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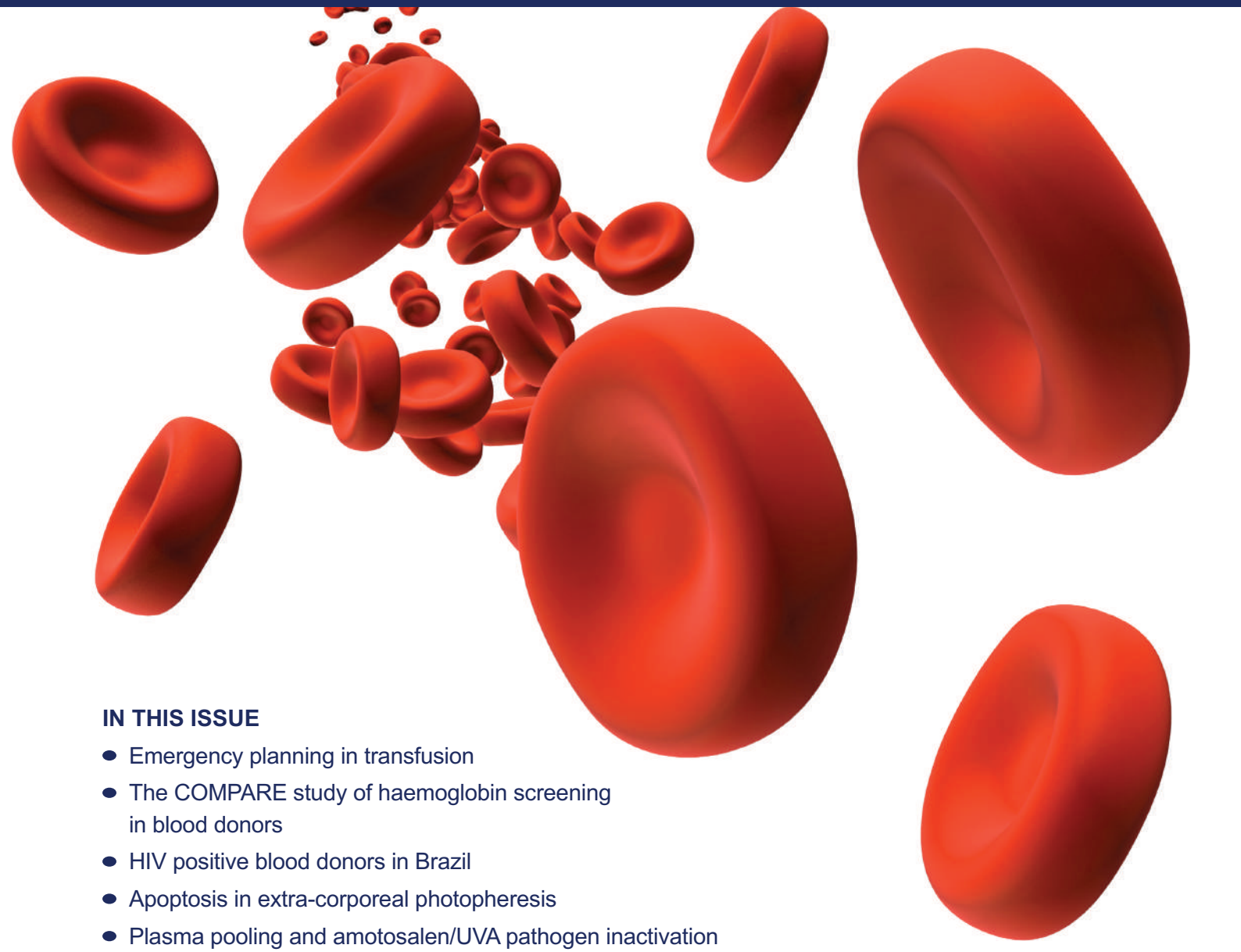

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Transfusion Medicine

An international journal published for the British Blood Transfusion Society

Aims

Transfusion Medicine will publish articles on transfusion medicine in its widest context, including blood transfusion practice (blood procurement, pharmaceutical, clinical, scientific, computing and documentary aspects), immunohaematology, immunogenetics, histocompatibility, medico-legal applications, and related molecular biology and biotechnology.
In addition to original articles, which may include brief communications and case reports, the journal will contain a regular educational section (based on invited reviews and state-of-the-art reports), technical section (including quality assurance and current practice guidelines), leading articles, letters to the editor, occasional historical articles and signed book reviews. Some lectures from Society meetings that are likely to be of general interest to readers of the Journal may be published at the discretion of the Editor and subject to the availability of space in the Journal.

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ORIGINAL ARTICLE

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London 2017: Lessons learned in transfusion emergency planning

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Abstract

Background and objectives: Hospitals prepare for emergencies, but the impact on transfusion staff is rarely discussed. We describe the transfusion response to four major incidents (MIs) during a 6-month period. Three events were due to terrorist attacks, and the fourth was the Grenfell Tower fire. The aim of this paper was to share the practical lessons identified.

Methods: This was a retrospective review of four MIs in 2017 using patient administration systems, MI documentation and post-incident debriefs. Blood issue, usage and adverse events during the four activation periods were identified using the Laboratory Information Management System (TelePath).

Results: Thirty-four patients were admitted (18 P1, 4 P2, 11 P3 and 1 dead). Forty-five blood samples were received: 24 related to nine MI P1 patients. Four P1s received blood components, three with trauma and one with burns, and 35 components were issued. Total components used were six red blood cells (RBC), six fresh frozen plasma (FFP) and two cryoprecipitate pools. Early lessons identified included sample labelling errors (4/24). Errors resolved following the deployment of transfusion staff within the emergency department. Components were over-ordered, leading to time-expiry wastage of platelets. Careful staff management ensured continuity of transfusion services beyond the immediate response period. Debriefing sessions provided staff with support and enabled lessons to be shared.

Conclusions: Transfusion teams were involved in repeated incidents. The demand for blood was minimal. Workload was related to sample handling rather than component issue. A shared situational awareness would improve stock management. A laboratory debriefing system offered valuable feedback for service improvement, staff training and support.

KEYWORDS
emergency planning, major incidents, stress, transfusion teams

1 | BACKGROUND

A Major Incident is defined as any occurrence that presents a serious threat to the health of the community or causes such numbers or types of casualties that require special arrangements to be implemented.[1] In 2004, The Civil Contingencies Act[2] stated that National Health Service (NHS) organisations are required to demonstrate that they can deal with Major Incidents (MIs) while maintaining

critical services. NHS England has established the framework for healthcare Emergency Preparedness, Resilience and Response (EPRR).[3] The EPRR framework affords a general response to MIs,[4] which can be adopted by different healthcare facilities depending on their capacity and capability. The most comprehensive of these is the major trauma centre (MTC), which equates to a U.S. Level 1 trauma unit. MTCs, with their associated trauma networks, were established in 2008 following the 2007 National Confidential Enquiry into Patient Outcome and Death report,[5] which identified serious failings in the clinical and organisational aspects of trauma care in England.

The successful delivery of healthcare in MIs involving multiple casualties is based on triage or "sorting" according to clinical priority for intervention. Triaging trauma patients starts with pre-hospital assessment and guides initial casualty dispersal. A joint understanding of clinical triage and the hospital receiving plan underpins transfusion support during an incident. Patients are described as Priorities 1–3, where Priority 1 (P1) require the most urgent intervention (Figure 1). This enables prioritisation of blood grouping and issue for bleeding P1 patients. The newly released NHS England clinical guidelines[6] for MIs and Mass Casualty Events (MCE) advises that MTCs should expect to receive predominantly P1 casualties, whereas a Trauma Unit should expect to receive P2 and P3 casualties. Each Trust is required to have their own MI plan, which includes the organisational aspects of the response.

Triage enables the best use of resources and targets effort where it is most needed. Doughty and Rackham[7] have proposed that a similar triage approach may be used for transfusion support in emergencies, prioritising not just patients but also sample handling, appropriate blood use and donor selection. This philosophy underpins the recently released emergency planning guidance for hospital transfusion teams.[8] The guidance also reflects the practical lessons identified by multidisciplinary Hospital Transfusion Teams in both Manchester and London. St Mary's Hospital, London was one of the receiving MTCs for three terrorist-related incidents and the Grenfell Tower fire disaster in 2017. We describe some of the transfusion emergency planning lessons identified by a single London hospital transfusion team and consider the implications for policy and practice.

2 | MATERIALS AND METHODS

2.1 | Setting

NHS Trusts are organisational units within the National Health Service in England, which provide acute and specialised secondary care in a geographical area. Imperial College Healthcare NHS Trust is a multisite organisation in northwest London consisting of five hospitals on four sites with two MI receiving sites. The three main hospital sites—

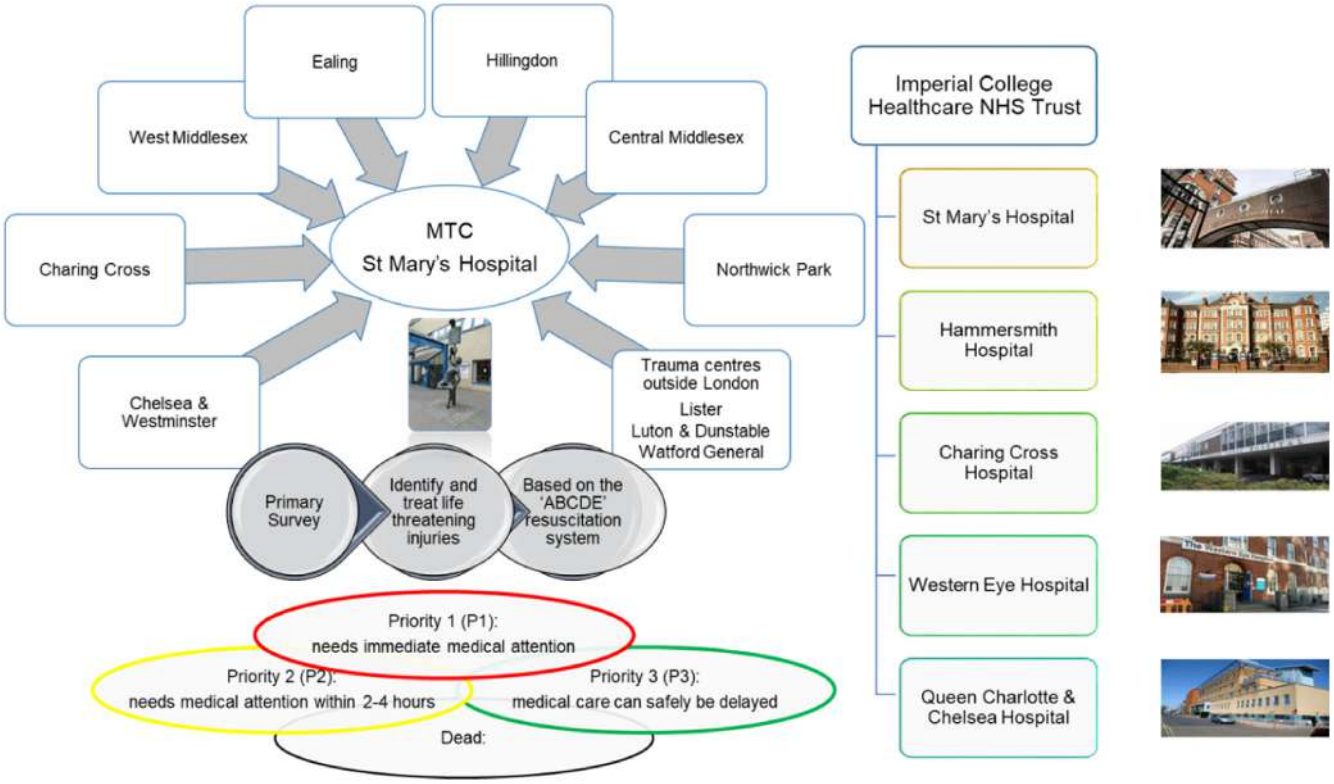

FIGURE 1 Imperial College NHS Trust consists of five hospitals in four locations in North West London. St Mary's Hospital is the MTC for the 10 trauma units in the North West London Trauma Network. Triage uses the Priority categories based on clinical need for intervention. Triage is a dynamic process as patients classified as P3 or P2 can become P1 patients without warning [Color figure can be viewed at wileyonlinelibrary.com]

St Mary's, Charing Cross and Hammersmith Hospitals—have incident control rooms from where incidents are managed. The Trust operates Gold (Strategic), Silver (Tactical) and Bronze (Operational) command and control system when responding to an incident. The Emergency Planning Team (EPT) meets every 4 months to review plans and rolls out regular training to the Trust. These plans are developed in partnership with relevant areas of the Trust and local partners to guarantee an integrated response to any incident. The EPT includes the Trust's communications team who lead on all communications internally and externally, including the media during incidents.

