; BSS, Bernard–Soulier syndrome; ITP, idiopathic thrombocytopenic purpura; L, lymphoma; MDS, myelodysplastic syndrome.

anti-mouse IgG antibody (incubated for 30 min at 37°C; apDia, 900005). After washing, horseradish peroxidase (HRP)-conjugated goat anti-human IgG (apDia, 900005) was captured by Human Platelet Antibody–Mouse Anti-Human Monoclonal Antibody–Platelet Glycoproteins Complex (30 min at 37°C). Non-adherent complexes were washed off; the substrate was added and followed by glycoprotein-specific antibody measurement (at 492 nm). As per the manufacturer's instructions, the Platelet Antibody Control Plasma/Serum Kit (apDia, 900003) was employed to validate the results, and the optical density (OD) after subtraction of the blank value exceeded 0·2 which was considered as a positive reaction.

Platelet immunofluorescence test by flow cytometry

To determine the platelet immunofluorescence among samples, the FC method was applied with some modifications according to the protocol originally described by Kiefel and Kohler [4,11]. For this purpose, freshly pooled platelets were rinsed with a washing solution containing 4% sodium citrate (Merck, 106448) and 0·1% bovine serum albumin (BSA; Sigma, A7030, Franklin Lakes, NJ, USA). Platelets were resuspended in washing solution after centrifugation (300 g/5 min), and the final concentration was adjusted to 3 × 105 platelets/μl. A hundred microlitres of platelet suspension (30 × 106 platelets) was treated with 100 μl of serum for 60 min at 37°C; then, the unbounded antibodies were eliminated through three consecutive washes (with 37°C washing solution) and centrifugation (300 g/5 min). Platelet pellets were resuspended in 50 μl of warm washing solution, and after the addition of 10 μl of rabbit immunoglobulin fraction antibody (Dako, X0903), the resulting mixture was incubated for 30 min at 37°C. After blocking, 10 μl of polyclonal fluorescein isothiocyanate (FITC)-conjugated F(ab')2 fragment rabbit anti-human IgG (Dako, F0185) was added to the platelet suspensions and left for 45 min at room temperature in the dark. The F(ab')2 fragment of IgG was used for minimizing background fluorescence. Finally, the washed platelet pellets were resuspended in 50 μl of washing solution, and 10 000 events were evaluated through the BD FACSCaliburTM flow cytometer and the CellQuestTM software (Becton Dickinson, Franklin Lakes, NJ, USA). We used two factors for the identification of positivity and negativity%, FC-related threshold, and ROC-specified cut-off value. The negative control (a serum that did not have any antibodies) was used for the threshold setting in the FC histogram. This sample was exposed to the pooled platelets and the experimental conditions which were used for patient samples. Considering this, mean fluorescence intensity% (MFI) fewer and higher than threshold level was regarded as negative cell population (M1) and positive cell population (M2), respectively. The assay was performed in triplicate for each sample, and the mean of corresponding MFIs was calculated. It should be noted that each positive cell population% does not mean PR. Using the best cut-off, determined by the ROC curve, we discriminate PR patients from other subjects. Namely, positive cell population% greater and lower than the cut-off value are correspond to PR patients and non-PR subjects, respectively. The negative and pooled positive control sera (apDia, 900003) were included in each determination.

Slot blotting

As described above, 100 μl of the sensitized platelet suspensions (washed platelets that treated with patients' serum) was prepared and then loaded into wells of slot blot apparatus (Hoefer™ PR 648, Thermo Fisher Scientific, Waltham, MA, USA) that were equipped with nitrocellulose membrane (GE Healthcare Life Sciences). The vacuum pump was set up at 13–25 cmHg and turned on. After complete transferring of the sensitized platelets onto the membrane (10 min), the non-specific sites were blocked (1 h at room temperature) using 5% (w/v) skimmed milk (Merck Millipore Corporation) in Tris-Buffered Saline Tween-20 (TBST), at pH 7·4. The membrane was then washed with TBST and incubated (1·5 h at room temperature) with horseradish peroxidase-conjugated goat anti-

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Human IgG (Fab')2 (1:80 000; Abcam, ab98535). Finally, after adding enhanced chemiluminescence reagent (Amersham ECL Prime Western Blotting Detection Reagent, RPN2232), immunolabelling was recorded (3 min exposure time) using Chemi Doc XRS + imaging system (Bio-Rad Company) and analysed by the Image Lab 3 software. It is necessary to mention that all incubations were carried out under continuous agitation, and also 5%–blocking buffer was used for antibody dilution. Appropriate controls were included in each determination, and the results were expressed as the mean percentage of band density decrease compared to the mean density of pooled positive control (aDdia, 900003). Three slots were considered for each sample, and the mean band density percentage was calculated using the following formula. As FC, the threshold value for SB was selected using a negative control reagent. Samples with Mean band densities (%) lower and higher than the threshold level were considered as negative and positive samples, respectively. Eventually, using the best cut-off point, determined by the ROC curve, we discriminate PR patients from other subjects.

Mean band density (%) = (mean density of three bands corresponding to each sample)/(mean density of three bands of pooled positive control) × 100.

Chloroquine-treated platelets

In another set of experiments, the efficiency of SB and FC assays in discrimination of platelet-reactive antibodies, in patients with positive MAIPA results, was evaluated. Chloroquine removes HLA class I antigens from platelets, thereby these cells no longer react with HLA class I antibodies, whereas reactions with HPA antibodies are unchanged [24]. Given this fact, chloroquine-treated platelets were prepared according to the Langenscheidt method [17]. For this purpose, acidified chloroquine was prepared by dissolving 10g of chloroquine diphosphate (Sigma, C6628) in 100 ml of PBS (0·2 M) and adjustment of pH at 4·0 (with 5N Hydrochloric acid). In the following, 5 ml of this solution was added to the 2 ml of platelet suspension, and the obtained mixture was incubated for 20 min at room temperature in the dark with continuous mixing. After being washed twice and centrifuged (250 g for 5 min), platelets were resuspended with the washing solution and then were used in the FC and SB assays, as described for untreated platelets.

Statistical analysis

Statistical analysis was performed using SPSS version 23 (IBM, New York, NY, USA). A receiver operating characteristic (ROC) curve analysis was performed to establish sensitivity and specificity corresponding to each cut-off value. According to the highest sensitivity and specificity, the best cut-off point was confirmed and, on that basis, the results were categorized as either true/false positive/negative. Subsequently, the performances of the FC and SB tests were compared with the MAIPA assay. In order to recognize the efficiency of FC and SB tests relative to MAIPA test, the positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LR+), negative likelihood ratio (LR–), the area under the ROC curve (AUC) value, 95% confidence interval for AUC and Youden's index for each test were calculated. The Youden's index is an approach for summarizing the performance of a diagnostic assay and ranges from 0 through 1. A zero value implies that the test is futile and not capable of discriminating among diseased and non-diseased groups. On the other hand, a value of 1 indicates that the test is perfect and there are no false positives/negatives. Furthermore, Spearman correlation analysis was applied for checking the correlation between the results of the MAIPA test (as a gold test) and that of the two other tests. The efficiency of each assay was also compared with the other methods by the Z test. $P < 0.05$ was considered as a significance level.

Results

Platelet antibodies were explored in the sera of 118 patients with suspected PR (Table 1) and also in commercially available control samples, by MAIPA, SB and FC. Respectively, the negative and pooled positive serum control reagents showed negligible and significant antibody binding with the mentioned methods (Fig. 1). The cut-off values were determined to be 13·2% and 13·09%, for FC and SB based on the patients' results and by employing the ROC curve analysis.

Out of the 118 patients, three patients gave a false-positive result in the FC, and eightpatients did in the SB. In addition, seven patients with false negativity in the SB and nine patients in the FC were definitely positive in the MAIPA assay (Table 5). It should be noted that the false-positive/negative results were confirmed using additional follow-up (post-transfusion response). Of the studied sera, 42 and 44 cases showed true positive, and 64 and 59 cases exhibited true negative reactions for FC and SB, respectively (Table 5). It is better to indicate that the concordance means similarity between MAIPA, FC and SB results, while discrepancy means FC and SB results are different from MAIPA findings. As described in Materials and Methods, test validation was performed through the evaluation of the sensitivity, specificity, predictive values, accuracy and likelihood ratios. The mentioned factors were calculated considering the MAIPA as a reference and using the cut-off values. As presented in Table 2, the

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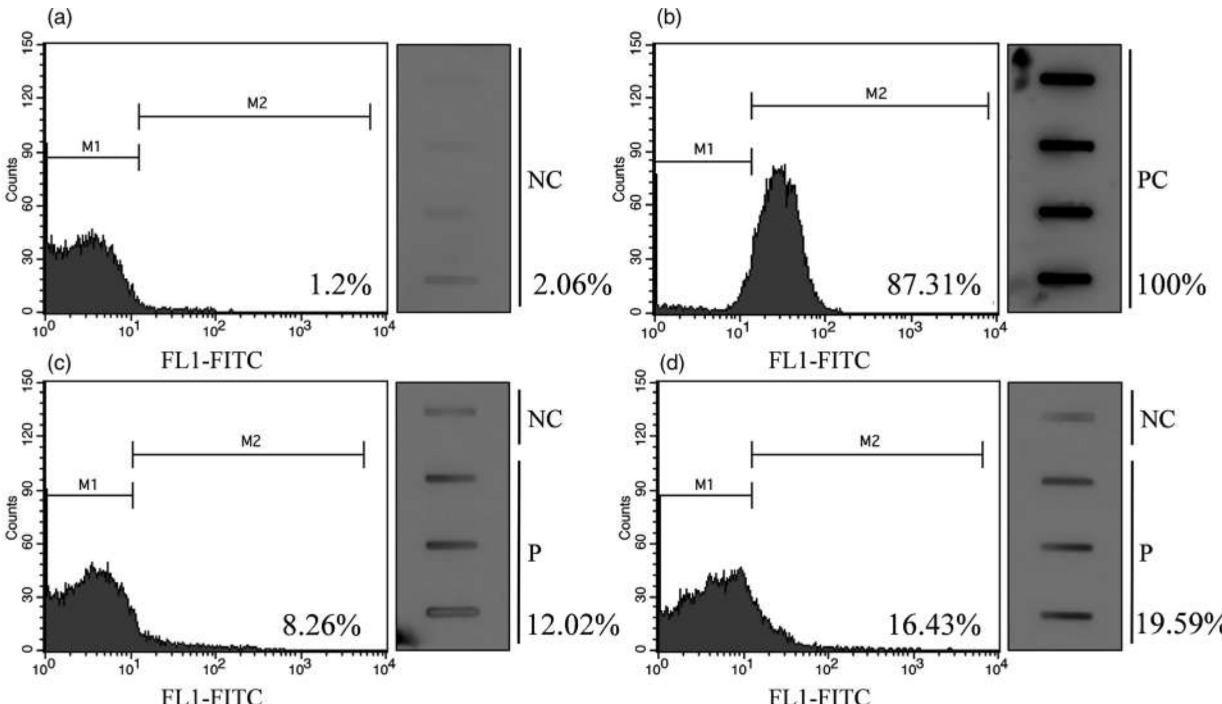

Fig. 1 The FC and SB results following the incubation of patients' and controls' sera with untreated platelets. Higher intensities (%) compared with the cut-off value were considered positive and vice versa. In consistent with the MAIPA, the negative control (a) depicted essentially negligible antibody binding with FC and SB methods. A positive control (b) along with two representative negative (c) and weak positive (d) patients are shown. The three 'P' bands represent triplicates for each sample. M1, negative cell population; M2, positive cell population; NC, negative control serum; P, patient serum; PC, positive control serum.

performance (sensitivity, specificity, negative predictive value, positive predictive value, negative likelihood ratio, positive likelihood ratio and accuracy) of both methods was satisfactory for PR screening.

The ROC curve analysis (Fig. 2) confirmed the satisfactory accuracy of our methods (Z valueAUC-0·5/standard error of AUC >1·96) and indicated insignificant (Z test P-value >0·05) superiority of the FC test over the SB, as the AUC and Youden's index values obtained for the former test (0·944 (0·896–0·993) and 0·779) were slightly better than those obtained for the latter method (0·93 (0·881–0·978) and 0·744). In addition, the results of the FC and SB showed a significant ($P < 0{\cdot}001$) correlation with MAIPA assay ($r = 0{\cdot}763$, $r = 0{\cdot}738$).

In another set of experiments, the HPA/HLA class I differentiation applicability of FC and SB in comparison with MAIPA assay was also analysed (Fig. 3). Patients with positive MAIPA results (PR cases) were taken up for study (Table 3).

Using MAIPA as a reference, antibodies were detected in 51 sera, with specificity for human leucocyte antigens (HLA) and human platelet antigens (HPA) or both HLA/HPA in 27, 18 and 6 patients, respectively (Table 3). Consistent with other studies, approximately 69·23% of patients had alloimmunization against HLA and 30·76% against HPA antigens when ITP and BSS patients were not taken into account [10,12]. In respect to ITP, out of 17 patients, 11 cases possess autologous antibodies that two of them co-expressed HLA and HPA antibodies (Table 4).