Blood components for all sites are obtained from the NHS Blood & Transplant (NHSBT) processing and manufacturing centre based at Colindale, which is 8 miles northwest of St Mary's Hospital. St Mary's is a 484-bed hospital situated in Paddington, in the City of Westminster, London, which is only accessible by road. This hospital became a MTC in 2010; it provides a 24-hour consultant-led service for adults and children as part of the North West London (NWL) Major Trauma Network (*see* Figure 1), and the purpose of this network is to deliver safe, effective major trauma care to patients in this region. The network covers a daily average population of 3.9 million residents, tourists and commuters (from Westminster to just outside the M25 London orbital motorway). More than 3000 severely injured major trauma patients are seen annually. St Mary's has a medium-sized emergency department (ED), with a resuscitation room, and can take 20 casualties, including 6 Priority 1 casualties in the first hour, followed by further casualties each hour. The capacity across the whole Imperial College Healthcare NHS Trusts is currently stated as 25 Priority 1, 100 Priority 2 and 100+ Priority 3 within the first 24 hours. Beds can be created in the MTC for MI patients who need the expertise of the trauma service by transferring non-MI patients to Charing Cross and Hammersmith Hospitals.

Each site of this NHS Trust has its own transfusion laboratory. The transfusion Laboratory Information Management System (LIMS) at St Mary's laboratory is the TelePath Cache version 2.0. The laboratory analyses over 42 000 samples for Group & Save and issues approximately 17 000 red cell units yearly. The emergency stock-holding capacity is approximately 50 emergency RBC in saline-adenine-glucose-mannitol solution (SAGM), including O negative together with 10 pools of adult therapeutic doses of platelets, and for each group, 20 units of fresh frozen plasma (FFP) and 10 units of cryoprecipitate. Day time staffing from 09:00 to 17:00 includes a Blood Transfusion Laboratory Manager with responsibility for day-to-day running of the laboratory, six Biomedical Scientists (BMS) specifically trained in laboratory medicine and one Medical Laboratory Assistant (MLA). A lone BMS covers at night, supported by MLA cover for the late evening. Transfusion staffing is supplemented during incidents by an on-site haematology BMS until additional transfusion staff members are available. The laboratory is supported by a Consultant in Transfusion Medicine jointly employed by NHSBT together with three Transfusion Practitioners from a scientific/nursing background. The multidisciplinary team is referred to as the Hospital Transfusion Team.

2.2 | Study design

A retrospective qualitative and quantitative review of the four MIs was performed using hospital, ED and laboratory debriefings and reports for each event. Details were collected from the point of activation for the first incident to the stand-down of the last, that is, 22 March to September 15, 2017. Patients admitted were identified using the MI numbering system. Demographics and injury type were confirmed using paper-based records from the London Ambulance Service (LAS) and hospital electronic (CERNER) records. Blood components issued and used were identified using the LIMS (TelePath). Adverse events were identified through haemovigilance reports. The review forms a part of internal quality improvement measures. Ethics approval was not required.

3 | SUMMARY OF THE FOUR MAJOR INCIDENTS

3.1 | Westminster Bridge

This attack was initiated on Wednesday 22nd March 2017, at 14:40. A single perpetrator drove a car onto the pavements of the south side of Westminster Bridge and Bridge Street. He then crashed the car into the perimeter fence of the Palace grounds and ran into New Palace Yard, where he fatally stabbed an unarmed police officer who tried to stop him. Seconds later, he was shot by an armed police officer. St Mary's was placed on standby at 15:27 and received the first casualty at 15:29 but was not formally declared as a receiving site until 15:55. The last casualty arrived at 16:50. A total of 10 casualties were received: 6 P1, 2 P2 and 2 P3 casualties; the perpetrator was among the P1 casualties, and he was confirmed dead on arrival. The site was stood down at 18:45. The filming crew from the BBC series "Hospital" was present in the ED to film the second episode. Ironically, they had been pre-scheduled to follow the Trauma ED Lead that day. Two samples taken from one MI patient were identified by the laboratory as "Wrong blood in tube" (WBIT), labelled and taken at the same time. Fifty people were injured, and 5 people were killed. Casualties were distributed across the four London MTCs and some associated trauma units.

3.2 | London Bridge and Borough Market

This attack took place on Saturday, 3rd June, at 21:58. Three attackers drove in a van across London Bridge. The first emergency call went out at 22:07, after the van hit pedestrians on the bridge. The men abandoned the van on the south side of London Bridge and then ran into Borough Market, stabbing people in their paths. At 22:16, the police shot dead the three attackers; by that time, 48 people had been injured. A total of eight people (including a senior nurse from Guys and St Thomas' Hospital) were killed. St Mary's was placed on standby at 00:22 and formally declared at 00:39. As this attack

was out of normal working hours, transfusion managers co-ordinated the incident off site. Only two P1 casualties were sent to St Mary's Hospital; the first arrived at 00:04, 18 minutes before London Ambulance Service (LAS) placed the hospital on standby, and the second at 05:36. Charing Cross Hospital was placed on standby at 00:46, and this was subsequently cancelled at 02:26. St Mary's was stood down at 02:05.

3.3 | Grenfell Tower Fire

Less than 2 weeks after the London Bridge incident, St Mary's was stood up for a third MI, the Grenfell Tower fire, on Wednesday, 14th June, at 02:35. The fire started at 00:54 and rapidly rose from the fourth floor. The first casualty was received at 03:29 and the last at 15:35. In this incident, 11 P1 and 4 P3 casualties were treated at St Mary's. Two of the admitted P1 paediatric casualties were later identified as siblings. One subsequently died from inhalation injuries. St Mary's was stood down at 16:00, by which time it had become apparent that many of the residents of Grenfell Tower had not managed to escape from the building. Charing Cross Hospital was also declared and received four P3s before being stood down. In the months to come, it was recognised that 72 people were killed and more than 70 others were injured in the fire.

3.4 | Parsons Green Train Station

The final MI of 2017 occurred on Friday, 15th September, at 08:20, that is, during the morning rush hour in London. In this incident, a bomb was detonated in Parsons Green station. The device failed to detonate correctly. One of St Mary's Transfusion Practitioners was on the train and alerted the transfusion laboratory before LAS officially declared this as an incident. St Mary's was formally placed on standby at 09:07 and was declared a receiving site at 09:30. Twenty-nine people were injured in this attack; the injuries were minor crash injuries. St Mary's received only two P2 and five P3 category patients before being stood down at 11:27. No patients were admitted. The clinical transfusion response was co-ordinated remotely.

4 | RESULTS

The four incidents resulted in the injury of almost 200 individuals and the death of 85; 2 of these casualties were members of the emergency service, and 4 were the individuals who perpetrated two of the attacks. St Mary's was "stood up" as a receiving hospital each time. Each MI successfully triggered the activation of the MI plan, with communication cascading through all departments, including the transfusion laboratory. The Trusts' priorities were to alert all departments, to establish security and provide optimal medical care for those already in the Trust and enhance healthcare capacity for those involved in the MI. Patients requiring level 2 care were accommodated in the Trusts' high-dependency units and other medical beds created through distribution and appropriate discharge of patients. Theatre space and surgical beds were created by cancelling non-emergency elective surgery, thereby conserving pre-allocated blood components and increasing the reserve of emergency components for MI casualties.

4.1 | Patients

Thirty-four of the MI casualties (33 victims plus perpetrator) were admitted at St Marys Hospital. A total of 19 were P1 (1 confirmed dead on arrival), 4 were P2 and 11 P3. In addition, the ED continued to receive other patients unrelated to the four incidents. These admissions included clinical conditions with implication for transfusion, including haematemesis, knife wound and thalassaemia major. Twenty-one patients had transfusion samples taken and sent to the laboratory during the activation periods; 9 were from MI casualties (all P1s), and the rest from non-MI patients. The demographic details showed that the gender divide was equal between men and women. The mean age of the nine MI casualties was 23 years, with a range of 5–46. Traumatic injury type was predominantly blunt rather than penetrating trauma. In addition, patients were admitted with smoke inhalation-associated conditions.

4.2 | Workload

Forty-five transfusion samples were processed by the laboratory during the four events' combined activation time, and 24 samples were received from nine (47%) P1 casualties. The number of samples received from each patient ranged from one to six. The hospital uses the two-sample rule and only released group-specific blood after duplicate samples were received. One MI casualty had two samples sent together; these were identified as a WBIT. Consequently, repeat samples were required to confirm the correct ABO and RhD group. If this had not been identified, group A blood would have been issued to a group O patient. Repeat samples were taken once patients received their permanent hospital number. Normally, this is undertaken on confirmation of identity and/or patient admission to the Trauma ward. Thirty-five blood components were issued. Three P1 casualties from Westminster Bridge and one P1 casualty from the Grenfell fire were transfused blood components. The total amount of blood used was modest, with a total of six units of SAGM red cells, six units of FFP and two units of cryoprecipitate (Figure 2). None of the casualties met the criteria for activation of the Major Haemorrhage Protocol, and no platelets were transfused.

4.3 | Incident activation

The laboratory was notified of Standby through the MI communication cascade. Standby allows preparation time to optimise staffing and to undertake an internal stock-take of available emergency stock in the blood bank and satellite fridges so that, when formally declared as a receiving site, stock and staff can be deployed to the ED. The

timeframe between being placed on standby to receiving casualties was 17–28 minutes for the three terrorist-related events. For Grenfell, the hospital went immediately to receiving patients.

St Mary's MTC had a mature MI plan, which worked well in each event.

However, it was the first year that the transfusion service had been activated repeatedly. Switchboard staff located at the MTC put out an automatic cascade call-out to staff on this site only on declaration of each MI; this cascade takes up to 20 minutes. The first incident at Westminster Bridge highlighted that junior members of medical staff were not on this automated cascade. All departments amended their MI action card to address this; some formed a WhatsApp group to co-ordinate staffing, whereas others ensured that the contact details of all staff, including Transfusion Practitioners, were available in key areas.

4.4 | Security and safeguarding

Security emerged as a common theme during these four events. Site security was achieved through an immediate "Lockdown." This did not prevent receipt of additional blood stock delivered by NHSBT vans through pre-designated routes. Safeguarding was identified as an important aspect of the Grenfell Tower fire. Many members of the public arrived at the hospital looking for injured and lost family members, including children. The presence of media on site also contributed to the need to "lockdown" the hospital. Staff are always advised to be security conscious, to be clearly identified and wearing hospital ID, especially during MIs.

4.5 | Sample labelling

Sample labelling due to human error was a problem following the Westminster Bridge and Grenfell Tower fire incidents. Despite the trauma team being used to dealing with unknown patients, four blood transfusion samples from casualties in these incidents were identified as labelled incorrectly, with errors in spelling the MI names in two casualties. This was despite training undertaken immediately after and in between each incident. Subsequent root cause analysis revealed the cause to be the modified MI naming system for the CERNER records system. The assigned MI name was found to be confusing and too long to be handwritten on transfusion sample bottles. This has been rectified, and the pre-allocated MI hospital numbers are associated with appropriate names to prevent this. The lessons identified informed the development of the subsequent patient safety alert issued in 2018.[9]

4.6 | Staffing

Transfusion Practitioners played a key role in supporting patient safety during these incidents. The main role for Transfusion Practitioners was to issue emergency stock and oversee collection of baseline blood samples. However, they also provided confidence and a visible connection to the laboratory. However, most transfusion practitioners (TPs) are only available during core hours and are therefore not on the automated MI call-out list. The BT laboratory manager or Transfusion Consultant directed the Transfusion Practitioner (via mobile phone) to the clinical areas needing support.

Medical staff supported the transfusion response from within ED and the emergency theatres. During the incidents, arrangements were made to provide continuation of haematology clinical care. In addition, transfusion staff transported patient samples and blood components as needed, which reduced the pressure on porters. Additional porters are a valuable but often scarce resource out of hours, and it may be difficult to get additional staff to attend on site due to traffic and public transport problems. The departmental guidance has been updated to reflect the changes in practice for transfusion staff (Table 1).

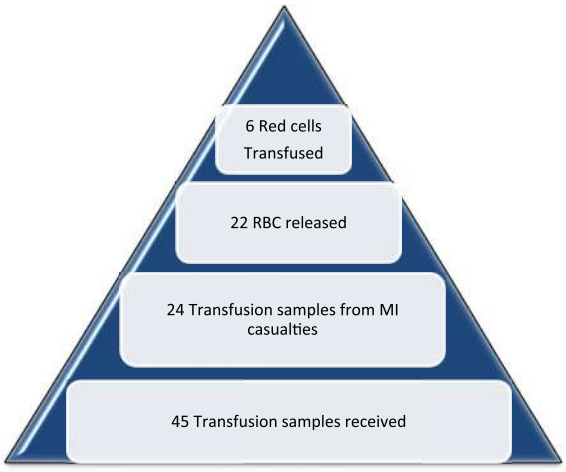

FIGURE 2 Transfusion activity during activation periods (red cells only) [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Organisation of blood stock and staff

1. Undertake a quick stock take of blood bank fridges and all satellite fridges. De-reserve stock for cancelled procedures
2. Once expected casualty numbers and type estimated, calculate stock requirements. Contact blood provider for resupply and inform them of the Major Incident
3. Move emergency stock to key areas (ED and theatres) and maintain cold chain and traceability
4. Optimise laboratory staffing levels. Consider moving staff to ED and theatres to co-ordinate blood sampling and component usage
5. Promote appropriate use of components. Use group O positive for unknown males and women beyond childbearing age to conserve O negative usage
6. Obtain a confirmatory blood sample for blood grouping to enable decision to use group specific units if appropriate
7. Maintain an incident log and document key decisions and summary of handovers

4.7 | Stock management

Blood use at other hospitals during these attacks is unknown, but the low blood use at St Mary's resonates with previously reported findings by Ramsey.[10] Blood was only used in four patients, three of whom had received significant blunt trauma. The modest blood component use may partly reflect the success of the pre-hospital response and the adoption of effective damage control resuscitation and early damage control surgery. However, it is notable that there was significant over-ordering of blood by both clinical and laboratory staff. The crossmatch: transfused ratio was 3.6:1, and 50 units of emergency RBC were ordered from the blood service as a precautionary measure. Casualty numbers were often uncertain as pre-hospital teams sometimes took patients to the nearest hospital instead of the assigned MTC.

A large amount of universal blood was moved into ED but not used. The red cell units were recovered into stock and reissued or subsequently re-distributed to other hospitals within the trust. Plasma and cryoprecipitate were only issued on request. Platelets ordered were not required but could not be redistributed due to local road closures and other security measures. There is now a shared understanding of the numbers and types of casualties that can be received at each hospital that, together with revised national guidance, should improve both clinical and laboratory demand planning.

4.8 | Staff support

Following the Grenfell Tower fire, it became apparent that the psychological toll due to the number of incidents over a short period of time was felt by not just frontline clinical staff, some of whom had military experience, but also other members of the non-clinical community, including laboratory staff. Two hospital staff, one of whom worked in the laboratory, lived in the Grenfell Tower; they had lost all their possessions in the fire. Grenfell Tower sits in the middle of a triangle cornered by our three main hospitals: St Mary's, Hammersmith and Charing Cross. The geographical proximity to the tower block and the need for laboratory staff to pass the burned building when travelling from home to work daily, together with knowing that a member of the laboratory team had lost their home and belongings in the fire, contributed to the psychological impact. The death of a young patient and the knowledge that many others had died in the fire compounded the emotional stress felt by all.

5 | DISCUSSION

Transfusion emergency preparedness is increasingly recognised as an important element in the healthcare response to MIs and MCEs.[10] However, the workload and psychological impact on laboratories has rarely been described. This is the first report of the accumulated effects of MIs on laboratory staff and has served to inform national guidance.[8] The emerging literature focusses primarily on blood use. Reviews of blood use in MCEs published in 2013 and 2017 suggested that blood use per patient admitted was modest, at 2–3 units of red cells. However, we note that the haemostatic support per patient may be increasing as both transfusion practice and mechanisms of injury change. The most recent analysis by Ramsay recommended three RBCs, one plasma and a quarter platelet dose per admission.[11] He confirmed the rising plasma usage and platelet needs, especially in large mass shootings. In contrast, only 10% of our patients required blood, and the average use of RBC was 2 per admission. Too much blood was ordered in anticipation of traumatic haemorrhage. On reflection, this was felt to be due to a lack of awareness of the hospital plans and the nature of the events. Our review demonstrates that not all P1, that is, critically injured, patients need blood, whereas others admitted during same period may need blood.

Although the demand for blood during these four incidents appears modest, it belies the work behind the scenes to process blood samples and manage stock. Six units of red cells were transfused for the 45 samples grouped. Of these, four samples (10%) were mislabelled. These errors caused additional work when staffing levels were low. We describe a case of WBIT, which is one of the most common causes of error within the transfusion chain. The event was identified by the laboratory using a second sample for ABO confirmation. The "second sample" rule is designed to identify sampling errors and is most often picked up by laboratory staff. Laboratory staff must be alert for, and correct errors during a potentially stressful period. Other members of the hospital transfusion team can support them by bridging the clinical and laboratory divide. Transfusion Practitioners were sent to the ED to support transfusion practice in the last two incidents. A similar practice was reported during the Manchester bombing in 2017.[12] This new concept in managing transfusion support has been incorporated into national guidance. The Transfusion Practitioner is emerging as the key link in this patient safety process.[13]

The study describes the experience of a single hospital site within a capital city. There are limitations to generalisability. We recognise that each hospital site has its own unique set of challenges and that teams should prepare their own EPRR plan. The blood use was lower than expected and should not be used as a generic guide. We have used a variety of sources to inform this paper and reduce errors. Post-incident review is important, but data collection is difficult. Patient tracking may be incomplete, and patients are admitted before hospitals have been formally "stood up". In this small study, one P1 casualty was admitted before the hospital was placed on standby for London Bridge and was not initially captured through the MI numbering system. Anecdotally, this has been reported elsewhere. Despite these limitations, we hope that the practical lessons learned will inform others. Our final comments are to highlight that the psychological stress caused by these events should not be underestimated. The impact may be worsened where there are chronic staff shortages and use of temporary team members. All efforts should be made to minimise the impact. Good leadership and training, together with staff welfare facilities, are essential. Peers provide valuable support; however, some colleagues may also require signposting to professional psychological support. Operational debriefs support service

improvement and recognise the essential role of the hospital transfusion team.

6 | CONCLUSIONS

All hospital transfusion laboratories should plan and be prepared for MIs. Clinical and laboratory staff should be aware of their predetermined casualty capacity and plan accordingly. We have reviewed the transfusion team involvement in four events and have identified key areas for transfusion EPRR planning. These include security, sample handling, staffing, stock management and staff support. Although this report has described the experience of a MTC in an urban setting, the lessons learned are transferable to smaller trauma units.

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F.C. reviewed the response to the four events and wrote the paper. H.D. reviewed the literature and contributed to the writing and review of the manuscript. N.B. contributed to review and revision of the manuscript. The authors acknowledge the Hospital Transfusion Laboratory team at Imperial College Healthcare NHS Trust, particularly their colleagues Nina Bell, Lorry Phelan and Denise McKeown.

CONFLICT OF INTEREST

FC and HD are both lead members of the Emergency Planning Working Group of the National Blood Transfusion Committee. No conflicts of interest have been identified.

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ORIGINAL ARTICLE

TRANSFUSION MEDICINE WILEY

Blood collection failures from a blood establishment perspective

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Summary

Objectives: The objective of this study was to show experience of the Croatian Institute of Transfusion Medicine in monitoring and analysing collection failures caused by the venepuncture technique or occurred as a result of adverse reactions and complications experienced by donors during donation.

Background: Collection failures represent one of the leading nonconformities in blood establishments. Apart from being a negative motivating factor for blood donors, they also affect the blood components supply and have a negative financial impact.

Methods: Nonconformity records referring to collection failures were analysed retrospectively over a 6-year period (2013–2018) with regard to their frequency, causes, donor characteristics (age, gender, number of donations), place of occurrence (blood establishment, mobile sessions) and trends during the analysed period.

Results: A total of 5166 collection failures out of 618 251 donations (0.84%) were recorded during the analysed period. The leading cause was haematoma at puncture site (1676, i.e., 32.4%). Collection failures which are primarily attributed to the venepuncture technique or vein selection accounted for 91% of all cases, whereas collection failures which occurred as a result of discontinued punctures due to adverse reactions in donors accounted for 9% of all cases. A much higher frequency of all collection failure types was recorded in female donors, whereas younger donors experienced adverse reactions more frequently (median age of 24).

Conclusion: The analysis and monitoring frequency of collection failures play an important role in planning of staff training activities, work organisation and timely implementation of corrective actions.

KEYWORDS
blood collection, blood donors, collection failures

1 | INTRODUCTION

Blood collection is one of the key activities in the transfusion chain. Both donor satisfaction and quality and safety of blood components depend on it. Collection failures reduce the blood components supply for transfusion treatment, and they can also be a negative factor for blood donors demotivating them from repeating the donation. Frequency of adverse reactions occurring during and after blood donation depends equally on experience of the blood collection staff, the venepuncture technique and overall engagement with donors. Therefore, the reputation of a transfusion establishment is also highly dependent on the above factors.

According to Croatian Institute of Transfusion Medicine (CITM) experience, collection failures are the leading nonconformity of the blood collection department. According to a study published in 2019,[1] as many as 47.3% of all nonconformities of the CITM Department of Promotion of Blood Donation and Blood Collection referred to collection failures, which speaks enough about their significance.

Therefore, each blood collection centre should make continuous efforts to keep collection failures at the lowest possible level. This can be achieved by initial and continuous staff education, but also with other quality assurance elements in this segment of transfusion activity. This primarily refers to monitoring the frequency of collection failures and factors influencing collection failures, as well as to implementing appropriate corrective actions based on the obtained results.

Since studies on this topic are extremely rare, we believe that the results presented will draw attention to the importance of this issue, enable data comparison and help create strategies for reducing the frequency of this nonconformity type.

2 | MATERIALS AND METHODS

Records referring to collection failures that occurred at the Croatian Institute for Transfusion Medicine in Zagreb over a 6-year period (2013–2018) were analysed in this retrospective study. Above-mentioned records and the IT system of the Croatian blood transfusion service (e-Delphyn) provided data on donor characteristics (age, gender, number of donations), as well as on time, cause and place (in an establishment or during mobile sessions) of collection failure occurrences.

The frequency of collection failures was analysed according to age, gender, number of donations and place of donation. The analysis was performed for the two largest groups of collection failures: those mostly attributable to the venepuncture technique (Group 1) and those occurring as a result of donor reactions to blood collection (Group 2). This division is colloquial due to the possible overlap of two groups and their causal interdependence (staff experience and similar). There are other types of collection failures that can be attributable to technical reasons, errors, accidents, and similar; however, they are much less frequent than the first two groups.

Group 1 covers the following: double puncture (a failed puncture attempt resulting in a second puncture using a new blood collection system), double failed puncture (two consecutive failed venepuncture attempts), discontinued puncture due to haematoma, discontinued puncture due to prolonged duration (inappropriate needle position in the vein, choosing an inadequate vein and other events cause a slow blood flow so, as a consequence, the donation is not or would not be collected within the set time), failed puncture due to donor veins (donor veins are thinner and/or have more fragile walls), arterial puncture (an artery punctured instead of a vein).

Group 2 covers the following: discontinued donation due to the vasovagal reaction without losing consciousness (blood donors experience symptoms as discomfort, weakness, dizziness during donation), discontinued donation due to a short-term loss of consciousness without other signs or symptoms and discontinued donation due to a long-term or complicated loss of consciousness. Employees responsible for performing venepuncture and attending physicians enter all information about failed collections into a nonconformity report.