The cut-off values were estimated to be 10·59% and 10·87%, for FC and SB in chloroquine-treated platelets. Accordingly, it was observed that the former method reported true positives in 22 sera and true negatives in 26 sera. Two samples exhibited a negative reaction by FC but a positive reaction by MAIPA (false negative), and one serum yielded a false-positive result (Table 5). Consequently, the FC performance characteristics were calculated (Table 2).

When comparing SB to MAIPA, the SB was positive when the MAIPA was negative in 2, while the former was negative when the MAIPA was positive in four of the cases. Of the studied sera, 20 and 25 cases showed true positive and negative reactions, respectively (Table 5). Subsequent analysis revealed that the sensitivity of SB was 83·3%, and the specificity was 92·6% with a positive predictive value of 91% and a negative predictive value of 86%. The accuracy of the method was 88%, and the

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Table 2 Performance characteristics of FC and SB assays

	FC		SB	
	Before treatment	**After treatment**	**Before treatment**	**After treatment**
Sensitivity (%)	82·4	91·7	86·3	83·3
Specificity (%)	95·5	96·3	88·1	92·6
NPV (%)	87·7	92·9	89·4	86
PPV (%)	93·3	95·7	84·6	91
NLR	0·184	0·086	0·16	0·18
PLR	18·31	24·78	7·25	11·26
Accuracy (%)	89·8	94	87·3	88

FC, flow cytometry; NLR, negative likelihood ratio; NPV, negative predictive value; PLR, positive likelihood ratio; PPV, positive predictive value; SB, slot blotting.

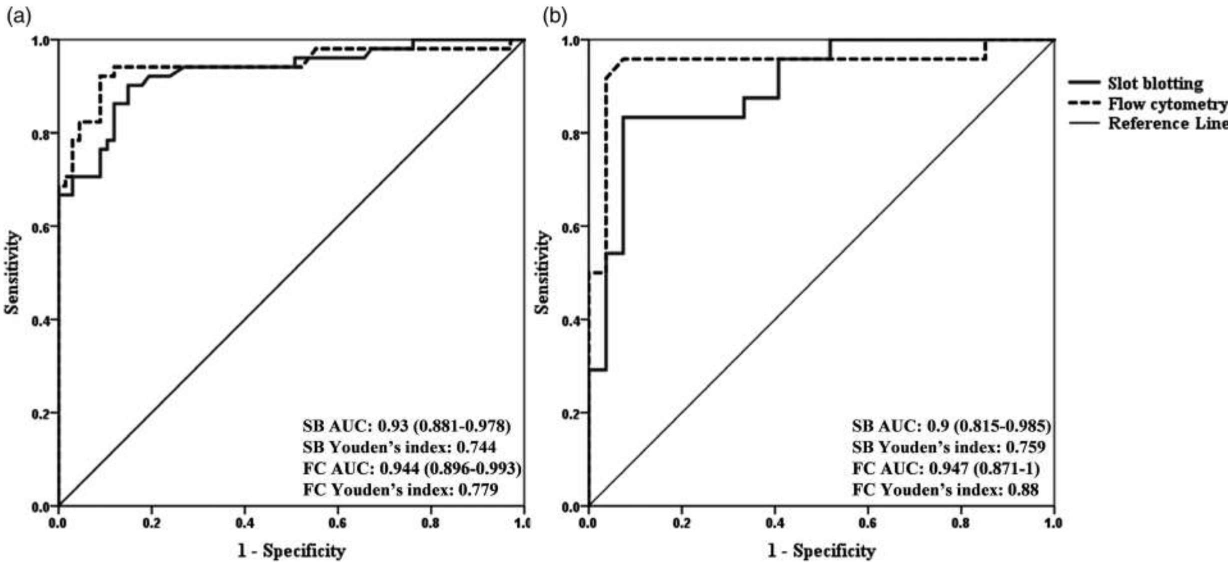

Fig. 2 ROC curves of the two methods, corresponding to before (a) and after (b) stripping. These curves and the corresponding AUCs show that FC and SB techniques had appropriate overall diagnostic accuracy to detect and discriminate platelet-reactive antibodies. The top-leftmost points of these curves, which have the highest sensitivity and specificity and the cut-off values that give these points in FC and SB curves, are 13·2% (a), 10·59% (b), 13·09% (a) and 10·87% (b), respectively. The AUC and Youden's index values are indicated in each graph. AUC, area under the curve.

positive and negative likelihood ratios were 11·26 and 0·18, respectively (Table 2). Furthermore, the AUC and Youden's index values for FC and SB after stripping were 0·947 (0·871–1), 0·88 and 0·9 (0·815–0·985), 0·759, respectively (Fig. 2). The satisfactory accuracy of our methods and insignificant ($P > 0·05$) superiority of the FC test over the SB was confirmed using Z value calculation (>1·96) and Z test analysis, respectively.

Finally, the FC results showed a significant correlation with MAIPA assay ($P < 0·001$, $r = 0·773$), whereas the corresponding values for the SB test were slightly lower than the FC test ($P < 0·001$, $r = 0·69$; Fig. 4).

Discussion

Achieving an adequate count increment after transfusion of platelets is a challenging issue that may significantly improve the patients' responsiveness and outcome. Optimal post-transfusion response depends on antibody screening for patients facing frequent platelet intake [2,25]. Currently, a variety of methods, with different pros and cons, have been suggested. However, the choice of methods that can be successfully used in refractory patients continues to be a problem [10]. The MAIPA, as a reference method, is a very complicated and time-

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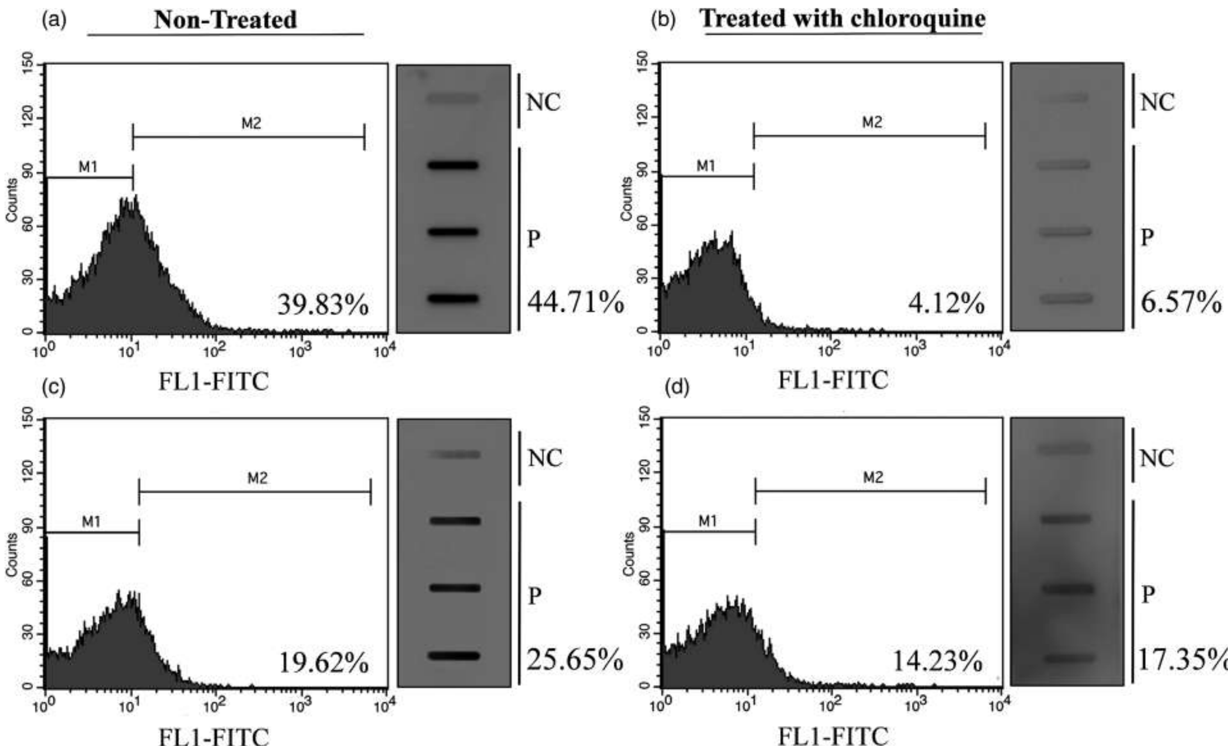

Fig. 3 Representative results of the FC and SB methods, before and after chloroquine treatment. Illustrations a and b indicate a patient with anti-HLA class I antibody, while c and d represent a patient with anti-HPA antibody. Accordingly, positive results after stripping (d) confirmed the presence of HPA antibodies in the patient (c), while a marked reduction in the antibody binding (negative results) was observed in the presence of HLA class I antibodies (b) in the corresponding subject (a). The three 'P' bands represent triplicates for each sample. M1, negative cell population; M2, positive cell population; NC, negative control serum; P, patient serum; PC, positive control serum.

Table 3 Demographic characteristics of HLA class I and HPA antibodies in the MAIPA-positive patients

Antibody specificity	Number	%
HLA class I only	27	52·9
HPA only	18	35·3
HLA class I + HPA	6	11·8
Total	51	100

HLA, human leucocyte antigen; HPA, human platelet antigen; MAIPA, monoclonal antibody-specific immobilization of platelet antigens technique.

Table 4 Glycoprotein (GP) specificities in HPA positive patients

GPs	Reactive sera
IIb/IIIa	7
Ib/IX	3
IIb/IIIa + Ia/IIa	9
IIb/IIIa + Ib/IX	3
IIb/IIIa + Ia/IIa + Ib/IX	2
Total	24

consuming approach for it to be used routinely [12,26,27]. Therefore, introducing a feasible, cost-effective and efficient screening method is imperative. The present study aimed to evaluate and compare the efficiency of the FC, SB and MAIPA assays, among 118 subjects with suspected PR, respecting various issues. Namely, these issues could be the determination of the presence, nature of platelet-reactive antibodies in patients' sera, and also the correlation rate of the first two tests with MAIPA assay. The present study is the first screening comparison of SB and FC for PR detection, but in the previous limited researches, SB was considered a relatively streamlined, sensitive, easily adaptable and applicable assay for detection of plasmodium-infected mosquito specimens, type I Glanzmann thrombasthenia and bovine herpesvirus type 1 [21,22,28].

The FC and SB results following incubation of the untreated platelets with sera of controls and patients are illustrated in Fig. 1. As evidenced, considerable differences in the antibody labelling of the positive control and PR patients were noted as compared with negative controls and PR-negative patients. Moreover, the PR-

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Table 5 Distribution of screening and discrimination of results using FC and SB assays

		Reactive samples in MAIPA (n = 51)			Non-reactive samples in MAIPA (n = 67)		
		SB	FC	SB + FC	SB	FC	SB + FC
Before treatment	Concordance	44	42	39	59	64	56
	Discrepancy	7	9	3	8	3	1
		Reactive samples in MAIPA (HPA positive samples, n = 24)			Non-reactive samples in MAIPA (HPA negative samples, n = 27)		
		SB	FC	SB + FC	SB	FC	SB + FC
After treatment	Concordance	20	22	18	25	26	24
	Discrepancy	4	2	0	2	1	0

MAIPA, monoclonal antibody-specific immobilization of platelet antigens technique; FC, flow cytometry; SB, slot blotting

Based on the MAIPA findings, the number of discrepancy (D) and concordance (C) results is also outlined. In terms of concordance, the FC + SB indicates the number of patients that provide the same FC and SB results with MAIPA.

In contrast, the FC + SB related to the discrepancy part means that both FC and SB results are different from MAIPA.

With regard to the C/C + D ratio, the specificity of SB and the sensitivity of FC tests were increased after chloroquine treatment.

There was no appreciable change in the FC specificity, while the sensitivity of SB was decreased after treatment.

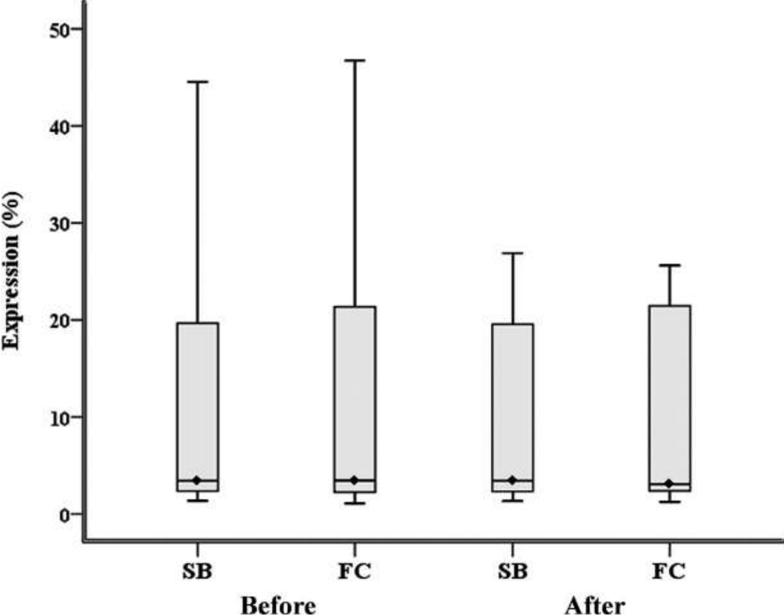

Fig. 4 Boxplots for the expression percentages of anti-platelet antibodies in patients' sera before and after chloroquine treatment. The central boxes exhibit the median (indicated with diamond) and the interval between the 1st and 3rd quartiles. FC, flow cytometry; SB, slot blotting.

screening results of FC and SB were correlated with reactivity in MAIPA. Estimated cut-off values for the FC assay (13·2% and 10·59%) were slightly different from that of the previous study, which reported a cut-off value of 12·0% [29]. As expected, FC as an applicable screening test had appropriate performance (Table 2), a result consistent with the previous studies [11,12]. For instance, Bonet Bub et al. reported good sensitivity, accuracy and high positive predictive value for this method in the detection of anti-HLA antibodies [10]. Kohler and colleagues also reported values of 94·7% and 96·3% for its sensitivity and specificity [12].