Frequency of each type of collection failures was compared with regard to gender (M/F), number of donations (first-time or repeat donors), age groups (18–25, 26–35, 36–45, 56–64 and >65) and place of donation (blood establishment or mobile session).

In addition, the frequency of collection failures was analysed for the monitored 6-year period on an annual basis, as well as on a monthly basis in order to determine possible seasonal fluctuations.

Staff experience was impossible to analyse due to a high turnover rate of employees, making processing of such data more difficult. This study does not cover collection failures caused by technical factors (blood bags, poor welds, scales, etc.), errors and accidents (e.g., failure to close a clamp after puncture or needle slipped out of the vein during blood collection) and similar.

3 | RESULTS

A total of 5166 collection failures out of 618 251 (0.84%) donations (both successful and unsuccessful) was recorded during the analysed period. Their frequency during the analysed period ranged between 0.79% and 0.97% per year (Figure 1). The only significant deviation from the average annual frequency was recorded in 2016.

Three causes accounted for 87% of collection failures: haematoma at puncture site (1676, i.e., 32.4%), double venepuncture (1552, i.e., 30%) and discontinued donation due to prolonged duration (1270, i.e., 24.6%). All collection failure causes with an overview of their frequency are shown collectively in Figure 2.

Events primarily attributed to the venepuncture technique or the vein selection accounted for 91% of all collection failures, whereas events which occur as a result of adverse reactions in donors accounted for 9% of all collection failures. Out of the total number of donations, a significantly higher frequency of all collection failure types was recorded in female donors (Table 1).

Distribution of collection failures according to donor age is shown in Table 2. A higher frequency of adverse reactions during blood donation was recorded in younger donors (median age of 24). Interestingly enough, this frequency is independent of the number of donations. Namely, the total of 493 collection failures during first-time donations were recorded in the 18–25 age group, out of which 149 (30%) were caused by donor reactions, and the total of 147 collection failures during first-time donations were recorded in the 26–35 age group, out of which 37 (25.2%) were caused by donor reactions. This relation is much smaller in the 35–46 and 46–55 age groups: 7 (9.5%) out of 74 and 2 (6.1%) out of 33, respectively. Not a single case of collection failure due to donor reaction during first-time donation was recorded in the 56–65 age group, and no donor reactions whatsoever were recorded in the 66–69 age group. A detailed analysis of collection failures belonging to Group 1 (venepuncture technique)

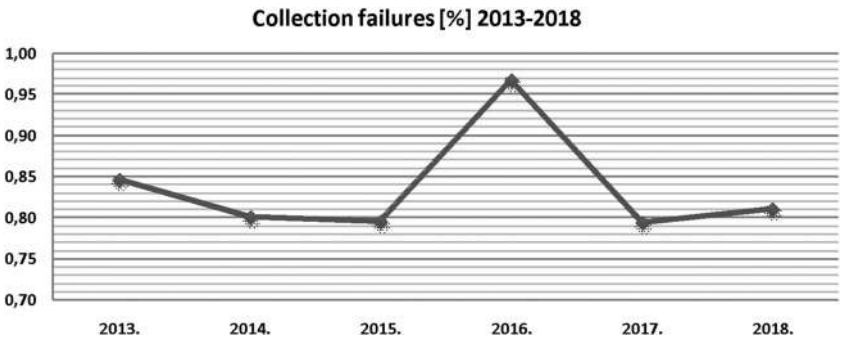

FIGURE 1 Collection failures (%) 2013–2018

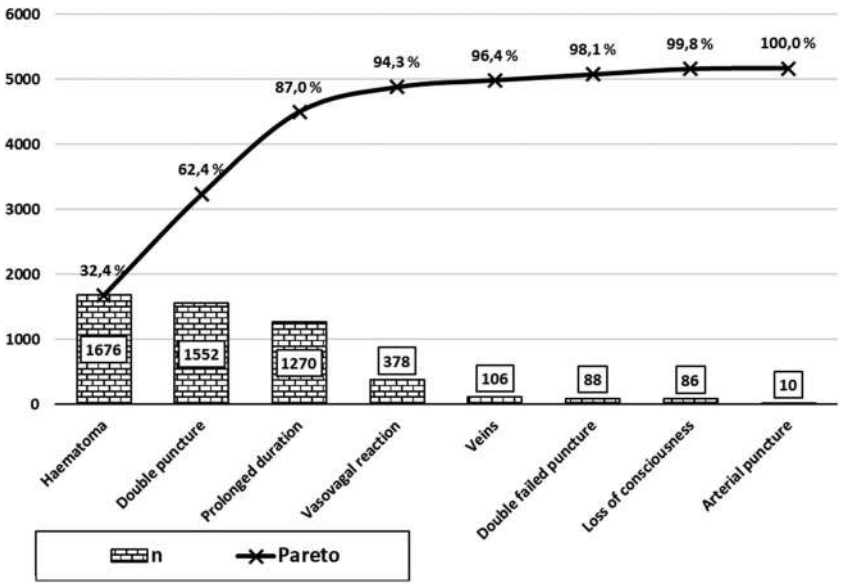

FIGURE 2 Pareto chart of collection failures according to their cause

TABLE 1 Collection failures—frequency according to type and gender (both in absolute figures and expressed out of the total number of donations per gender)

	Gender		
Failed collection type	**Both**	**M**	**F**
Donation discontinued due to fatigue	378 (0.061%)	260 (0.050%)	118 (0.121%)
Donation discontinued due to a loss of consciousness	86 (0.014%)	61 (0.012%)	25 (0.026%)
Donation discontinued due to prolonged duration	1270 (0.205%)	870 (0.167%)	400 (0.411%)
Donation discontinued due to haematoma	1676 (0.271%)	1169 (0.224%)	507 (0.520%)
Donation discontinued due to donor veins	106 (0.017%)	31 (0.006%)	75 (0.077%)
Double puncture	1552 (0.251%)	1038 (0.199%)	514 (0.528%)
Double failed puncture	88 (0.014%)	33 (0.006%)	55 (0.056%)
Arterial puncture	10 (0.0016%)	8 (0.0015%)	2 (0.0021%)
Total	5166	3470	1696

showed that double punctures and failed punctures due to donors' veins were more frequent in older donors than in donors with collection failures due to haematoma and prolonged duration of donation (40 and 42 years vs. 38 and 39 years). Although the frequency of collection failures from Group 1 shows a continued upward trend from the 26–35 age group to the age group of over 65 years, the highest frequency of such collection failures is recorded in the youngest age group. This observation contributes to the fact that a part of those collection failures also occurred as a result of vasovagal changes (drop in blood pressure, lowered heart rate), but without manifestation of other symptoms which would be recorded as donor reactions.

TABLE 2 Collection failures according to donor age (both in absolute figures and expressed out of the total number of donations per age groups)

Age	18–25	26–35	36–45	46–55	56–65	>65
Venepuncture technique	983 (1.06%)	950 (0.59%)	1172 (0.71%)	1074 (0.85%)	567 (0.85%)	42 (0.03%)
Donor reactions	213 (0.23%)	100 (0.06%)	43 (0.03%)	17 (0.01%)	5 (0.01%)	0
Total	1196	1050	1215	1091	572	42

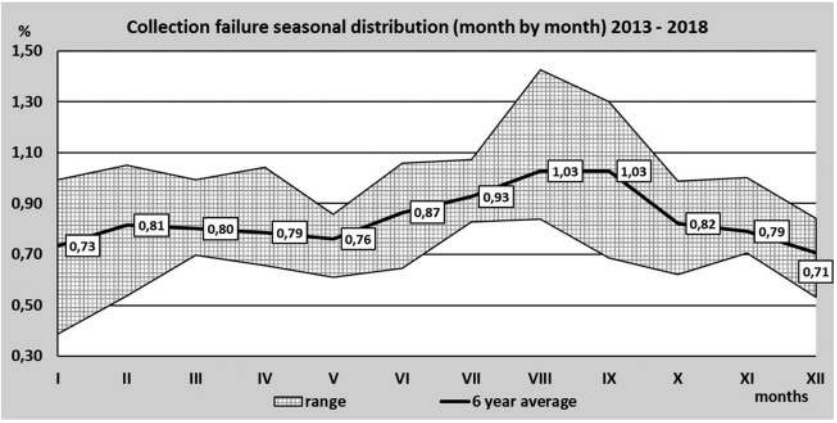

FIGURE 3 Distribution of collection failures per months. *x* axis = months represented by roman numbers, I–XII; *y* axis = collection failure frequency (%); bold line = average monthly (I–XII) frequency of collection failures for the whole study period (2013–2018); Shaded area = frequency range for each month of the study period

When it comes to location of blood collection, failed collections in the establishment were predominant (0.89% vs. 0.81%).

The analysis of collection failure occurrences in certain months of a year showed that the highest number of collection failures occur during summer months (Figure 3).

4 | DISCUSSION

Despite regular occurrence of collection failures, literature data on such events during collection of blood and blood components is scarce. The majority of available data stems from studies on donor vigilance, which primarily focus on adverse reactions and complications in blood donors.[2]

This retrospective study analyses types and frequency of collection failures, as well as factors affecting their occurrence on a sample of more than 600 000 of whole blood and apheresis donations. Those donations were collected in the 6-year period characterised by a relatively stable total frequency of collection failures. This comes as a consequence of measures implemented for reducing the frequency of nonconformities with regard to blood collection, primarily through staff education and continuous quality monitoring, with root cause analysis of identified deviations and implementation of appropriate corrective actions. The efficiency of those measures is demonstrated in a paper published in 2004.[3]

The leading causes of collection failures in this study were haematoma at puncture site (32.4%) and double puncture (30%). Predominance of those two causes of collection failures highlights the importance of the venepuncture technique, thus also the significance of staff education in achieving a low frequency of collection failures. This is also supported by our study on correlation between experience of the blood collection staff and the frequency of collection failures caused by the venepuncture technique.[4] That study demonstrated that it takes up to a year for new employees to reach the same level of collection failures as their more experienced colleagues.

The third most common cause of collection failures in this study were donations discontinued due to prolonged duration (24.6%). The exact causes of prolonged duration of blood collection time were not determined in this study. However, the literature data on phlebotomy suggests that needle insertion technique and selection of the vein are of crucial importance to establish and maintain an adequate flow.[5,6] This type of collection failures can also be caused by vasovagal changes in donors, when a drop in blood pressure and slowing down of heart rate result in reduced blood flow. According to the aforementioned 2015 study, experience and skills of staff influence not only the venepuncture technique, but also the frequency of vasovagal reactions in blood donors.[4] Therefore, one can assume that a prolonged donation time can occur as a result of both of those causes or as a combination thereof.

The collection failure analysis according to donor age showed that double punctures and failed punctures due to donors' veins are more frequent in older donors, which can be explained with reduced elasticity and more fragile vein walls, caused by ageing.[7,8] Even though the majority of studies on the effects of ageing on the cardiovascular system focus on arteries, veins undergo similar structural and functional changes, as well.[8] Therefore, the frequency of collection failures

primarily attributable to the venepuncture technique increases with the advancing donor age, with the exception of the youngest age group, in which this cause was recorded as the most frequent. This could also be connected with the aforementioned discussion on the likely connection between the prolonged donation time and vasovagal changes in donors, even in cases when those changes do not manifest themselves as adverse reactions.

As expected, collection failures due to vasovagal reactions in donors were much more frequent in the youngest age group of 18–25 years. This is because younger people are more prone to experience vasovagal reactions, which is strongly confirmed by literature data.[9-12] Those data point to the importance of pre- and post-donation fluid intake, as well as other measures of ensuring a comfortable and relaxed atmosphere during blood donation, especially when it comes to the more vulnerable population of younger donors, data on which can also be found in literature.[13-15]

According to location of occurrence, collection failures were somewhat more frequent in the establishment than at mobile sessions (0.89% vs. 0.81%). We did not find any specific reason for this occurrence during our study.

As emphasised above, there were no significant variations in the frequency of collection failures during the monitored period, with the exception of 2016, when a significant rise of their frequency was recorded.

The cause of this increase could not be related to any specific factor. However, based on the results of our study from 2015,[4] which demonstrated a significantly higher frequency of collection failures among new employees and the fact that in 2016 the largest number of blood collection staff was employed (12) compared to other study years (0–8), this could be considered at least as one of the possible causes.