In the SB test, we determined the cut-off values to be 13·09% and 10·87%, before and after treatment and the

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authors found that the SB method demonstrated satisfactory PR-screening performance, which is almost similar to another used method (FC, Fig. 2). Apart from high sensitivity and specificity, the assessment of negative predictive value, positive predictive value, negative likelihood ratio, positive likelihood ratio and accuracy of this method yield reliable results (Table 2). As a remarkable finding, SB possesses a greater sensitivity than the FC test for screening of reactive antibodies in untreated platelets (Table 5). This can be the consequence of the higher sensitivity of chemiluminescence detection method rather than fluorescence-based assay. Accumulating evidence indicates that enhanced chemiluminescence technique is an ultra-sensitive approach that can detect a low concentration of the biological molecules and have a wide dynamic range and linear relationship between intensity and the concentration of the protein [30,31]. To keep the prozone phenomenon from occurring in false-negative samples, we have made serial dilutions from related serum samples and reperformed the tests. The results did not differ from those of the undiluted samples.

The evaluation of HPA detection performance was another contribution of the present study. In consistent with another study [10], the results of our gold standard method proved a more alloimmunization risk against HLA class I than HPA among the PR patients (Tables 3 and 4). Regarding the HLA/HPA discrimination, previous studies offer a promising option for HLA stripping and recommend that the treatment of platelets with chloroquine solution can yield HLA-free cells [18,24]. On the other hand, Srivastava and coworkers proposed that acidified chloroquine treatment through a configurational change of membrane glycoproteins and also HLA class I antigen removal can slightly improve the number and accessibility of available sites for HPA [25]. Accordingly, in the present study chloroquine-treated platelets were incubated with selected sera from the patients for whom MAIPA assay was positive, and then, the results of the SB and FC techniques were compared. As a general rule, the positive and negative results after stripping of platelets indicate that the serum antibodies were targeting HPA or HLA class I, respectively (Fig. 3). The concentration and pH of chloroquine solution (0·2 M, pH 4·0) were selected in accordance with the previous study [25] and were also confirmed using MAIPA evaluation of treated and untreated platelets which were incubated with control sera (apdia, 900003). In the present study, chloroquine treatment along with appropriate stripping causes minimal damage to platelets, and as outlined in the results section, the PR-screening performance of SB and FC assays was satisfactory after treatment. Namely, samples with anti-HLA antibodies show negative reactions while those with anti-HPA or anti-HLA + HPA exhibit positive results with chloroquine-treated platelets in FC and SB assays. Generally, false reactions may occur due to inappropriate chloroquine treatment of platelets (pH, concentration and incubation time), unsuitable preparation process, non-specific applied antibodies and use of an inappropriate number of platelet donors. In all mentioned instances, our methods can give suboptimal results. Instead, it seems that the use of labelled monoclonal secondary antibody and the removal of additional blocking/incubation/washing steps can contribute to maximizing the performance and rapidity of these methods. Given the ideality of chloroquine treatment for stripping, sensitivity reduction of SB tested with treated platelets is controversial. We are unable to determine the definitive cause of this phenomenon, but the plausible explanation could be the abundance of manipulation during the preparation process of SB, which may have affected the labile epitopes on the platelet membrane and enhances the false-negative results. ROC curve analysis further confirmed the insignificant inferiority of SB performance to that of the FC assay, and one can conclude that both of tests are useful for discriminating anti-HPA from anti-HLA class I antibodies (Fig. 2). Correlation analysis underscored this conclusion as the FC and SB results (Fig. 4) were consistent with those of the MAIPA assay. Totally, the present study established that chloroquine treatment can effectively eliminate HLA class I antigens from the platelets surface and both SB and FC are sensitive and suitable enough to screen and differentiate HLA class I from HPA antibodies. Nevertheless, these assays could not identify the specific platelet antibodies. Subsequent testing for antibody identification using other assays (MAIPA, Single antigen bead test, etc.) is recommended. Furthermore, history- and clinical symptoms-based interpretation of results is suggested. Because chloroquine treatment of platelets gives no different results for negative samples, so, HLA and HPA discrimination should be done on positive FC/SB samples. Although FC had somewhat more overall specificity and sensitivity than SB assay, the major obstacles are unavailability, need to remarkable technical expertise and expensive instrumentation. It is noteworthy that lower sensitivity and specificity and less automatic procedure of the SB may be outweighed because of its superior simplicity, practicability, rapidity and cost-effectiveness. However, avoidance of unnecessary manipulation in the sample preparation process and determination of the reference range and cut-off value in each individual laboratory are critical for accurate reporting of SB results.

Conflict of Interest

The authors declare no conflict of interests.

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Authors contribution

A.F proposed the original concept and designed the experiment and supervised all aspects of the work. R.V, Z.A, M.K and A.S equally participated in data acquisition and practical work. R.V, T.D, M.K and Z.A contributed to the data analysis. All authors contributed to writing the manuscript and final approval of the version to be submitted.

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Daratumumab interference in flow cytometric anti-granulocyte antibody testing can be overcome using non-human blocking antibodies

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Vox Sanguinis

Background Daratumumab (DARA), a human IgG1$_K$ monoclonal antibody targeting CD38, is used to treat refractory multiple myeloma patients. CD38 is expressed on many cell types (RBCs, granulocytes, lymphocytes, etc.), and thus, DARA can interfere with serological tests. Information regarding how DARA affects anti-granulocyte antibody (AGA) testing and optimal neutralization of DARA will help laboratories perform accurate testing.

Methods Screening of AGA was performed by the granulocyte agglutination test (GAT) and the flow cytometric granulocyte immunofluorescence test (Flow-GIFT). Samples were tested from patients on DARA (n = 7), non-transfused blood donors (healthy controls, n = 7) and AGA reactive samples (positive controls, n = 5). Two neutralization experiments, CD38 removal with DTT and DARA epitope blockage with mouse anti-CD38, were evaluated.

Results Positive reactivity of human IgG binding was observed in 5/7 DARA cases when tested by Flow-GIFT; however, all 7 cases had negative GAT agglutination results. Further studies by Flow-GIFT revealed DARA concentrations >0·63 μg/ml bound to granulocytes. DARA binding was negated by DTT though a reduced Flow-GIFT sensitivity was observed in positive control samples due to increased background detection of human IgG. Mouse anti-CD38 neutralized the detection of human IgG observed in DARA-treated patient serum without effecting controls.

Conclusion We established that DARA can interfere with AGA testing, leading to false positive Flow-GIFT results without causing GAT agglutination. DTT treatment increased background binding of secondary antibodies causing a decrease in Flow-GIFT sensitivity. In comparison, blockage of the DARA binding epitope using mouse anti-CD38 antibody was effective in neutralizing DARA interference while maintaining Flow-GIFT sensitivity.

Key words: Daratumumab, DARA, granulocyte, anti-granulocyte antibody.

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Introduction

Daratumumab (DARA) is a human IgG1$_K$ monoclonal antibody that targets the CD38 glycoprotein located on the cellular surface of neoplastic plasma cells in multiple myeloma (MM). While CD38 has proven to be an effective therapeutic target [1–2], its expression is not exclusive to MM; it is found on multiple other haematologic cell types including red blood cells (RBCs), medullary thymocytes, activated B and T cells, natural killer cells, monocytes, granulocytes and others [3–4]. This ubiquitous and varied expression has led to interference in laboratory testing for patients currently taking DARA – particularly in transfusion medicine where

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its expression on RBCs significantly complicates serologic evaluations leading to false positive results in both typing and screening [5–9]. More specifically, this interference is due to the secondary antibody in these assays reacting with the DARA bound to CD38 on the surface of the panel cells, resulting in pan reactivity preventing identification of underlying allo-antibodies.

Daratumumab's prevalence and implications to red cell testing has led AABB to recommend removal of CD38 with dithiothreitol (DTT) pretreatment [10]. However, while this effectively neutralizes DARA related signal, it destroys Kell blood group antigens in the process. For this reason, prospective Kell typing before beginning DARA treatment or the use of K antigen negative RBC units is recommended. Despite this off-target limitation, DTT treatment has proven to be a very successful method for managing DARA interference with RBC antibody testing.

Daratumumab interference may not be exclusive to red cell testing and could similarly impact other serologic assays. MM patients often take DARA in combination with other chemotherapeutic agents, predisposing them to bone marrow suppression with multi-lineage cytopenias. An autoimmune processes mediated by anti-granulocyte antibodies (AGAs) should be ruled out. The granulocyte immunofluorescence test (GIFT), a serologic based assay, is often used in this context [11]. Briefly, patient and control sera are incubated with donor granulocytes and bound antibodies are detected using a fluorescently labelled anti-human IgG secondary antibody analysed by microscopy or flow cytometry (Flow-GIFT) [12]. Results are interpreted as positive when the patient serum's fluorescence intensity is greater than the negative control. A major limitation inherent to secondary anti-human IgG antibody capture is decreased specificity and concomitant increased risk of false positive results due to non-AGA IgG reactivity. False negative results of clinically significant AGAs directed against human neutrophil antigens (HNA), particularly anti-HNA-3a associated with transfusion-related acute lung injury, are also known to be a limitation of GIFT and the inclusion of the granulocyte agglutination test (GAT) to assess aggregation upon antibody binding is recommended for more accurate screening [13].

We hypothesized that AGA testing on patients currently taking DARA would lead to false positive results. We evaluate two methods to overcome DARA interference on granulocytes including DTT removal of CD38 target antigen and blockage of the DARA epitope with mouse anti-CD38.

Materials and methods

Study serum samples

Anti-granulocyte antibody testing compared serum collected from patients undergoing DARA treatment ($n = 7$) against healthy non-transfused male paid donors ($n = 7$). Positive controls ($n = 5$) included serum from patients with defined AGA reactivity (P1 = anti-HNA-1b, P2 = anti-HNA-2, P3 = anti-HNA-3a, P4 = anti-HNA-4a, P5 = pooled positive AGA serum). Previously tested serum without AGA reactivity collected from healthy non-transfused male paid donors was used as negative controls ($n = 5$).

Donor granulocytes

A granulocyte donor panel ($n = 3$) typed for HNA by sequence-specific primer PCR (HNA Genotyping Tray, One Lambda, West Hills, CA) was used to detect AGA test positivity (panel HNA types: A = HNA-1b, -2, -3a, -4a, -4b, -5a, -5b; B = HNA-1b, -2, -3a, -4a, -5a; C = HNA-1a, -1b, -2, -3a, -4a, -5a, -5b). Residual EDTA whole blood from de-identified healthy paid donors was obtained as part of routine clinical control collection from the Mayo Clinic Normal Values Group. Granulocytes were isolated by centrifugation of leucocyte-rich plasma formed by sedimentation with methyl cellulose (Sigma, St. Louis, MO) layered over a double phase density gradient using lymphocyte separation medium (MP Biomedicals, Solon, OH) and omnipaque (GE Healthcare, Marlborough, MA). Collected cells were washed 3 times with Dulbecco's Phosphate-Buffered Saline (DPBS) (Life Technologies, Grand Island, NY) containing 0·2% bovine serum albumin (BSA) and counted on a XP-300 Automated Hematology Analyzer (Sysmex, Lincolnshire, IL). Granulocyte purity was >90% and adjusted to a 5×10^3 cells/μl concentration for use.

Granulocyte agglutination testing

In GAT, granulocytes (5×10^3 cells/well) were incubated with 3 μl of serum for 4 h at room temperature in an oiled Terasaki plate. Agglutination results were observed on an inverted microscope and characterized as negative (–), weakly positive (+), positive (++) and strongly positive (+++).

Flow cytometric granulocyte immunofluorescence testing

In Flow-GIFT, granulocytes ($1{\cdot}25 \times 10^5$ cells/well) were incubated with 25 μl of serum for 30 min at 37°C. Cells were washed four times with DPBS containing 0·2% BSA and then incubated with goat F(ab')2 anti-human IgG FITC (Jackson ImmunoResearch, West Grove, PA) for 30 min at room temperature. An additional 3 washes were done prior to analysis by flow cytometry (FACS-Canto II using FACSDiva software, BD Biosciences, San

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Jose, CA). Results were interpreted as negative (–), weakly positive (+), positive (++) and strongly positive (+++) based on the median fluorescence intensity (MFI) of a test serum compared to the standard deviation of negative controls.