According to data analysis of failed collections with regard to seasonality, a significant rise in the frequency of collection failures was recorded during summer months (July, August, September). This seasonal increase in frequency could be related to higher temperatures during the summer months, and consequently to donor dehydration and fatigue. Adequate vein selection and maintaining the blood flow can be more difficult in these conditions. Also, in the summer period, less staff is present at work due to holidays while the number of donations and working hours remains roughly the same as the rest of the year. Consequently, a higher strain is being put on the working staff and it can be speculated that the higher strain can be a cause for the rise in collection failure frequency.

5 | CONCLUSIONS

The results of this study emphasise the importance of monitoring and analysing collection failures from different perspectives of a blood bank. However, data obtained is also applicable to venous blood sampling in laboratories. Monitoring the frequency of collection failures as a quality indicator in blood banks enables a timely detection of deviations from the set goals, root cause analysis of such deviations and implementation of efficient corrective actions, with the aim of keeping the frequency of collection failures at the lowest possible level. This is important not only for protecting the blood component supply as a unique resource in patient treatment, but also for ensuring the most positive possible donor experience during donation of blood and blood components. Initial and continuous staff education can be planned based on knowledge of the structure and frequency of collection failures, also taking into account needs of certain donor groups with regard to their age, gender and other characteristics. Publishing of results of the collection failure analysis enables benchmarking among establishments of similar characteristics. Reducing the frequency of collection failures is important from the economical perspective, as well, since it leads to less destruction of both input materials and finished products.

CONFLICT OF INTEREST

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

Marko Karlo Radovčić and **Tomislav Vuk:** Designed the study, collected, analysed and interpreted the data and wrote the paper. **Tihomir Očić:** Analysed the data. **Julijana Ljubičić** and **Irena Jukić:** Revised the paper and assisted with editing.

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Comparison of four methods to measure haemoglobin concentrations in whole blood donors (COMPARE): A diagnostic accuracy study

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Abstract

Objective: To compare four haemoglobin measurement methods in whole blood donors.

Background: To safeguard donors, blood services measure haemoglobin concentration in advance of each donation. NHS Blood and Transplant's (NHSBT) customary method have been capillary gravimetry (copper sulphate), followed by venous spectrophotometry (HemoCue) for donors failing gravimetry. However, NHSBT's customary method results in 10% of donors being inappropriately bled (ie, with haemoglobin values below the regulatory threshold).

Methods: We compared the following four methods in 21 840 blood donors (aged ≥18 years) recruited from 10 NHSBT centres in England, with the Sysmex XN-2000 haematology analyser, the reference standard: (1) NHSBT's customary method; (2) "post donation" approach, that is, estimating current haemoglobin concentration from that measured by a haematology analyser at a donor's most recent prior donation; (3) "portable haemoglobinometry" (using capillary HemoCue); (4) non-invasive spectrometry (using MBR Haemospect or Orsense NMB200). We assessed

Steven Bell and Michael Sweeting are joint first authors.

David J. Roberts, John Danesh and Emanuele Di Angelantonio are joint last authors.

sensitivity; specificity; proportion who would have been inappropriately bled, or rejected from donation ("deferred") incorrectly; and test preference.

Results: Compared with the reference standard, the methods ranged in test sensitivity from 17.0% (MBR Haemospect) to 79.0% (portable haemoglobinometry) in men, and from 19.0% (MBR Haemospect) to 82.8% (portable haemoglobinometry) in women. For specificity, the methods ranged from 87.2% (MBR Haemospect) to 99.9% (NHSBT's customary method) in men, and from 74.1% (Orsense NMB200) to 99.8% (NHSBT's customary method) in women. The proportion of donors who would have been inappropriately bled ranged from 2.2% in men for portable haemoglobinometry to 18.9% in women for MBR Haemospect. The proportion of donors who would have been deferred incorrectly with haemoglobin concentration above the minimum threshold ranged from 0.1% in men for NHSBT's customary method to 20.3% in women for OrSense. Most donors preferred non-invasive spectrometry.

Conclusion: In the largest study reporting head-to-head comparisons of four methods to measure haemoglobin prior to blood donation, our results support replacement of NHSBT's customary method with portable haemoglobinometry.

KEYWORDS

gravimetry, haemoglobin screening, HemoCue, inappropriate bleeding, inappropriate deferral, non-invasive haemoglobin measurement, whole blood donor

1 | INTRODUCTION

Blood services are mandated to measure haemoglobin concentrations of potential whole blood donors in advance of each donation. The rationale is to protect the health of donors (ie, to prevent collection from anaemic donors and mitigate the possibilities of rendering the donor anaemic) as well as to ensure the quality of blood products.[1,2] European legislation on selection criteria of blood donors (EU directive 2004/33/EC Article 4) states that haemoglobin concentration should be ≥125 g/L for women and ≥ 135 g/L for men before allowing blood donation.[3] There is, however, substantial variation across national blood services in methods of haemoglobin measurement.[4,5] This has resulted in part because the timing of blood sampling and sample material for assessing blood donors is not defined by legislation, and partly because there is little evidence about the comparative performance of different rapid measurement methods.[6-11]

The customary approach of National Health Service Blood and Transplant (NHSBT, the national blood service of England) has been a gravimetric method (copper sulphate test) carried out on finger-prick capillary blood taken immediately before donation, followed by a spectrophotometric test (HemoCue) with venous blood for those who fail the copper sulphate test.[12] Recent data, however, indicate that NHSBT's customary method may allow about 10% of donors to give blood despite having baseline haemoglobin concentrations below the minimum regulatory threshold.[13,14] By contrast, blood services in some countries (eg, the Netherlands and Finland) assess haemoglobin concentration before blood donation using a spectrophotometric test on capillary blood obtained by a finger-prick.[4] Other services (eg, France and Denmark) use haemoglobin values obtained from the most recent prior donation ("post donation" approach), employing automated haematology analysers of venous blood.[4,15] Other services (eg, Bavaria, Ireland, Spain) have employed non-invasive spectrometry that does not require obtaining a blood sample.[4,16]

We conducted a within-person comparison of four haemoglobin measurement methods using performance metrics relevant to the blood donation context and comparing each method to the reference standard of a haematology analyser.

2 | METHODS

2.1 | Study design

This study evaluated four haemoglobin measurement approaches used by blood services in high-income countries (see "Diagnostic tests" below) against a haematology analyser reference standard. The study involved participant recruitment into two stages (Figure 1). Stage 1 involved direct comparisons of invasive and non-invasive methods in the same participants. Stage 2 involved an indirect comparison of two non-invasive spectrometry devices described below. Allocation of the non-invasive device between teams was done by "cross-over" randomisation. Participants in Stage 1 were not eligible to join Stage 2. The study protocol is provided in the Annex. The study was registered with ISRCTN (ISRCTN90871183), and approved by the National Research Ethics Service (15/EE/0335).

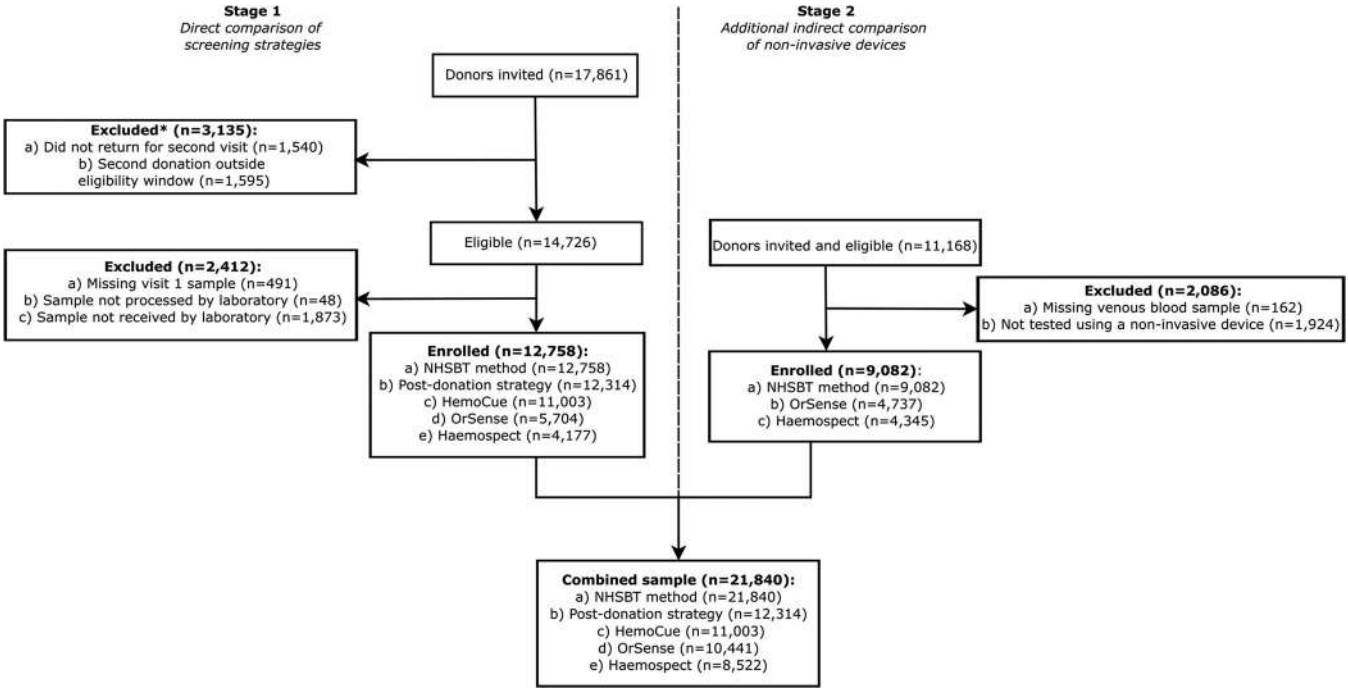


FIGURE 1 CONSORT flowchart showing.
Note: 30% drop-out rate expected between Stage 1 visit 1 and visit two donors as per study design

2.2 | Diagnostic tests

We used haemoglobin concentration measured by a Sysmex XN-2000 haematology analyser at a central laboratory (UK BioCentre, Milton Keynes, UK) as the study's reference standard.[17] We evaluated four rapid diagnostic tests against that standard: (1) gravimetry/venous HemoCue ("NHSBT's customary method" at the time of this study), i.e., a copper sulphate gravimetric test carried out on finger-prick capillary blood, followed by spectrophotometry (HemoCue AB, Ängelholm, Sweden) on venous blood for those failing gravimetry[12]; (2) "post donation" approach, that is, estimating current haemoglobin concentration from that measured by a haematology analyser at a donor's most recent prior donation (ie, about 12–16 weeks earlier); (3) "portable haemoglobinometry", using a Hemocue 301 device using finger-prick capillary blood[18]; and (4) one of two hand-held non-invasive spectrometer devices - the MBR Haemospect (MBR Optical Systems GmbH & Co. KG, Wuppertal, Germany)[19] or the Orsense NMB200 (OrSense Ltd, Petah-Tikva, Israel).[20]

2.3 | Study participants

Between February 2016 and March 2017, donors were eligible for recruitment into COMPARE if they: were aged 18 years or older; fulfilled routine criteria for donation (with the exception of pre-donation haemoglobin concentration measured using the NHSBT testing method); had an email address and access to the internet to respond to web-based questionnaires; and were willing to undergo additional haemoglobin concentration measurements at one of the 10 "mobile" donor centres of NHSBT, the sole blood provider to the NHS in England, UK. After reading study information leaflets and participating in a discussion with donor carer staff, eligible donors were asked to complete the study consent form and provide a blood sample. Soon after enrolment, participants received online health and lifestyle questionnaires, including the Fitzpatrick Skin Score.[21]