Quantification of CD38 expression

Mouse anti-CD38 FITC (BD Biosciences, clone HB7) was used to assess CD38 expression on granulocytes compared to mouse IgG1κ FITC isotype control (BD Biosciences, clone MOPC21). Granulocytes ($1{\cdot}25 \times 10^5$ cells/well) were incubated for 30 min at room temperature and washed 3 times with DPBS containing 0·2% BSA before flow cytometry analysis.

DARA binding studies

Granulocytes ($1{\cdot}25 \times 10^5$ cells/well) were incubated for 30 min at room temperature with increasing concentrations (0·04–500·0 μg/ml) of DARA (Janssen Biotech, Horsham, PA) compared to human IgG1κ isotype control (Southern Biotech, Birmingham, AL). Cells were washed 3 times with DPBS containing 0·2% BSA and incubated with goat $F(ab')_2$ anti-human IgG FITC for 30 min at room temperature. An additional 3 washes were done prior to flow cytometry analysis.

DARA neutralization studies

Parameters for removal of CD38 by DTT (Sigma-Aldrich, Saint Louis, MO) treatment were established by titration (0·1–1 M DTT) with increasing incubation times (10–30 min) at 37°C with granulocytes (5×10^6 cells) from 3 donors. Cells were washed 3 times and evaluated for CD38 expression as described above, which determined minimal conditions used in Flow-GIFT tests for effective CD38 removal at 0·2 M DTT for 20 min at 37°C.

Parameters to block DARA binding by mouse anti-CD38 (BD Biosciences, clone HB7) treatment were established by incubating granulocytes ($1{\cdot}25 \times 10^5$ cells/well) in 25 μl of increasing concentrations of mouse anti-CD38 (6·25–25 μg/ml, 20 min at room temperature) followed by incubation with increasing concentrations of DARA (0·63–500 μg/ml, 30 min at room temperature). DARA binding was measured as described above to determine conditions for effective inhibition.

Anti-granulocyte antibody testing was repeated in experiments to overcome DARA interference with donor granulocytes (n = 3) split, treated and tested in parallel with untreated cells. Treated granulocytes included cells incubated in 0·2 M DTT for 20 min at 37°C followed by 3 washes with DPBS containing 0·2% BSA or incubated with 25 μl of pure mouse anti-CD38 at 25 μg/ml for 20 min at room temperature.

Results

DARA treatment can cause false positive anti-granulocyte antibody results by Flow-GIFT, but did not cause agglutination in GAT

Positive reactivity was observed in 5 of the 7 DARA-treated patients tested by Flow-GIFT, while no reactivity was observed in GAT (Table 1). Healthy controls (n = 7) tested in parallel had no reactivity observed in either test. Representative data from granulocyte donor A in Table 1 illustrate the Flow-GIFT positive detection of anti-human IgG from AGA positive control sample P3 (Fig. 1a) and DARA sample D2 (Fig. 1b) collected from a patient 30 days after a 16 mg/kg DARA dose.

Quantification of CD38 on granulocytes and DARA binding

The presence of CD38 on granulocytes was quantified and compared to isotype control. Data from granulocyte donor A in Table 1 showed the level of DARA binding detected (Fig. 1b) compared similarly to CD38 expression (Fig. 1c). DARA binding to granulocytes was studied using titration (0·04–500·0 μg/ml) with pure DARA drug. DARA concentrations >0·63 μg/ml bound significantly to granulocytes as detected by anti-human IgG by flow cytometry (Fig. 2a – Untreated line).

CD38 removal by DTT increases background binding of secondary antibody to granulocytes

Though DTT treatment was found to remove CD38 expression and reduce DARA binding, overall Flow-GIFT sensitivity was reduced in AGA positive controls (Table 1). An increase in background binding of secondary anti-human IgG antibody was observed in negative control serums, as illustrated in Fig. 2b by the shift in negative control serum MFI in DTT treated compared to untreated cells (dotted line). This caused calculated values for assigning test positivity to increase when compared to untreated cells.

Blocking with mouse anti-CD38 reduces detection of DARA binding while maintaining test sensitivity

Daratumumab binding inhibition studies revealed reduced DARA binding in a concentration-dependent manner when pre-incubated with pure mouse anti-CD38 (Fig. 2a – Mouse

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Table 1 Results of anti-granulocyte antibody detection in the granulocyte agglutination test (GAT) and the granulocyte immunofluorescence test (GIFT) +/– treatment with DTT or mouse anti-CD38 (HB7) A granulocyte donor panel (A, B and C) was tested against positive AGA controls (P1-P5), DARA patient serums (D1-D7) and healthy control serums (H1-H7) for negative (–), weak positive (+), positive (++) and strong positive (+++) reactivity

		GAT			GIFT			GIFT + DTT			GIFT + HB7		
		A	B	C	A	B	C	A	B	C	A	B	C
Positive Controls	P1	++	++	+	+++	+++	+++	++	++	++	+++	+++	+++
	P2	+	+	+	++	++	++	+	+	+	++	++	++
	P3	++	++	++	+	+	+	–	–	–	+	+	+
	P4	+	+	+	++	+++	++	+	+	+	++	++	++
	P5	+++	+++	+++	+++	+++	+++	+++	++	++	+++	+++	+++
DARA Samples	D1	–	–	–	+	+	–	–	–	–	–	–	–
	D2	–	–	–	+	+	+	–	–	–	–	–	–
	D3	–	–	–	–	–	–	–	–	–	–	–	–
	D4	–	–	–	+	+	–	–	–	–	–	–	–
	D5	–	–	–	–	–	–	–	–	–	–	–	–
	D6	–	–	–	+	+	+	–	–	–	–	–	–
	D7	–	–	–	+	+	+	–	–	–	–	–	–
Healthy Controls	H1	–	–	–	–	–	–	–	–	–	–	–	–
	H2	–	–	–	–	–	–	–	–	–	–	–	–
	H3	–	–	–	–	–	–	–	–	–	–	–	–
	H4	–	–	–	–	–	–	–	–	–	–	–	–
	H5	–	–	–	–	–	–	–	–	–	–	–	–
	H6	–	–	–	–	–	–	–	–	–	–	–	–
	H7	–	–	–	–	–	–	–	–	–	–	–	–

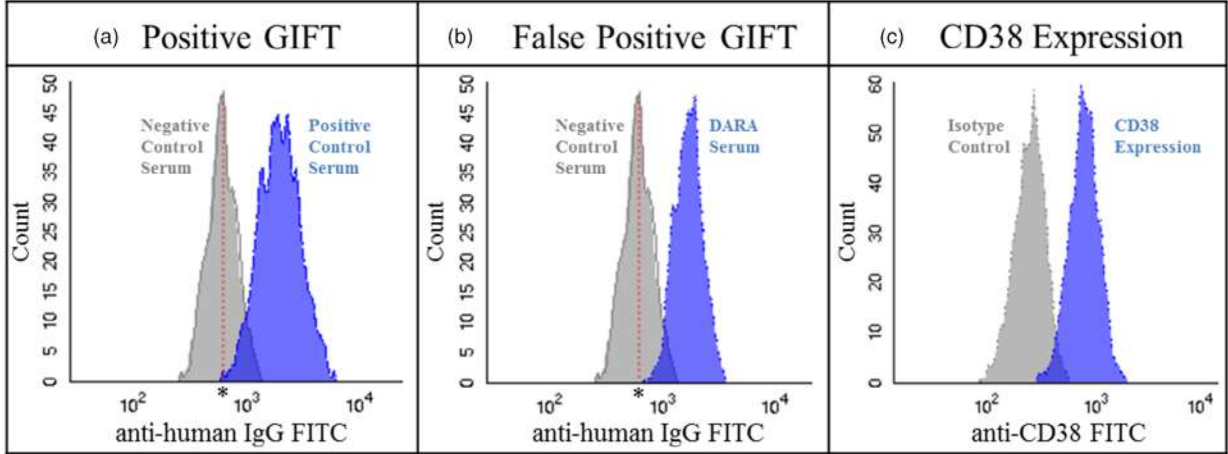

Fig. 1 A representative case of DARA interference in Flow-GIFT. (a) Positive assessment of anti-granulocyte antibody detected by anti-human IgG FITC secondary antibody after incubation of granulocyte donor A with positive AGA control P3. (b) Positive assessment by anti-human IgG FITC secondary antibody after incubation of granulocyte donor A with DARA sample D2. (c) CD38 expressed on granulocyte donor A detected with anti-CD38 FITC compared to isotype control. *Note: The dotted line denotes the background MFI observed in untreated granulocytes incubated with negative control serum for comparison of treatments in Fig. 2.

anti-CD38 lines). In Flow-GIFT, negative and positive control MFI values were comparable in treated vs. untreated cells indicating mouse anti-CD38 did not affect the ability to detect AGA (Table 1). DARA sample D2 tested after treatment with mouse anti-CD38 showed reduced anti-human IgG detection (Fig. 2c) and had negative AGA results.

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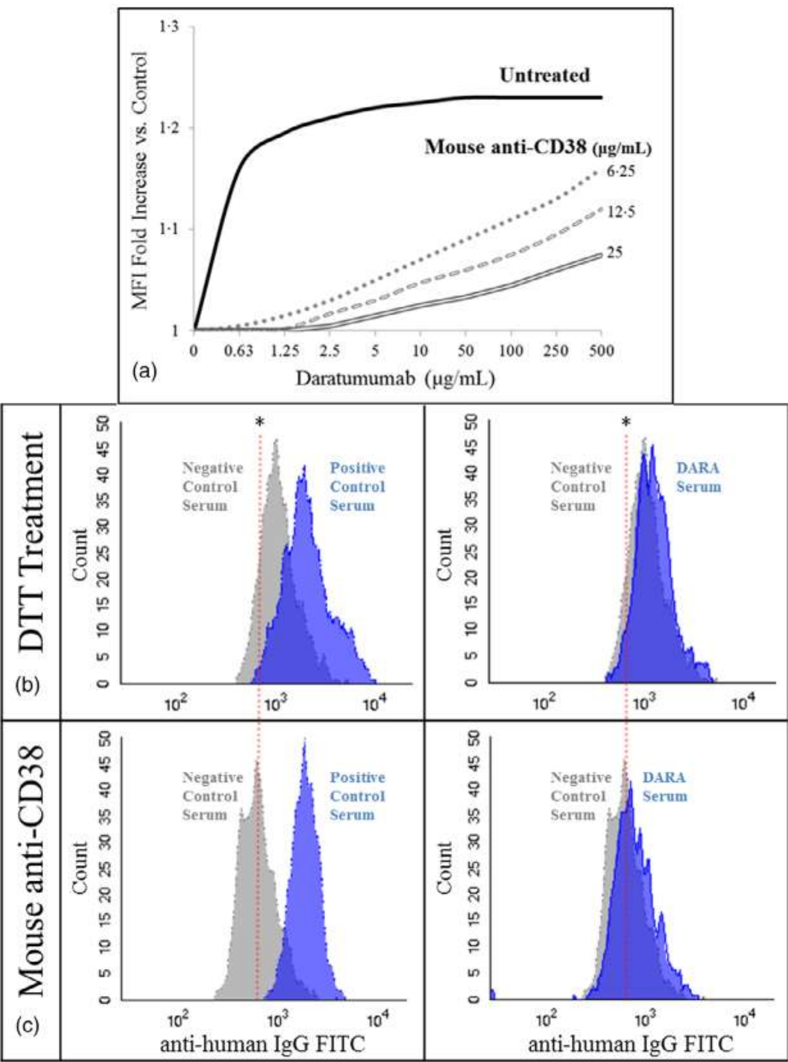

Fig. 2 Evaluation of granulocyte treatment to overcome DARA interference in Flow-GIFT. (a) DARA binding curve observed in untreated (black line) and mouse anti-CD38 treated (dashed lines) granulocytes showed a concentration-dependent response. (b) DTT treatment reduced test sensitivity and caused a negative result in positive AGA control P3 due to increased background of anti-human IgG FITC secondary antibody. (c) Mouse anti-CD38 treatment maintained test sensitivity observed in positive AGA control P3 and had a negative result on serum from DARA sample D2. *Note: The dotted lines in B and C signify the background MFI observed in untreated granulocytes incubated with negative control serum from Fig. 1.

Discussion

DARA targets CD38 expressed on plasma cells treated in MM; however, CD38 is also expressed at various levels on other cell types. This off-target binding can affect diagnostic testing when using secondary anti-human antibodies. This DARA-mediated interference is most often recognized in red cell antibody testing and several approaches to overcome DARA-mediated interference exist. CD38 antigen destruction by DTT is performed by most reference laboratories across the country because of the ease of use and low cost [5]. Similar to other cleaving agents, DTT's effect is not restricted to CD38 and several other cell surface proteins are impacted. Recently, targeted approaches for overcoming DARA interference have been developed: soluble CD38, anti-idiotype antibody and F(ab')$_2$ fragments of DARA. Unfortunately, these alternate methods are expensive or not commercially available [5, 7, 14, 15].

Our AGA testing results demonstrated that DARA interfered with Flow-GIFT but not GAT. As Flow-GIFT detects the presence of bound antibodies with a secondary antibody while GAT directly measures agglutination, one can speculate that the level of DARA binding to granulocytes

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does not trigger the more biological process of chemotaxis. Because granulocytes express lower levels of CD38, DARA interference may be less obvious and more concentration-dependent. Our drug titration experiment accounted for this variability and included a range of DARA concentrations including clinically routine bioavailable levels.

We demonstrated an approach using mouse anti-CD38 can successfully block DARA from binding CD38 expressed on granulocytes. Murine antibodies have a unique Fc portion that is not recognized by anti-human IgG secondary antibodies. The different Fc prevents test interference, while mouse anti-CD38 clone HB7 effectively binds an epitope (serine at position 274) that is important for DARA binding [1, 16]. We leveraged this feature to more specifically block DARA interference. While we demonstrated that this method is dose dependent and that high DARA concentrations can overcome this inhibition, it is important to note that even at the highest DARA levels tested, the murine anti-CD38 treatment significantly reduced signal. This suggests clinical utility through the expected range of DARA concentrations (241–905 μg/ml) found in patient serum [2].

Our approach provides the first commercially available targeted solution to DARA interference that is inexpensive, easy to use and does not remove surface antigens with its associated increase in assay background. Additional humanized antibody drugs are under development, and their introduction will likely further complicate serologic testing [17]. By selecting non-human antibodies specific to overlap of epitope targets, this method may be adapted to prevent clinical antibody testing interference caused by future drugs.

In conclusion, we confirmed that DARA can interfere with AGA test results. We evaluated granulocyte DTT treatment and, while it successfully removed CD38 from granulocytes, it unexpectedly made test results difficult to interpret by increasing background signal by non-specific binding of secondary antibodies. In comparison, our novel approach using murine anti-CD38 antibody pretreatment was as effective in eliminating interference without increasing background.

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None.

Conflicts of interest

The authors declare no conflict of interests.

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GMP-grade CD34$^+$ selection from HLA-homozygous licensed cord blood units and short-term expansion under European ATMP regulations

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Vox Sanguinis

Background Based on a synergistic consortium, the cord blood (CB) bank Düsseldorf was responsible for the selection of HLA-homozygous (HLA-h) donors, contacting/re-consenting the mothers, Good Manufacturing Practice (GMP)-grade CD34$^+$ enrichment, followed by short-term expansion of CD34$^+$ cells and qualification of the resulting CD34$^+$ population as advanced therapy medicinal product (ATMP)-starting material. Among 20 639 licensed Düsseldorf cord blood units (CBUs), 139 potential HLA-h donors were identified with the most frequent 10 German haplotypes. 100% of the donors were contacted, and for 47·5%, consent was obtained. HLA-A, -B, -C, -DR, -DQ and -DP were determined by sequencing.

Methods Thawing/washing of the CBUs was performed in the presence of Volulyte/HSA with Sepax®, CD34$^+$ selection by automated CliniMACS®-system (Miltenyi), expansion with qualified GMP-grade cytokines and media in the GMP facility.

Results Here, we specify minimal criteria (≥5 x 10^5 viable CD34$^+$-count, ≥80% CD34$^+$-purity and ≥70% viability) and confirm that n = 10 CB units (max storage time 16 years) could be qualified for an ATMP starting material. The mean fold change expansion of isolated CD34$^+$ cells at Day 3/4 (d3/4) was 3·38 ± 3·02 with a mean purity of 86·90 ± 10·38% and a high viability of 96·07 ± 4·72%.

Conclusion As of March 2019, approval was obtained by the Bezirksregierung Düsseldorf for the GMP-compliant production. The production of HLA-homozygous expanded CD34$^+$ cells from cryopreserved CB under European ATMP regulations presented here describes the successful clinical translation and implementation of a qualified manufacturing process. This approach considers the main obstacle of rejection of transplanted cells (due to the immunological HLA barrier) by preselection of HLA-homozygous transplants.

Key words: blood component production, blood donation testing, expansion, hematopoietic stem cell, HLA, quality control.

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Introduction

In 2018, our cord blood bank (CBB) got involved into the 'establishment of a Good Manufacturing Practice (GMP)-compliant induced pluripotent stem cell (iPSC) bank of HLA-homozygous (HLA-h) umbilical cord blood (CB) for the production of allogeneic cell therapeutics', funded by the 'The Federal Ministry of Education and Research grant' 031B0760B (2019-2021) 'KMU-Innovativ-21: HLA-iPS-GMP'. The compliance with GMP as the essential part of the pharmaceutical quality system and the procurement of licensed cryopreserved CB units (CBUs) is one of the main advantages of allogeneic CB to

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be qualified as starting material for ATMP´s (advanced therapy medicinal products) [1]. Within the project, our CBB provided HLA-h CD34+ haematopoietic stem cells (HSC) for iPSC reprogramming. The iPSC derived from the CB (CD34+) has the potential for anti-tumour approaches and NK-cell therapy [2,3]. Clinical-grade iPSC can serve as a potential therapy for age-related macular degeneration (AMD) for regeneration of retinal pigment epithelium (RPE) [4–6].

The unrelated CBUs were produced under GMP compliance, have a licensure by the Paul-Ehrlich-Institute, a production permission by the Bezirksregierung Düsseldorf and are accredited by the International Foundation for Accreditation of Hematopoietic Cell Therapy (FACT). As of March 2019, approval was obtained by the Bezirksregierung Düsseldorf (DE_NW_03_MIA_2018_0019/24.05.05.02-HHU-ITZ) for the production of HLA-h expanded CD34+ cells from cryopreserved CB as a starting material for an iPSC-derived ATMP. All methods required for immunological, genetic and hematopoietic characterization were established in the group before, for expansion in a clinical/research setting [7,8] and for iPSC in research projects [9,10]. Few highly selected homozygous donors could provide the maximum practical benefit for HLA matching [11–13]. Homozygous HLA genotypes are present in an individual due to parents transmitting identical haplotypes to their offspring, and some HLA haplotypes are found more frequently in some populations. Based on the HLA homozygosity, no rejection reaction is expected in a transplant situation [14,15]. The 10 selected HLA-h CBUs have already undergone routine testing as cell count, CD34+-count, blood group, viability, colony-forming assays, sterility testing, exclusion of viral markers as CMV, Parvo B19, Hepatitis B, C, HIVI/II, HLTVI/II, Lues, and hemoglobinopathy on the mother and the CBU. Regarding the preselection of appropriate HLA-homozygous CBUs: generic HLA class I typing (A, B, C) and HLA class II subtyping DR, DQ was primary available. Maternal HLA-typing information for all homozygous CBUs was analysed, where the mother provided the re-consent. The homozygous CBUs were sequenced for HLA-A, -B, -C, -DRB1, -DQB1 and -DP; however, HLA-DP is known for its linkage equilibrium of about 20%. Although an autologous stem cell resource would be the best option for iPSC, the use of autologous iPSC will be lower since they derive from older donors with co-morbidities (ageing effects, mutation of skin cells) [16]. The biological age is therefore relevant for the application of CD34+/IPSC [15,17–21].

Enriched CD34+ cells instead of mixed mononuclear cells (MNC) were chosen (pure population is preferred by our regulatory authorities) which can be easily reprogrammed [10,22,23], in addition to the fact that efficient reprogramming of CD34+ cells into iPSC relies on a high proliferating potential making them a preferred starting population in comparison with differentiated MNC or T cells [24]. Therefore, the CD34+ population will be expanded for few days to keep them in a proliferating status without differentiation. CB CD34+ cells are preferred over normal skin cells, since skin-derived iPSC harbour mutations that were likely caused by UV-light exposure representing the mutational burden of iPSC [25,26].

HLA-homozygous CD34+ cells were isolated from cryopreserved CBUs applying the GMP-compliant CliniMACS® system [27,28] and expanded until Day 3/4 to keep the cells in proliferation for reprogramming with plasmids [29]. Plasmids are still preferred since a limitation of lentivirus systems is that some pre-integrated factors become non-inducible leading to a low reprogramming efficiency [30,31]. Special consideration is given for labelling to allow bidirectional tracking (ATMP6.6-EU-GMP) from donation through manufacturing was established, since CB is specified as a blood product in Germany.

It was essential for the project (1) to analyse the results for CD34+ expansion and release to reprogramming, (2) to keep the cells in a more proliferative state, (3) to define minimal release criteria and (4) to describe all methods required for qualification of starting material.

Materials and methods

Important prerequisites prior to processing

Details about informed consent/re-consent, testings, labelling and qualification of reagents/material as in direct contact with the product are presented in the supplemental online data chapter 1-4.

Thawing/washing of cryopreserved CBUs

CBUs were volume-reduced in the presence of Hetastarch and cryopreserved in Dextran/DMSO as described [32,33]. They were removed from liquid nitrogen (–196°C) and stored for at least 4 h in the gas phase of the liquid nitrogen tank. Thawing was performed in a sterile overwrap bag (WhirlPAK; neoLab Migge GmbH, Germany) at 37°C in a water bath under agitation. Afterwards, the CB bag was installed in the automated Sepax® system (GE Healthcare, Eysins, Switzerland) for washing applying a 1:1 solution of Volulyte 6% (Kabi Pac, Germany; PZN–2796285) and HSA 5% (Octapharma, Germany).

GMP-grade CliniMACS® CD34+ enrichment

For magnetic labelling of washed CBUs, one vial (7·5 ml) of CliniMACS® CD34 reagent (Miltenyi, Bergisch Gladbach, Germany) was applied. After 30 min at room

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temperature (RT), the excess CD34$^+$ antibody was removed by two consecutive washing steps with CliniMACS® PBS/EDTA buffer (Miltenyi, Bergisch Gladbach, Germany) supplemented with 0·5% HSA (Octapharma, Germany). After centrifugation (RT/200 rcf/15 min without brake), the supernatant was removed with a plasma extractor. After the second washing step, the CBU was installed in the automated CliniMACS® device with an LS tubing set (Miltenyi, Bergisch Gladbach, Germany) as recommended by others [27]; CD34$^+$ selection was performed following the device 'CD34 SELECTION 2' programme. The amount of TNC at process steps 'prior to cryopreservation', 'after thawing' and 'after Sepax wash' was determined applying the CellDyn-Ruby (Abbott Diagnostics, Chicago, USA).

GMP-grade expansion of CliniMACS® enriched CD34$^+$ cells

CliniMACS® enriched CD34$^+$ cells were collected at a volume of 80ml and concentrated by centrifugation (10°C/500rcf/10 min). The cell count was determined applying a Neubauer improved chamber. CD34$^+$ cells were seeded at a density of 2-5x10^5/2ml/well on 24-well plates (Sigma-Aldrich Chemie Gmbh, Munich, Germany) and expanded at standard cell culture conditions (37°C, 5% CO_2, humidified air) in SCGM media (Cellgenix GmbH, Germany) supplemented with the prequalified human recombinant GMP cytokines rh-SCF, rh-TPO, rh-IL-3, rh-IL-6 and rh-FLT3-L (Cellgenix GmbH, Germany) at a final concentration of 100 ng/ml. Cells were expanded for 3–4 days in a volume of 2 ml. On Day 3/4 and Day 8, respectively, total nucleated cells (TNC) were enumerated by Neubauer improved chamber.

Colony-forming unit (CFU) assay

To test functionality, 100 (n = 2) to 1000 (n = 8) CD34$^+$ cells were seeded in StemMACS HSC-CFU methyl-cellulose medium (Miltenyi, Bergisch Gladbach, Germany) and cultivated at 37°C with 5% CO_2 in a humidified atmosphere for 14 days. Resulting colony-forming cells (CFC) were counted under a microscope distinguishing red (burst/colony-forming unit erythrocytes (B/CFU-E)), white (colony-forming unit granulocytes/macrophages (CFU-GM) or mixed colonies (colony-forming unit granulocytes/erythrocytes/macrophages/monocytes (CFU-GEMM).

Determination of viable CD34$^+$-count, CD34$^+$-purity, CD34$^+$7AAD$^-$-viability and CD45$^+$7AAD$^-$ leucocyte viability by flow cytometry

Approximately 5 x 10^4 cells were stained with 5 µl each of CD34-PE clone 8G12 and CD45-FITC clone 2D1 (both BD Biosciences, Heidelberg, Germany) and the viability dye 7AAD (Beckman Coulter Inc., France) for 30 min in the dark. Using a no-wash lysis approach for whole-blood samples, 0·5 ml of Versa Lyse (Beckman-Coulter, Krefeld, Deutschland) was added and incubated for 15 min at RT in the dark. Flow cytometric determination of CD34$^+$ cells was performed applying the CytoFlex flow cytometer (Beckman Coulter Inc., France) according to the International Society of Hematotherapy and Graft Engineering (ISHAGE protocol) [34].