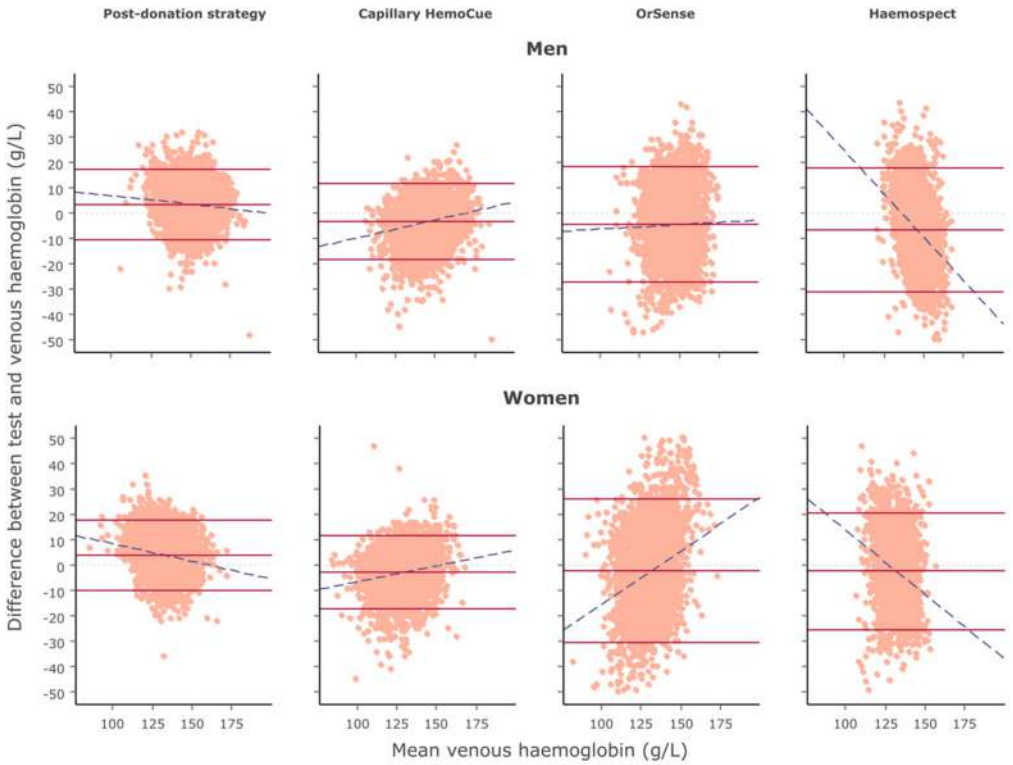
2.4 | Outcomes

The primary endpoint was the proportion of donors in the study who would have been inappropriately bled by each method (ie, the proportion of donors for whom a given method would not identify them as having sub-threshold haemoglobin levels as measured by the reference standard). Secondary endpoints included sensitivity, specificity, the proportion of donors who would have been excluded from blood donation ("deferred") incorrectly, variability of the performance of different methods by donors' personal characteristics (eg, repeat vs first-time donor, and skin colour tone), and the acceptability of different methods according to donors.

2.5 | Statistical analysis

The statistical analysis followed a prespecified plan. Briefly, Bland-Altman[22] plots were used to assess systematic difference between haemoglobin screening methods when compared against the reference standard, and supplemented by linear regression models to examine proportional biases (ie, how much the difference between

two methods is dependent on the magnitude of the measurement). The percentage of donors who would have been bled below the threshold (ie, <125 g/L for women and <135 g/L for men) was calculated by taking the number of donors categorised as having adequate haemoglobin levels by the screening method but should have been deferred according to the reference standard, and dividing by the total number of donors in the analysis population. The proportion of donors incorrectly deferred above the threshold was calculated similarly. Differences between each screening method and the reference standard were assessed using a McNemar's test for paired within-person comparisons. For direct comparisons between strategies, donation outcomes were standardised by sex and haemoglobin level to a reference population (ie, returning Stage 1 donor population). Each observation was assigned a weight based on the relative frequency of the sex-specific haemoglobin level appearing in the reference population relative to the estimation sample. The proportions for each of the four donation outcomes (bled below haemoglobin threshold, bled above haemoglobin threshold, deferred below haemoglobin threshold, deferred above haemoglobin threshold) were then weighted accordingly. Sensitivity (the probability of correctly identifying donors with a low haemoglobin level) and specificity (the probability of correctly identifying donors with sufficient haemoglobin levels) of each screening method were calculated and used to define the area under a receiver operating characteristic curve (AUC) to illustrate the diagnostic ability (ie, how well a test discriminates between donors with low and sufficient haemoglobin levels) of a screening method at different haemoglobin thresholds. Sex-specific sample size was estimated to provide 80% power, at a 5% significance level, to detect a 10% relative difference in the false pass rate (ie, percentage of donors who would have been bled below the threshold) between the NHSBT customary method and any of the other tests (Annex). Analyses were conducted separately for men and women using Stata v14. The analysis adhered to the Standards for Reporting Diagnostic Accuracy Studies (STARD).[23]



	Post-donation strategy	Capillary HemoCue	OrSense	Haemospect
Mean bias (95% LOA)				
Men	3.6g/L (-10.4, 17.6)	-3.1 g/L (-18.0, 11.8)	-4.2 g/L (-27.3, 18.8)	-6.3 g/L (-30.7, 18.0)
Women	4.0 g/L (-9.9, 18.0)	-2.4 g/L (-16.9, 12.1)	-2.2 g/L (-30.5, 26.2)	-2.2 g/L (-25.1, 20.8)
Proportional bias (95% CI)				
Men	-0.07 g/L (-0.09, -0.05)	0.15 g/L (0.13, 0.17)	0.04 g/L (-0.00, 0.07)	-0.69 g/L (-0.74, -0.64)
Women	-0.14 g/L (-0.16, -0.12)	0.13 g/L (0.11, 0.14)	0.42 g/L (0.38, 0.46)	-0.51 g/L (-0.56, -0.46)

FIGURE 2 Bland–Altman plot of each haemoglobin testing strategy by sex using venous haemoglobin values as the reference test. *Note:* Dotted light grey lines represent zero bias. Solid red lines represent the mean bias of the testing strategy (middle) and accompanying 95% limit of agreement (LOA; upper and lower) of the mean bias. Dashed blue lines depict proportional bias estimated using linear regression. Men—*Post-donation strategy*: N = 5920, 4.3% outside the LOA. *Capillary HemoCue*: N = 5279, 5.1% outside the LOA. *OrSense*: N = 4861, 5.7% outside the LOA. *Haemospect*: N = 4352, 5.5% outside the LOA. Women - *Post-donation strategy*: N = 6394, 5.2% outside the LOA. *Capillary HemoCue*: N = 5724, 5.0% outside the LOA. *OrSense*: N = 5580, 5.2% outside the LOA. *Haemospect*: N = 4170, 5.5% outside the LOA [Color figure can be viewed at wileyonlinelibrary.com]

2.6 | Role of the funding source

The academic investigators and representatives of NHSBT, a funder of the study, participated in the study design and oversight. The investigators at the study's academic coordinating centre had sole access to the study database, and had final responsibility for data collection, data integrity, data analysis, and data interpretation, as well as manuscript drafting and the decision to submit the manuscript for publication. All authors gave approval to submit for publication.

3 | RESULTS

A total of 29 029 participants were consented to participate in the COMPARE study (17 861 in Stage 1 and 11 168 in Stage 2), of whom 21 840 (75.2%) provided data for the current analysis (Figure 1). Table S1 shows baseline characteristics of the participants. Compared with NHSBT's general donor population, participants were, on average, older, more likely to be male, less ethnically diverse, and had a longer donation career (Tables S2 and S3). Baseline characteristics were similar between participants recruited in Stages 1 and 2, although haemoglobin concentration was approximately 3-4 g/L lower in returning donors.

Figure 2 and Figure S1 show the mean and proportional biases between the haemoglobin readings of each test and the reference standard. On average, the "post donation" approach over-estimated haemoglobin values by 3.6 g/L (95% limit of agreement −10.4, 17.6; SD 7.1) and 4.0 g/L (−9.9, 18.0; SD 7.1) for men and women, respectively, while each of the other methods tended to under-estimate haemoglobin values; −3.1 (−18.0, 11.8; SD 7.6) and −2.4 (−16.9, 12.1; SD 7.4) g/L for portable haemoglobinometry, −4.2 (−27.3, 18.8; SD 11.7) and −2.2 (−30.5, 26.2; SD 14.5) g/L for OrSense and −6.3 (−30.7, 18.0; SD 12.4) g/L for Haemospect. There was evidence of proportional bias for each test, with the "post donation" approach and Haemospect over-estimating, and portable haemoglobinometry and OrSense underestimating haemoglobin levels at the lower end of the distribution. Mean biases for non-invasive devices were larger in donors recruited in Stage 2 (Figure S2). Figures 3 and S3 show scatterplots of haemoglobin concentration measured by each testing method against the reference standard.

Figure 4 shows the AUC for each test by sex. Portable haemoglobinometry had the highest AUC, for both men and women, across all haemoglobin thresholds examined, followed by the "post donation" approach, the OrSense, and Haemospect. The sensitivities of the different methods at minimum donation thresholds for men and women were 26.0% and 34.7% for NHSBT's customary method, 27.9% and 35.5% for the "post donation" approach, 79.0% and 82.8% for portable haemoglobinometry, 44.4% and 51.3% for OrSense, and 17.0% and 19.0% for Haemospect The specificity of each method at the same haemoglobin thresholds for men and women were 99.9% and 99.8%, respectively, for NHSBT's customary method, 98.8% and 96.6% for the "post donation" strategy, 87.6% and 82.1% for portable

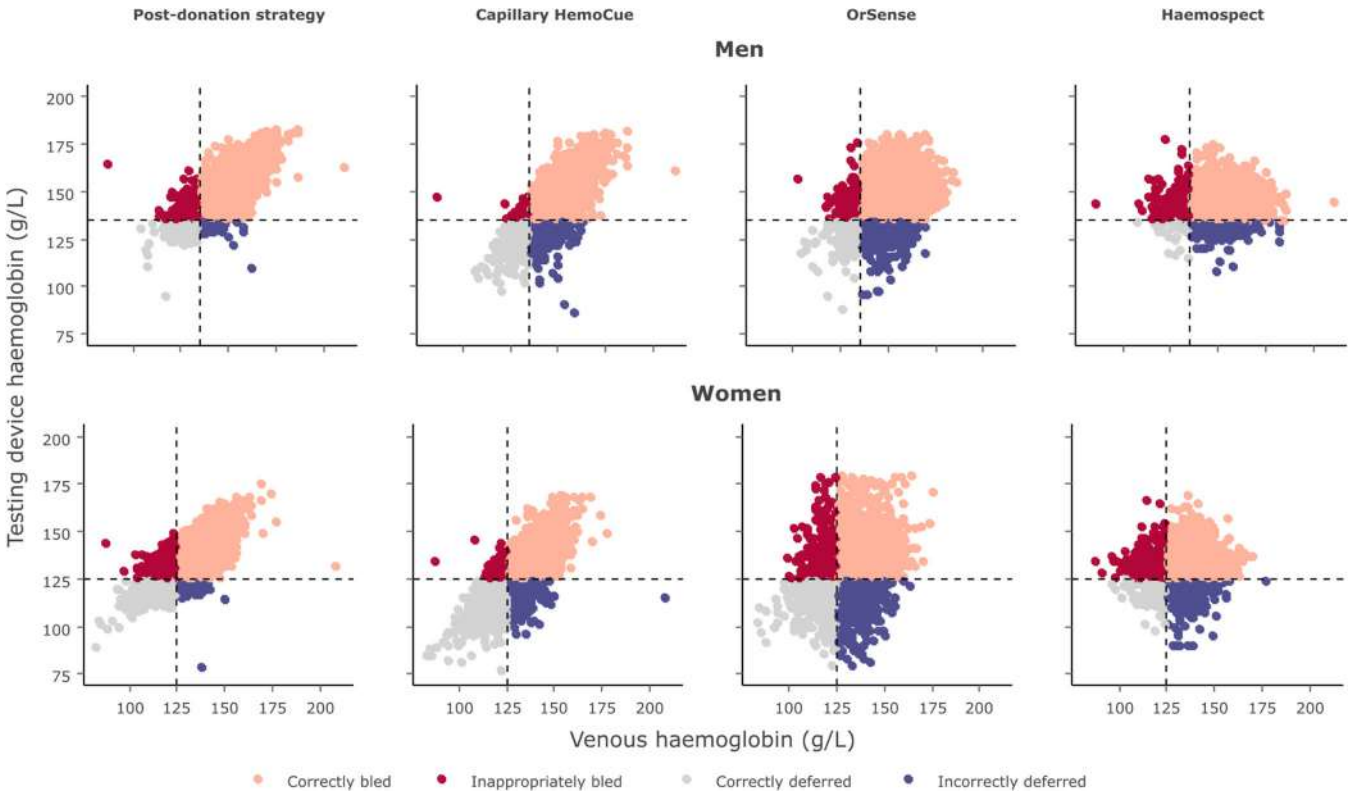
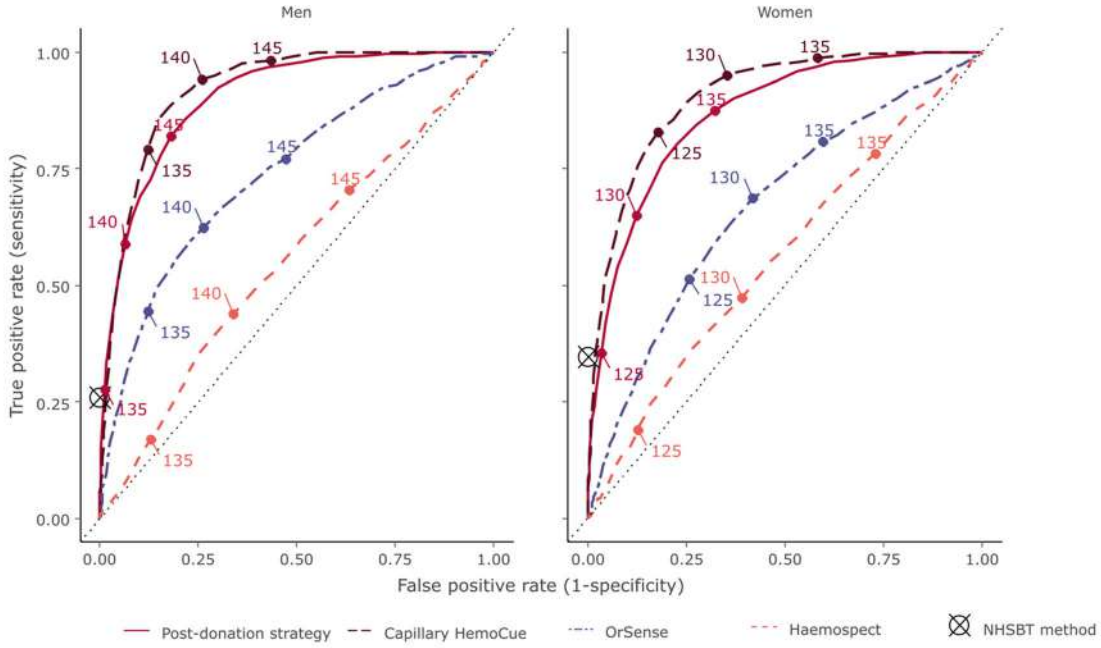