Statistical analysis

Data are presented with GraphPad Prism version 5.01 as arithmetic means with a standard deviation of n = 10 samples. Two-tailed unpaired t-tests were conducted with GraphPad Prism Version 5.01. P-values lower than 0·05 were considered as significant (* means $P < 0{\cdot}05$; ** means $P < 0{\cdot}01$; and *** means $P < 0{\cdot}001$). In case of TNC, CD34$^+$ and CFC fold change expansion, values in the Day 0 column are by definition 1.

Results

Selection process and quality of HLA-homozygous CBUs

Our CBB inventory (n = 20 639 licensed CBUs) contains CBUs with a TNC range of 5 x 10^8 up to 5·5 x 10^9 (data not shown). Only 0·04% of the CBUs reveal a TNC count lower than 5 x 10^8, followed by 19·53% of CBUs with a TNC range between 5 x 10^8 and 10 x 10^8 (Fig. 1a). The main percentage of CBUs (47·11%) show a high TNC count above 10 x 10^8 up to 15 x 10^8, and 33·32% of CBUs have a TNC count >15 x 10^8. In Fig. 1b, the frequency of distribution of TNC counts from transplanted CBUs clearly shows that with 48.87% preferentially CBUs with a very high TNC count of >15 x 10^8 are requested. The frequencies of distribution for CD34$^+$ cells in CBU are given in Fig. 1c,d.

Table 1 presents the 10 most common HLA haplotypes in Germany (ZKRD). Nine out of 10 HLA haplotypes were also presented as homozygous in our inventory. A total of 139 secondary consent forms were distributed with a positive re-consent for n = 66 CBUs. It is important to note that n = 42 other homozygous haplotypes were present, reflecting the more international inventory of the CBB. Since also HLA-DP was sequenced, as expected heterogeneity of HLA-DP was observed due to the linkage disequilibrium.

In Table 2, parameters of n = 10 HLA-selected CBUs after collection and after volume reduction prior to cryopreservation are summarized. Basic parameters are

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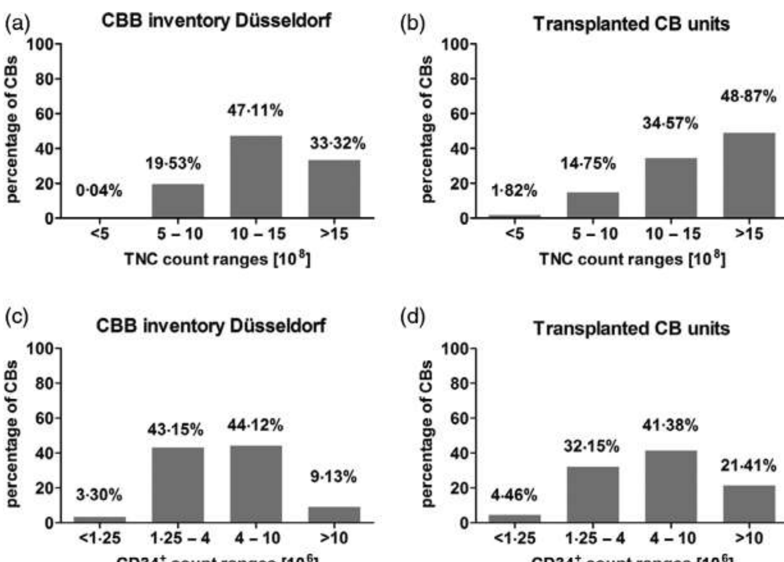

Fig. 1 Characteristics of CBB inventory Düsseldorf. (a-d) Frequencies of distribution are given for TNC count with categories <5 x 10^8, 5–10 x 10^8, 10–15 x 10^8 and > 15 x 10^8 and for viable $CD34^+$-count with categories <1·25 x 10^6, 1·25–4 x 10^6, 4–10 x 10^6 and >10 x 10^6. (a) TNC count; n = 1460, (b) TNC count; n = 1322, (c) Viable $CD34^+$-count; n = 14627 and (d) viable $CD34^+$-count; n = 1314. Data from CBB inventory are presented in the left panel, and data according to already transplanted CB units are presented in the right panel.

presented for collected CB volume (126·59 ± 21·99 ml), TNC count (17·26 ± 3·01[10^8]) and CB volume after volume reduction (n = 9 samples 42 ml; n = 1 sample 24 ml); TNC count after volume reduction (15·44 ± 2·91 [10^8]), TNC recovery (89.28 ± 5.26[%]), viable $CD34^+$-count (4·40 ± 2·25[10^6]) and storage time up to max 16 years (2088 ± 1875 days). No significant storage time-dependent influence was detected (Fig. S1).

$CD34^+$ CliniMACS® enrichment

Parameters of $CD34^+$ processing steps are presented in Fig. 2, and according significance is given in supplemental online Table S1A. A mean TNC count (Fig. 2a) of 15.43 ± 2.98[10^8] after thaw and of 13·75 ± 2·36[10^8] after Sepax results in a mean TNC recovery (Fig. 2c) of 89·73 ± 6·49[%] after Sepax. After CliniMACS®, only residual TNC were observed (0·03 ± 0·01[10^8]). CBUs after thaw revealed a viable $CD34^+$-count (Fig. 2b) range between 2·25–9·67[10^6] with a mean of 4·75 ± 2·08[10^6] showing a slightly lower value of 4·47 ± 2·01[10^6] after Sepax resulting in a final viable $CD34^+$-count of 2·05 ± 0·81[10^6] after CliniMACS®. It demonstrates that the main cell loss occurs during the magnetic isolation process. The $CD34^+$-recovery (Fig. 2d) after CliniMACS® versus prior to cryopreservation was 51·47 ± 23·12[%], ranging from 26 to 104[%]. The flow cytometric determination of viable $CD34^+7AAD^-$ cells resulted in a high mean viability of 91.17 ± 4.33[%] after thaw, 92·61 ± 4·92[%] after Sepax and 96·52 ± 1·39[%] after CliniMACS® (Fig. 2e). The lowest detected viability after CliniMACS was 95%. The significantly increased $CD34^+$-viability (P-value **0·0016; Table S1A) after CliniMACS® accounts for the loss of dying cells (in line with low recovery) leading to a higher overall viability of finally isolated $CD34^+$ cells. This is supported by the $CD45^+7AAD^-$ leucocytes viability (Fig. 2f) resulting in a lower overall viability of 64·42 ± 12·70[%] after thaw and 60·76 ± 10·72[%] after Sepax, but 18% higher with 78·88 ± 9·53[%] after CliniMACS. The mean purity of $CD34^+$ cells (Fig. 2g) was nearly unaffected after thaw with 0·32 ± 0·14[%] and after Sepax with 0·33 ± 0·14[%] reaching a mean of 59·02 ± 10·03[%] after CliniMACS. In order to evaluate the $CD34^+$ potency, CFC counts are given in Fig. 2h. Prior to cryopreservation, a mean CFC count of 3·54 ± 1·43[10^6] represents functional viable $CD34^+$ cells. The CFC count decreased after thaw to a mean of 2·18 ± 1·57[10^6], then 2·32 ± 1·45[10^6] after Sepax and 1·35 ± 1·13[10^6] after CliniMACS®. Cloning efficiencies are presented depicting the ratio of CFC count in relation to the respective viable $CD34^+$-count in Fig. S2.

$CD34^+$ expansion

Relevant parameters of $CD34^+$ expansion are presented in Fig. 3, and according significance is given in Table S1B. The mean TNC fold change (Fig. 3a) was

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Table 1 Frequency of HLA haplotypes in Germany and CBU homozygous in the CBB Düsseldorf

HLA-A, -B, -C, -DRB1, -DQB1	Frequency (Data ZKRD)	Cumulative frequency (Data ZKRD)	HLA-h CBUs in total (Data CBB Düsseldorf)	HLA-h CBUs CBB Düsseldorf back with informed consent
0101-0801-0701-0301-0201	5·93%	5·93%	57	32
0301-0702-0702-1501-0602	3·68%	9·61%	15	5
0201-0702-0702-1501-0602	2·16%	11·77%	12	4
0301-3501-0401-0101-0501	1·62%	13·39%	4	1
0201-1501-0304-0401-0302	1·36%	14·75%	1	1
0201-4402-0501-0401-0301	1·30%	16·05%	1	1
2902-4403-1601-0701-0202	1·28%	17·33%	4	3
0201-4001-0304-1302-0604	1·06%	18·40%	2	1
0201-1302-0602-0701-0202	1·01%	19·41%	1	1
0101-5701-0602-0701-0303	1·01%	20·41%	0	0
Other haplotypes at CBB Düsseldorf:			42	17
Sum:			139	66

Table 2 Characteristics of the HLA-selected CBUs prior to cryopreservation

Sample ID	CB volume collected CB [ml]	TNC count collected CB [10^8]	CB volume reduced CB [ml]	TNC Recovery after volume reduction [%]	TNC count volume reduced CB [10^8]	$CD34^+$ count volume reduced CB [10^6]	Storage time N_2 Tank [days]
Donor 1	159·34	17·05	42	96·57	16·46	5·32	501
Donor 2	151·75	17·60	42	88·28	15·54	10·08	333
Donor 3	134·69	15·49	42	92·73	14·36	3·41	1771
Donor 4	100·57	17·00	42	88·96	15·12	4·83	222
Donor 5	130·90	21·21	42	88·33	18·73	3·75	3242
Donor 6	124·27	18·52	42	87·10	16·13	2·19	1015
Donor 7	87·30	16·06	42	85·76	13·78	2·20	2944
Donor 8	120·47	12·29	24	78·52	9·65	3·28	3862
Donor 9	140·38	22·60	42	90·31	20·41	4·56	1034
Donor 10	116·20	14·76	42	96·20	14·20	4·40	5960
Mean	126·59	17·26	40·20	89·28	15·44	4·40	2088
SD	21·99	3·01	5·69	5·26	2·91	2·25	1875
Min	87	12·29	24·00	78·52	9·65	2·19	222
Max	159	22·60	42·00	96·57	20·41	10·08	5960

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1·98 ± 1·60 at Day 3/4 of expansion augmenting to 12·13 ± 11·79 at Day 8. The according viable CD34$^+$

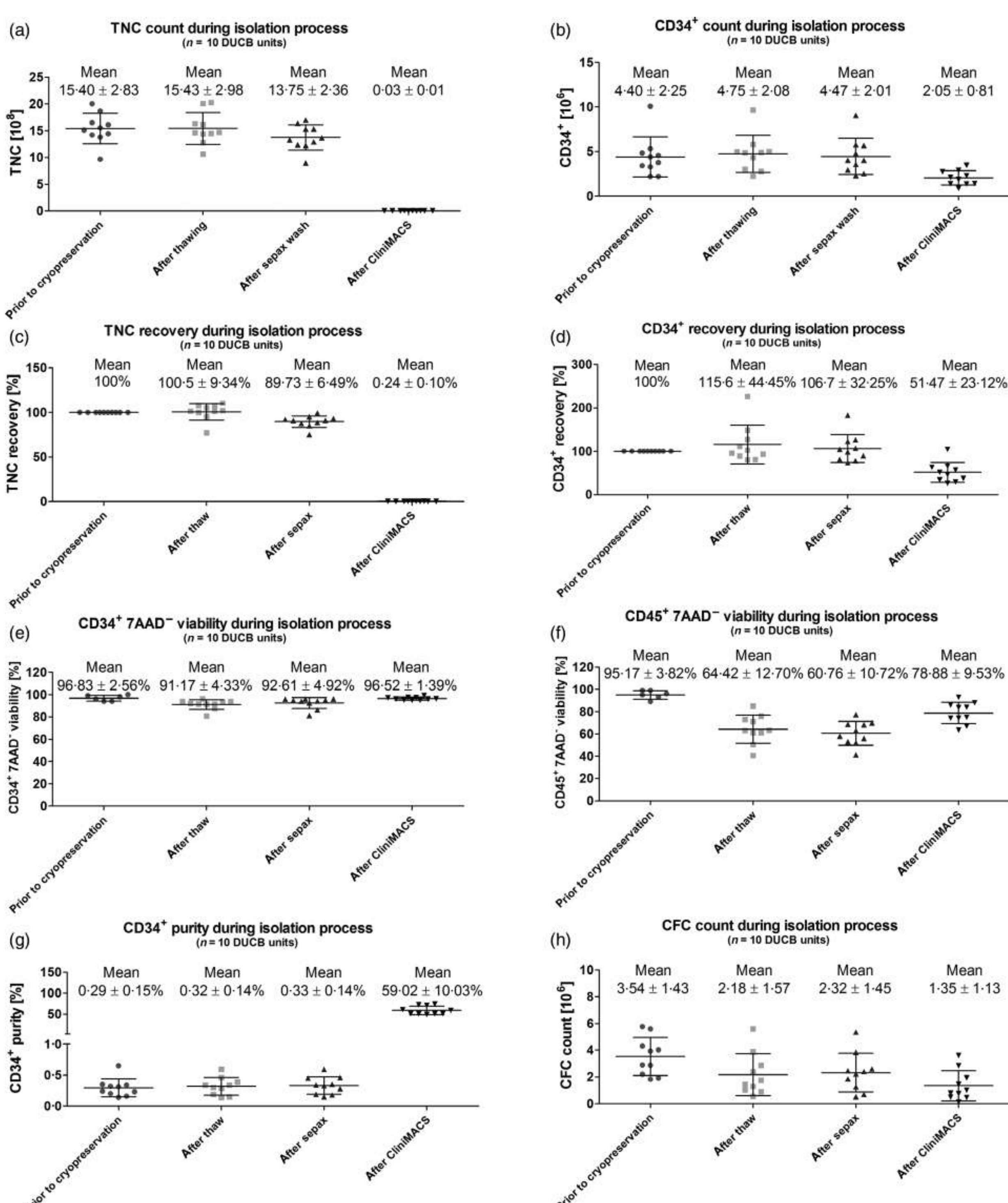

Fig. 2 Qualitative analysis during CD34$^+$ isolation. Samples are drawn 'prior to cryopreservation', 'after thaw', 'after Sepax' and 'after CliniMACS', respectively. (a) Total nucleated cell (TNC) count. (b) Viable CD34$^+$-count. (c-d) TNC- and CD34$^+$ recoveries referred to 'prior to cryopreservation'. (e-f) CD34$^+$7AAD$^-$ and CD45$^+$7AAD$^-$ viabilities determined by flow cytometry with 7-aminoactinomycin D (7AAD). (g) Viable CD34$^+$-purity. (h) Colony growth represented by colony-forming cells (CFC).