FIGURE 3 Scatterplot comparing testing device haemoglobin values to those obtained from venous blood samples by sex [Color figure can be viewed at wileyonlinelibrary.com]



AUC (95% CI)					
Men	90.2 (89.1, 91.4)	92.1 (91.2, 93.0)	74.1 (71.5, 76.7)	55.4 (52.3, 58.6)	N/A
Women	86.7 (85.7, 87.6)	90.4 (89.5, 91.2)	67.0 (65.2, 68.7)	56.2 (53.8, 58.5)	N/A
Performance at current NHSBT haemoglobin threshold					
Men					
Sensitivity	27.9 (24.3, 31.6)	79.0 (75.4, 82.3)	44.4 (39.5, 49.4)	17.0 (13.3, 21.3)	26.0 (23.1, 29.0)
Specificity	98.8 (98.5, 99.1)	87.6 (86.6, 88.6)	87.9 (86.9, 88.8)	87.2 (86.1, 88.2)	99.9 (99.9, 100.0)
Women					
Sensitivity	35.5 (33.0, 38.1)	82.8 (80.7, 84.8)	51.4 (48.4, 54.4)	19.0 (16.1, 22.2)	34.7 (32.6, 36.7)
Specificity	96.6 (96.0, 97.1)	82.1 (80.9, 83.2)	74.1 (72.8, 75.4)	87.2 (86.0, 88.3)	99.8 (99.7, 99.9)

FIGURE 4 Receiver operating characteristic curves for each haemoglobin testing strategy at different haemoglobin thresholds by sex. *Note:* Threshold values are shown in g/L. Sensitivity and specificity of NHSBT method has been superimposed as it only provides a pass/fail result rather than a quantitative readout [Color figure can be viewed at wileyonlinelibrary.com]

haemoglobinometry, 87.9% and 74.1% for OrSense, and 87.2% for both sexes with Haemospect (Figure S4).

The prevalence of donors who would have been inappropriately bled ranged from 2.2% in men for portable haemoglobinometry to 18.9% in women for MBR Haemospect (Figure 5 and Table S4). Compared to NHSBT's customary method, use of portable haemoglobinometry performed best in reducing the prevalence of inappropriate bleeding (−5.6%, −6.3, −4.9 for men and −11.1%, −11.9, −10.2 for women, $P < 0.0001$ for both: Figure 6). The proportion of donors who would have been deferred with haemoglobin concentrations above the threshold ranged from 0.1% in men for NHSBT's customary method to 20.8% in women for OrSense (Figure 5 and Table S4). In a sensitivity analysis, the proportion of donors who would have been bled with haemoglobin concentrations below the minimum threshold using the "post donation" approach decreased while the number of donors inappropriately deferred somewhat increased with longer time between donation (Figure S5). There were notable differences in the accuracy of methods between white and non-white donors, especially for the non-invasive devices (Figure S6). Stage 1 donors lost to follow-up tended to be on average younger, earlier in their donation career, and more likely to have had haemoglobin values beneath the threshold at their first visit (Table S6).

Regarding test acceptability, 72% of donors preferred the non-invasive devices, 20% preferred the finger-prick test, and 8% the "post donation" approach. However, 77% of donors reported that test accuracy was their most important consideration.

4 | DISCUSSION

In a study of over 21 000 whole blood donors in NHSBT, the national blood service of England, we conducted head-to-head comparisons of four rapid methods for the measurement of pre-donation haemoglobin levels, comparing each against the reference standard of a haematology analyser. Our key finding was that portable haemoglobinometry (ie, using the capillary HemoCue) had the highest accuracy across all haemoglobin thresholds examined for both men and women, as well as the smallest biases in comparison with the reference standard. Furthermore, pre-specified subgroup analyses indicated that portable haemoglobinometry performed similarly well among donors of different ages, ethnicities, and levels of blood donation experience. When compared to NHSBT's customary method (ie, gravimetry/venous HemoCue), use of portable haemoglobinometry reduced the prevalence of inappropriately bled donors, but increased

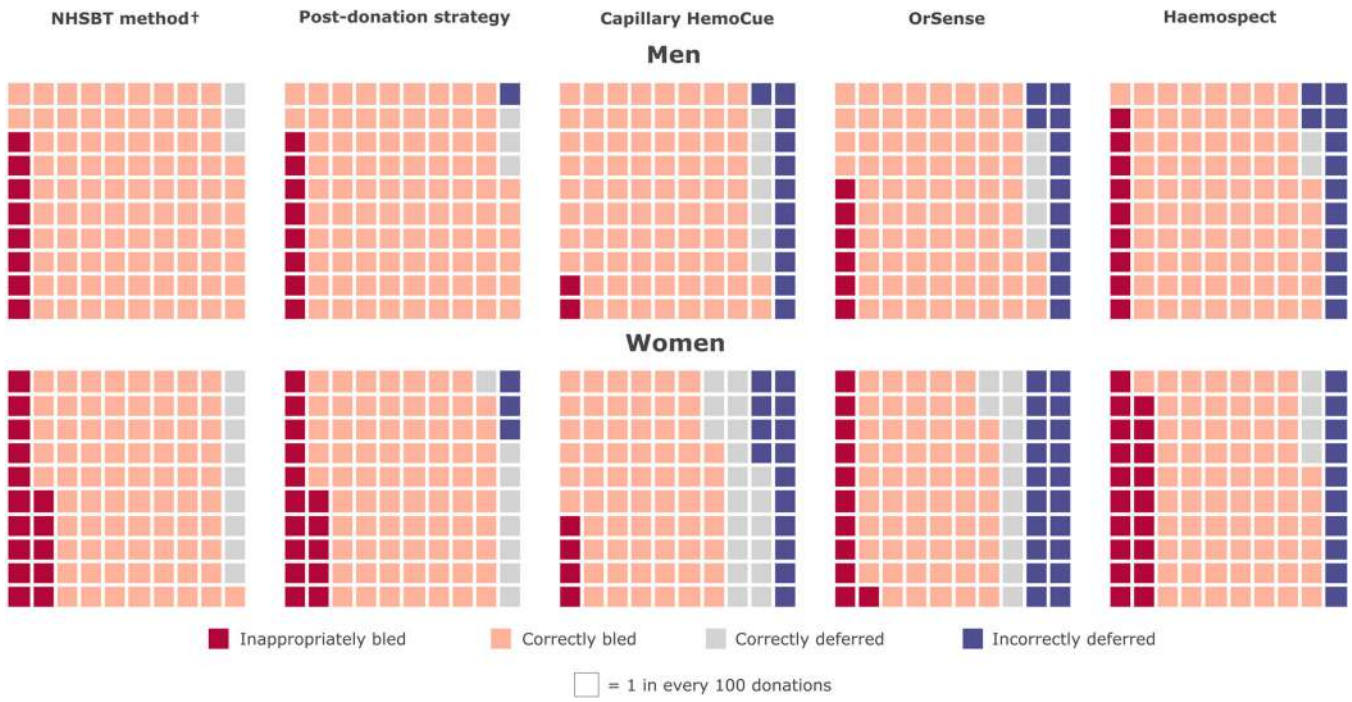


FIGURE 5 Donation outcomes by testing strategy and sex, per 100 donations standardised to the returning donor population in the COMPARE study.
Note: †1 in every 1000 donations for men, and 2 in every 1000 donations for women are incorrectly deferred using the customary NHSBT method [Color figure can be viewed at wileyonlinelibrary.com]

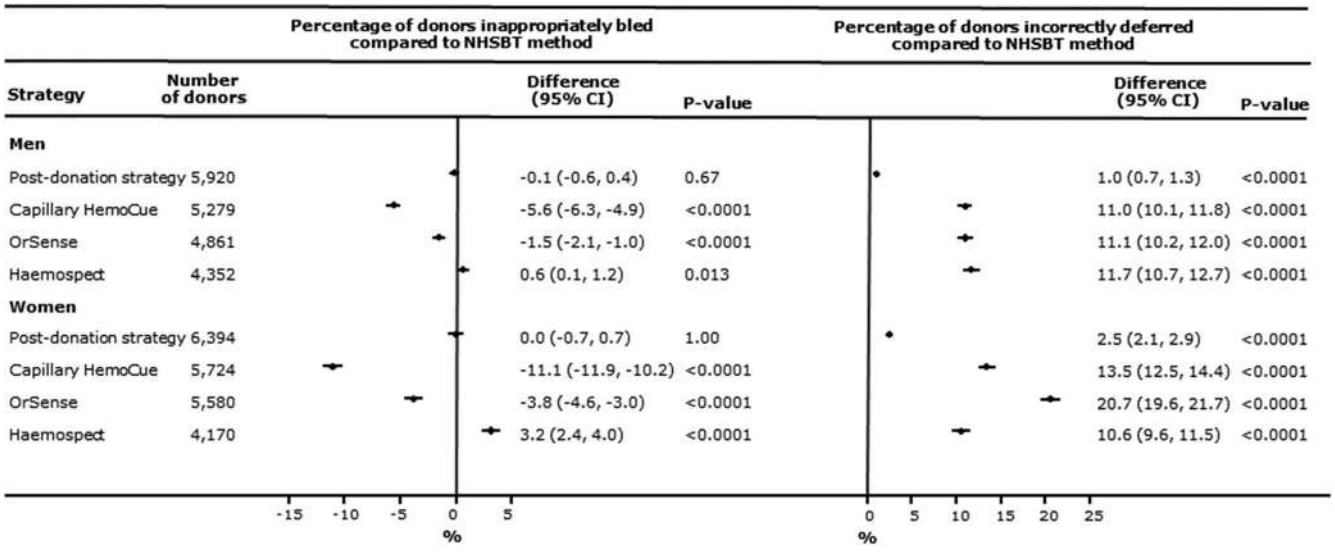


FIGURE 6 Percentage difference (95% confidence interval) in donors who would be bled below and deferred above the donation haemoglobin threshold for each testing strategy compared with the standard NHSBT test by sex.
Note: P-values calculated using McNemar's test

the proportion of donors incorrectly deferred (deferrals are disadvantageous because they demotivate donors and are costly for blood services[24]).