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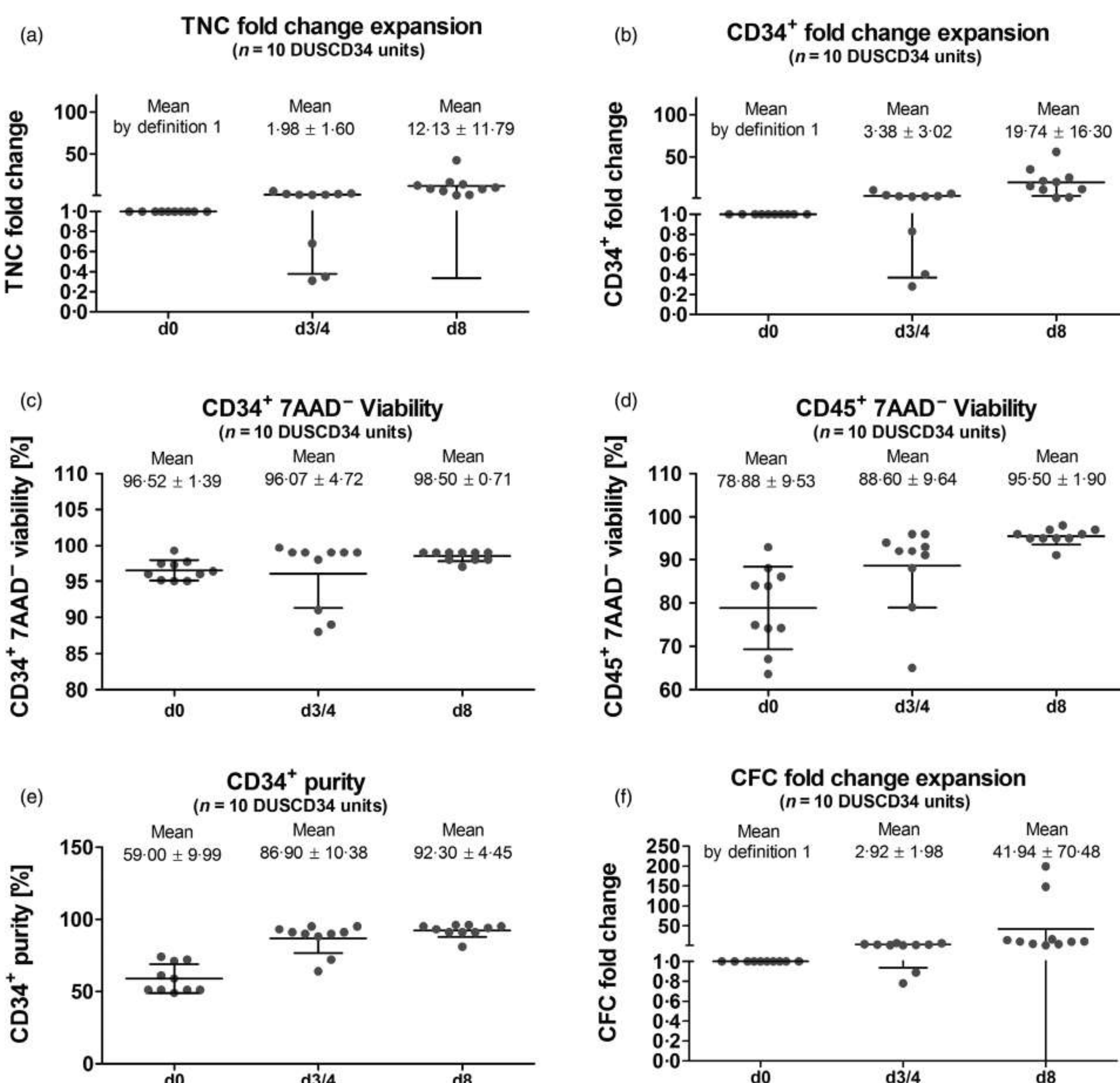

Fig. 3 Qualitative analysis during CD34$^+$ expansion. Samples are drawn at 'Day 0' (corresponds to 'after CliniMACS'), 'Day 3/4' and 'Day 8' of expansion, respectively. (a) Total nucleated cells (TNC). (b) Viable CD34$^+$-count. (c-d) CD34$^+$7AAD$^-$ and CD45$^+$7AAD$^-$ viabilities determined by flow cytometry with 7-aminoactinomycin D (7AAD). (e) Viable CD34$^+$-purity [%]. (f) Colony growth represented by colony-forming cells (CFC).

fold expansion (Fig. 3b) was 3·38 ± 3·02 at Day 3/4 of expansion and 19·74 ± 16·30 at Day 8. The differences in TNC vs. CD34$^+$ fold changes correspond to dying leucocytes. The CD34$^+$7AAD$^-$ viability (Fig. 3c) was 96·07 ± 4·72[%] at Day 3/4 and 98·50 ± 0·71[%] at Day 8. For CD45$^+$7AAD$^-$ viability, a mean of 88·60 ± 9·64[%] was detected at Day 3/4 increasing to 95·50 ± 1·90[%] (Fig. 3d). These results correspond to the CD34$^+$-purity (Fig. 3e), since at Day 3/4 a mean of 86·90 ± 10·38[%] was detected increasing to 92·30 ± 4·45[%] at Day 8. The mean CFC fold change (Fig. 3f) was 2·92 ± 1·98 and revealed a mean of 41·94 ± 70·48 at Day 8. As a quality control samples collected at Day 8 were confirmed negative for sterility in all samples tested. By single comparison of Day 3 vs. Day 4 (Fig. S3), the expansion until Day 4 leads to a substantial higher cell count of CD34$^+$ cells in line with a higher purity and viability, since residual leucocytes (mainly granulocytes) are lost in culture. Differentiation of cells at Day 8 is well-advanced as compared to Day 3 or Day 4, reflected by a wider divergent CD34$^+$ population with arising CD34^{high} cells. T cells are nearly depleted (less than 1%) in the final

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Table 3 DUSCD34 release criteria for transfer and transport

Parameters	Results	Timepoint
Informed primary consent for umbilical cord blood donation on file with option to contact the mother	Signed by the mother	Before transfer
Reconsent of the mother for new indications	Signed by the mother	Before transfer
Fulfilment of donor selection criteria (current maternal and family medical history questionnaire, collection site questionnaire incl. physical examination of the child, sterility of CB, repository samples of mother/child)	No risks identified	Before transfer
Confirmation of GMP-conform production (German GMP guidelines) air/particle count, settle/contact plates, water control incubator)	No serious events which could influence the safety and quality of the cord blood unit, deviations must be explained and clarified	Final release
Thawing, selection and expansion operation is completed correctly	No serious events which could influence the safety and quality of the CB unit, deviations must be documented and approved	Final release
Labeling	Unambiguous identification SEC, unique donor identification number	Before transfer
DUSCD34+		
AB0 Rhesus determination	Determined (CB)	Before transfer
HLA A[a], B[a], C[a], DRB1[a], DQB1[a], DP[a]Molecular genetic medium resolution or high resolution with NGS on segment on the bag	Determined (CB), declared as HLA homozygous for HLA-A, B, C, DR, DQ, DP	Before transfer
Anti-HIV 1/2 + 0 antibodies/p24 antigen[b]	Negative (maternal serum)	Before transfer
HIV 1-Genome (PCR) *	Negative (maternal plasma)	Before transfer
Anti-HCV antibodies[b]	Negative (maternal serum)	Before transfer
HCV-Genome (PCR) *	Negative (maternal plasma)	Before transfer
Anti-HTLV-I/II antibodies[b]	Negative (maternal serum)	Before transfer
HBsAg[b]	Negative (maternal serum)	Before transfer
Anti-HBc antibodies HBV-Genome (PCR)[a]	Negative (maternal serum) negative (maternal plasma, min. sensitivity < 12 IU/ml)	Before transfer
HBV-Genome (PCR)[a]	Negative (maternal plasma), min. sensitivity 50 IU/ml	Before transfer
Anti-Treponema pallidum- antibodies[b]	Negative (maternal serum)	Before transfer
Anti-CMV-IgG[b]	Diagnosed (maternal serum)	Before transfer
Anti-CMV-IgM[b]	Diagnosed (maternal serum)	Before transfer
CMV-Genome (PCR)	Negative (CB-whole blood)	Before transfer
Parvovirus B19-Genome (PCR)	Negative (CB-Plasma)	Before transfer
Hemoglobinopathy screening	Without pathological homozygous findings	Before transfer
Pack volume for transport 50 ml Falcon tube in sterile overwrapping	Perishable cells 6–20 ml, minimum 5 x 10^5 CD34+ cells	At transfer
Total cell nucleated cells count (TNC)	Determined	At transfer
CD34+ count	Target: ≥ 5 x 10^b	At transfer
CD34+ purity	Target: ≥ 80 % alive CD34+ cells	At transfer
Viability of CD34+ cells	Target: ≥ 70 % (7AAD-)	At transfer
Colony forming cells (CFU) for determination of biological potency of the expanded product	Determined/ declared n = ≥1 growth in the final release protocol	Final release
Sterility	Anaerobic and aerobic culture: sterile in the final release protocol	Final release
Visual sterility at day 3/4	Visual sterility -documented picture	At transfer

[a]The PCR for HIV, HBV and HCV could be performed either on CB or mother depending on the year of production of DUS cord blood.
[b]All virological/ serological parameters are defined before transfer.
[c]If the CD34+ count is not reaching its target dose, the product is transferred for reprogramming, since the homozygous CB is very rare and one cell to be reprogrammed would be sufficient.

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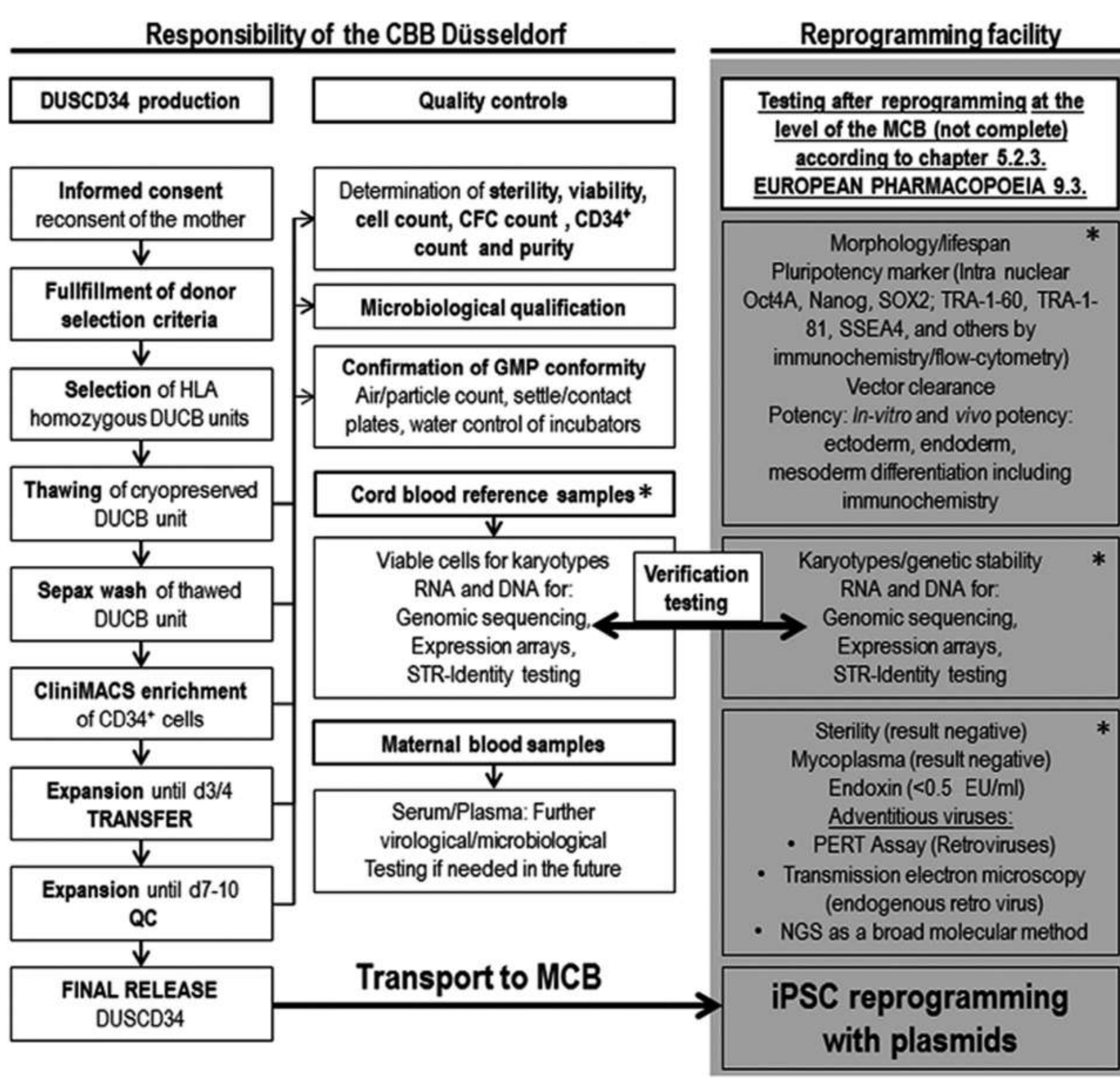

Fig. 4 Manufacturing process of DUSCD34 product as starting material for iPSC reprogramming. Process steps for DUSCD34 production (left). Required quality controls, microbiological qualification and relevant testing of CB reference samples and maternal blood samples (middle). Required steps after the transport of the final DUSCD34 product related to subsequent reprogramming at the level of the master cell bank (right). Asterisks (*) mark required specifications following the European Pharmacopeia guidelines.

product (Fig. S4). All DUSCD34 (product name for expanded CD34$^+$ cells) products met the requirements as minimal viable CD34$^+$-count of ≥5 x 10^5 cells, a purity of ≥80% viable CD34$^+$ cells and a viability of ≥70% according to our final release criteria together with additional criteria listed in Table 3.

The responsibilities during HLA-h CD34$^+$ manufacturing process of the CBB bank, quality controls and responsibilities of reprogramming facility including required quality criteria are summarized in Fig. 4.

Discussion

Alvarez-Palomo *et al.* [1] described 2019, how cord blood banks could be adapted for the provision of HLA-homozygous cells as starting material for iPSC. The results of the CBB Düsseldorf (showing as an example the responsibility of a CBB) are described for the detailed GMP-compliant generation/expansion of HLA-h derived CD34$^+$ cells from pharmaceutically qualified/licensed CBUs as a starting material for reprogramming. The

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regulatory advice to have (1) a more homogenous cell population (CD34$^+$) and (2) a stringent GMP compliance was followed in the approach here, despite that other references exist describing buffy coat/MNC [35] or rather laboratory conditions [29] for pre-clinical/clinical applications. Therefore, the process of thawing, isolation and expansion of CD34$^+$ cells was performed under GMP-compliant conditions and critical parameters were analysed. Our CD34$^+$ recovery with 51·47 ± 23·12% was in line with other published data like 53 ± 15% related to enrichment from cryopreserved CBUs [36] and 56 ± 2·5% from fresh CBUs [27], respectively. The CD34$^+$7AAD$^-$ viability post-enrichment was higher with 96·52 ± 1·39% as compared to published data with 74 ± 4·1% for enriched CD34$^+$ cells from fresh CBUs [27]. The high viability ≥96% confirms a stable starting material after expansion.

Although some differentiation of CD34$^+$ cells might occur during expansion, the efficiency of reprogramming correlates with the proliferative activity of cells [37], and the expansion rate observed at Day 3/4 seems to be sufficient with regard to quantity and quality aspects. At Day 8, the differentiation of CD34$^+$ cells is advanced, and at Day 4, expanded CD34$^+$ cells are the preferred starting material.

It could be further improved by methods applied in the clinics as nicotinamide [38], fucosylation [39], Notch ligand [40] or stem cell supporting agents [41,42].

According to EU-ATMP-guidelines, risk-based approaches should be considered to ensure the quality of the raw biological material applied. The risk of contamination of pretested biological material along the supply chain must be assessed with particular emphasis on viral and microbial safety and transmissible spongiform encephalopathy (TSE) [43].

For blood-derived cells compliance with Directive 2002/98/EC [44] and local law/guidelines [45–48] for donation, procurement and testing should be followed. Since by law, here, unrelated CB is under a licensure, the risk of infectious disease transmission is already minimized based on screening and detailed medical questionnaire of mother/child/family [45]. Besides the presence of BSE and the variant of Creutzfeldt–Jakob disease (vCJD), no other agents with TSE risk are known to be transmitted to humans. There is in addition no evidence for the presence of vCJD in CB [49].

Moreover, the manufacturing of an ATMP should follow the consideration of the European Pharmacopeia [50].

For the CBB, a risk-based approach for the selection and expansion of CD34$^+$ cells was used with the result that the sterility is tested on the product based on the matrix validation established in Europe many years ago. Mycoplasma [51], endotoxin [52] and adventitious virus testing [53] applying three different methods including next-generation sequencing (NGS) for virus determination [50] should be performed on the level of the iPSC master cell bank together with all other quality criteria for iPSC as summarized in Fig. 4 and in detail Ph.Eur.5.2.12 [50].

Conclusion

Although several studies have reported successful enrichment of CD34$^+$ cells from cryopreserved CBUs for iPSC [29], still few experiences exist regarding the adaption of procedures to a GMP setting as a starting material under the ATMP guidelines. The proposed manufacturing process described here holds great advantages overcoming restrictions of personalized medicine. Critical parameters need to be compared in the future with other cell sources in terms of subsequent reprogramming efficiency, or how liquid nitrogen influences relevant parameters [54].

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Conflict of interests

The authors declare no conflict of interests.

Author contributions

SL and GK conceived and designed the study, analysed the data and wrote the manuscript. SL, LK, JK and AD performed acquisition of data. All authors contributed to the final approval of manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1A: Statistical analysis of relevant CB parameters during CD34+ enrichment procedure. Table S1B: Statistical analysis of relevant CB parameters during CD34+ expansion procedure.

Figure S1: Qualitative analysis of relevant parameters related to storage time of CB units. Samples are drawn at distinct time points: "d0" (corresponds to "after CliniMACS"), "d3/4", and "d8" of expansion, respectively. (A) Total nucleated cell (TNC) fold change expansion were performed by automated hematology analyzer (CellDynRuby), or Neubauer chamber after CliniMACS® due to low sample volume. (B) Viable $CD34^+$ counts (flow-cytometry) were calculated by the viable $CD34^+$ purity ($CD34^+$ cells / total leukocytes x 100 [%]) x TNC count. (C-D) TNC- and $CD34^+$ recoveries are calculated referring to the time point "prior to cryopreservation". (E-F) $CD34^+$7AAD- and $CD45^+$7AAD- viabilities are determined by flow-cytometry with 7- aminoactinomycin D (7AAD) as fluorescent intercalator that undergoes a spectral shift upon association with DNA, leading to fluorescence detection in dead cells. (G) For determination of viable $CD34^+$ purity the viable $CD34^+$ cell count (flow-cytometry) is divided by total leukocyte counts x 100 [%]. (H) Colony growth as a functionality test for viable $CD34^+$ cells is determined by counting colony forming cells (CFC) after 14 days of incubation in a semi-solid medium enriched with hematopoietic growth factors, resulting in red (burst/colony forming unit

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erythrocytes (B/CFU-E)), white (colony forming unit granulocytes macrophages (CFU-GM) or mixed colonies (colony forming unit granulocytes erythrocytes macrophages monocytes (CFU-GEMM) from the according progenitors. Total CFC represent all distinct kinds of counted colonies.

Figure S2: Qualitative analysis of cloning efficiency during HLA-homozygous $CD34^+$ isolation process. Samples were drawn at distinct process steps: "prior to cryopreservation", "after thawing", "after Sepax wash", and "after CliniMACS", respectively. Cloning efficiency was calculated by the ratio of total CFC count in relation to the viable $CD34^+$ count and is given in percent.

Figure S3: Determination of $CD34^+$ expression during short-term expansion by flow-cytometry. (A) Representative dot plots of flow-cytometric $CD34^+$ determination at d0, d3, and d8 (quality control) of expansion. At d3 of expansion (middle dot plot) many dying leukocytes (black and red dots) are still present in the cell culture resulting in a low purity of $CD34^+$ cells with 88.21%. (B) Representative dot plots of flow-cytometric $CD34^+$ determination at d0, d4, and d8 (quality control) of expansion. At d4 of expansion (middle dot plot) only few residual leukocytes (black dots) are present and $CD34^+$ cells reveal a high purity of 97.42%. Therefore, d4 is the preferred expansion time prior to transfer to reprogramming facility.

Figure S4: Exemplified qualitative analysis of residual blood components after day 4 of $CD34^+$ expansion. (A) Dot Plot demonstrating the cut out of the debris determined by side scatter (SSC-A) versus forward scatter (FSC-A). (B) All populations (residual granulocytes, monocytes and lymphocytes), except the debris, determined by side scatter (SSC-A) versus forward scatter (FSC-A). (C) Populations of T and B cells determined by CD19 PC5.5-A expression versus CD3 PC7-A expression (D) Population of all $CD3^+$ T cells determined by CD4 APC-A750-A expression versus CD8 PE-A expression. (E) Population of NK and NKT cells determined by CD8 PE-A expression versus CD56 APC-A expression. (F) Population of monocytes determined by CD14 FITC-A expression versus side scatter (SSC-A). (G) Hierarchy and statistics of populations.

Supplemental online data chapter 1-4.

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LETTER TO THE EDITOR

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Operational protocol for donation of anti-COVID-19 convalescent plasma in Italy

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Coronavirus disease-19 (COVID-19) represents a public health threat worldwide, and Italy at the present time is considered the epicentre of this severe infection in the Western world [1,2]. Unfortunately, no standardized therapy does exist for COVID-19 and a number of investigational drugs for use in patients with life-threatening COVID-19 infections have been tried [3]. One investigational treatment being explored for COVID-19 involves the use of convalescent plasma collected from recovered COVID-19 patients [4]. Convalescent plasma, containing antibodies against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, the virus that causes COVID-19), might be effective against the infection. This treatment has been studied in particular during previous outbreaks of other respiratory infections, including the 2009–2010 H1N1 influenza virus pandemic, the 2003 SARS-CoV-1 epidemic and the 2012 Middle East respiratory syndrome (MERS)-CoV epidemic [4].

Based on the dramatic situation in Italy, but aware the convalescent plasma therapy is to be considered 'empirical' and not supported by robust scientific evidence and solid haemovigilance data on its safety [5], the Italian National Blood Center has decided to derogate from the current blood donor selection criteria in order to permit to individuals recovered from COVID-19 to donate convalescent plasma. Mandatory conditions that have to be met for donor eligibility are the following:

(1) Patient–donor, with virologically documented diagnosis of COVID-19, completely recovered by at least 14 days according to the clinical and laboratory criteria defined by the Superior Health Council on 28 February 2020 ('The recovered patient is the one who resolves the symptoms of COVID-19 infection and who is negative in two consecutive tests, carried out 24 h apart, for the search for SARS-CoV-2');
(2) Male patient–donor or a nulliparous female donor with a negative history of blood component transfusion;
(3) Careful clinical evaluation of the patient–donor with particular reference to the criteria provided for the current rules to protect the health of the apheresis donor;
(4) As at the moment no definitive scientific evidence supports the adoption of a defined titre of neutralizing antibodies in this specific setting, the presence of adequate levels of anti-SARS-CoV-2 neutralizing antibodies is recommended [a titre of at least 1:320 is recommended only for patients affected by primary or acquired (including patients treated with B-cell depleting monoclonal antibodies) immunodeficiencies];
(5) Negative results of the biological qualification tests provided for by the current rules;
(6) Negative results of the following additional tests performed at each donation, that is RNA testing for hepatitis A and E viruses and DNA testing for parvovirus B19.

In addition, each unit of plasma apheresis collected from convalescent patient–donors must be the following:

(1) Processed with a pathogen reduction method of recognized efficacy;
(2) Clearly labelled as 'Plasma unit collected from a convalescent patient–donor with a virologically documented diagnosis of COVID-19';
(3) Kept separately from other units for clinical use or industrial fractionation.

Finally, it is recommended to suitably adapt the donors' and recipients' informed consents, to strengthen haemovigilance (on patient–donors and recipients), and to keep the Italian National Blood Center and the Regional Blood Coordinating Centers informed on the start of the apheresis procedures and on the quantity of plasma units collected and available for transfusion to COVID-19-infected patients.

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13–16.11.2021	32nd Regional congress of ISBT, Brisbane, Australia