Based on these results, we offered two policies to NHSBT to improve its current haemoglobin screening practices. First, wholesale replacement of NHSBT's customary method with portable haemoglobinometry alone. We estimated that when projected across the approximately 1.4 million blood donations taking place annually in England, this policy would prevent about 65 000 donors annually from avoidable anaemia and potential iron deficiency and its potential

consequences. A second approach would be to use portable haemoglobinometry only in donors who failed a more methodologically rigorous use of the gravimetric test. NHSBT estimated that this second approach would prevent about 30 000 donors annually from experiencing anaemia and potential iron deficiency. The second approach would avoid the higher rates of inappropriate deferrals of donors associated with the first approach. In 2018, NHSBT adopted the second approach as national policy, implementing it swiftly across the whole of the blood service of England.[25,26]

We made several additional observations relevant to the policies and practices of blood services. We found that the "post donation" approach (ie, estimating current haemoglobin concentration from that measured by a haematology analyser at a donor's most recent prior donation) performed similarly to NHSBT's customary method when the interval between donations was about 12–16 weeks. However, the performance of this approach improved somewhat with longer intervals between donations, and when higher haemoglobin concentration at the first study visit was used to predict the donor's haemoglobin concentration at the next study visit. Blood services in several countries (eg, France, Denmark and Germany) have recently adopted the "post donation" approach due to its practical advantages, that is, it replaces the need for rapid on-site testing by using a haematology analyser at a central laboratory to measure venous blood taken from the donor's sample pouch.[15,27,28] Some blood services have started to supplement a post-donation approach with monitoring of serum ferritin, a measure of the body's iron stores, in selected blood donors.[29-31] Future work will seek to investigate the safety, cost-effectiveness, and practicability of the "post donation" approach in large, high-throughput blood services such as in England.

A further finding of our study was that non-invasive spectrometry devices (ie, MBR Haemospect and Orsense NMB200) did not generally perform well compared with the other methods, despite their obvious advantage of avoiding the need to take a blood sample. For example, these methods showed lower sensitivity for detection of haemoglobin concentration below the threshold for donation than portable haemoglobinometry, meaning higher numbers of donors would be inappropriately bled. Furthermore, non-invasive spectrometry devices, which measure haemoglobin by shining light on the skin of donors, performed inconsistently in people of different ethnicities and skin colour types, limiting the test's potential applicability to blood services in countries with a large and ethnically diverse pool of donors such as in the UK. Some blood services have suffered adverse consequences from introducing non-invasive spectrometry without such robust assessment.[16] Our study showed estimates of haemoglobin concentration by non-invasive methods, which would result in higher levels of inappropriate bleeding and/or higher levels of inappropriate deferral in blood donors when compared with portable haemoglobinometry. Nevertheless, further efforts are warranted to improve the performance of non-invasive spectrometry devices, given their potential to enhance the experience of blood donation by avoiding pain.

The current study had major strengths. It involved large numbers of participants, providing excellent statistical power and detailed comparisons of important sub-populations (eg, sex-specific results). The study design was a within-person comparison, enhancing validity by providing head-to-head comparisons of different methods to measure haemoglobin concentrations. It involved evaluation of four methods, making it wider in scope than previous efforts focusing on fewer methods.[7,9,32-34] It used a state-of-the-art haematology analyser in an accredited central laboratory as the reference standard. The study was embedded in NHSBT's routine blood service, enabling rapid recruitment of blood donors and resulting in findings of direct relevance to UK blood services.

Our study also had potential limitations. First, only about three-quarters of participants initially consented into the study returned for the second visit to allow measurements of haemoglobin concentration for the study purpose; however, a non-attendance rate of 30% at the second visit was originally factored into power calculations. Second, compared to the national donor population in England, participants in the study were older, more likely to be male, less ethnically diverse, and had a longer blood donation career. Hence, some caution is needed in extrapolating the findings to the general population of blood donors. Third, when assessing the post-donation approach we invited participants for a second visit about 12–16 weeks later, meaning our study had limited ability to assess this method for longer inter-donation intervals. Fourth, the study recruited only a limited number of non-white participants and relied on self-reported information for skin colour tone, limiting ability to assess potential differences by ethnic background.

In summary, in the largest study reporting head-to-head comparisons of four methods to measure haemoglobin prior to blood donation, our results support replacement of NHSBT's customary method with portable haemoglobinometry.

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CONFLICT OF INTEREST

John Danesh reports grants, personal fees and non-financial support from Merck Sharp & Dohme (MSD), grants, personal fees and non-financial support from Novartis, grants from Pfizer and grants from AstraZeneca outside the submitted work.

John Danesh serves on the International Cardiovascular and Metabolic Advisory Board for Novartis (since 2010); the Steering Committee of UK Biobank (since 2011); the MRC International Advisory Group (ING) member, London (since 2013); the MRC High Throughput Science Omics Panel Member, London (since 2013); the Scientific Advisory Committee for Sanofi (since 2013); the International Cardiovascular and Metabolism Research and Development Portfolio Committee for Novartis; and the AstraZeneca Genomics Advisory Board (2018).

AUTHOR CONTRIBUTION

All authors contributed to data collection, study design, data analysis, interpretation, and drafting of this paper.

David J Roberts, John Danesh, Emanuele Di Angelantonioon are joint last authors.

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ENDNOTE

[1] The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health and Social Care.

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

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HIV primary drug resistance and associated HIV risk factors among HIV positive blood donors in Brazil from 2007 to 2017

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Abstract

Background: Acquisition of HIV primary drug resistant (PDR) infection can lead to poor virologic and clinical outcomes in individuals and hampers public health efforts in epidemic control. Monitoring PDR in HIV-positive blood donors can be used to inform nationwide trends in the spread of drug-resistant HIV strains.

Methods: We conducted a cross-sectional study using genetic sequence analysis to assess HIV pol sequences, PDR, and risk factors for infection using audio computer-assisted structured interviews in four large blood centers in Brazil from 2007 to 2017.

Results: Of 716 HIV-positive blood donors, 504 (70.4%) were successfully sequenced. HIV clade B (73.2%) was the most prevalent subtype, followed by a mix of non-B (21.2%) sub-types. A twofold increase (from 4% to 8%) in recombinants prevalence was observed during the study period. Sixty-four (12.7%) presented PDR. Overall, HIV PDR prevalence remained stable during the study period. Drug resistance mutations for non-nucleoside reverse transcriptase inhibitors were found in 39 (7.7%) donors, while for nucleoside reverse transcriptase inhibitors were found in 26 (5.1%), and for protease inhibitors in 24 (4.8%) of HIV-infected donors. We did not find statistically significant differences in demographics, behavioural risk factors, or HIV genotypes when comparing volunteers with and without PDR.

Conclusion: The HIV PDR rate among donors remained stable during the study period. HIV-positive blood donors can be an informative population to monitor primary HIV resistance and ultimately may help to increase the knowledge and awareness of HIV risk factors and PDR.

KEYWORDS
blood donors, Brazil, HIV drug resistance, primary HIV drug resistance surveillance, transfusion safety

Cesar de Almeida-Neto and Brian Custer share the senior authorship for this study.

1 | INTRODUCTION

The increasing prevalence of pretreatment drug resistance and high rates of resistance-associated mutations found in many countries primarily, among specific populations, is the price for the widespread availability of the HIV treatment programs.[1,2]

HIV drug resistance (HIVDR) is due to changes in the genetic structure of the virus, affecting the ability of particular drugs or drug classes to block its replication effectively. There are two main categories: acquired HIV drug resistance, that occurs over time due to viral replication in individuals receiving antiretroviral therapy (ART), and transmitted HIV drug resistance or primary HIV drug resistance (PDR), that arises in people with no previous history of ART exposure, who were infected with an already drug-resistant strain HIV.[3]

PDR may lead to suboptimal viral suppression, followed by immunologic failure, poor clinical outcomes in individual patients, adding risk for communities from viremic patients.[4] Continued use of an already failed treatment will positively select for resistance mutations decreasing the efficacy of viral suppression by ART, favouring the risk of sexual and perinatal HIV transmission, and threaten the effectiveness of subsequent regimens.

The frequency and types of HIVDR are greatly dependent on the variety of drug classes currently proposed in each country, and according to the WHO, pretreatment HIVDR rates for non-nucleoside reverse transcriptase inhibitors (NNRTI) had increased worldwide from 11% to 29% since the global rollout of ART since 2001.[5] Also, the prevalence of NNRTI PDR is higher, notably in specific subpopulations, such as women, 11.8%, comparing to 7.8% among men initiating ART between 2014 and 2018.[3]

In Brazil, HIVDR mutations have evolved over the past 20 years, with an overall rate of 6.6% in 2001,[6] followed by 8.1% in a survey dated from 2007 to 2008,[7] and 16.3% was documented more recently in the year of 2017.[8] The prevalence of HIVDR varies across the country, ranging from 6.8%, in the Midwest, up to 11.2%, in the Southeast of Brazil.[9] Most HIVDR studies address newly diagnosed and before ARV onset.[9]

HIV infected blood donors are a readily accessible population that may, by proxy, act as a sentinel population for surveillance and monitoring drug-resistance trends. Although these donors may not represent the overall HIV population, we can infer that persons with a variable range of risk behaviours perform blood donation and can provide a picture of the currently circulating HIV strains, including genotypes, and the rates of PDR.

Our primary study aim was to evaluate the frequency of PDR among Brazilian blood donors from among a 10-year time frame (2007–2017), and secondarily to assess if PDR was associated with HIV different genotypes and donors behavioural risk factors.

2 | MATERIALS AND METHODS

2.1 | Study design and population

In this cross-sectional study, serologically confirmed HIV infection blood donors, during two phases of the NHLBI, NIH REDS program (Retrovirus Epidemiology Donor Study (II) and Recipient Epidemiology and Donor Evaluation Study (III)—International Component, Brazil REDS-II (2007–2012) and REDS-III (2012–2017)[10] were recruited to participate in molecular surveillance and behavioural risk factor assessments. Following the analysis of the work previously done, we extended that by describing in a more detailed and comprehensive narrative.

2.2 | Settings

HIV infected blood donors from four large Brazilian blood centers were included in the study. Fundação Pró-Sangue (FPS) is located in the state of São Paulo, Fundação Hemominas in Belo Horizonte, Minas Gerais; Fundação Hemorio, Rio de Janeiro; all in the southeastern part of Brazil which is the most densely populated, and Fundação Hemope in the state of Pernambuco, in the northeastern part of the country (Figure 1). In 2016, 3 796 776 blood donations were collected in Brazil, and 593 949 (15%) of these blood units were collected in these participating blood centers.

In Brazil, HIV infected donors are asked to return to the blood center for notification of the routine donation testing results, and for the collection of additional samples for new confirmatory testing.[11] In our study, all blood donors who returned to the blood bank were invited to participate.

A consecutive and sequentially convenience sample of HIV diagnosed blood donors enrolled in the research study. The participants collected blood samples for molecular surveillance and completed an audio computer-assisted structured interview (ACASI).[12] The ACASI instrument included questions on demographics, previous blood donation, motivational factors for donation, blood testing, HIV knowledge, and HIV behaviour risk factors (occupational, non-occupational and sexual exposure). Details of the study recruitment, HIV behaviour risk factor questionnaire, and HIV genotyping methods have been previously described.[11,13]

2.3 | Laboratory methods

2.3.1 | HIV-1 clade typing and drug resistance testing

Subtype and resistance analyses were performed at Fundação Pro-Sangue, São Paulo.[14] In brief, an HIV genome fragment encompassing the protease gene and approximately 700 base pairs of the reverse transcriptase gene were amplified and sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) following the manufacturer's protocol. Subsequently, the products of this reaction were analysed by an ABI3500 automated sequencer (Applied Biosystems). Only partial HIV pol sequences were obtained. The calibrated population resistance tool version 5.0 beta (available through the Stanford University HIV Drug Resistance Database http://cpr.stanford.edu/cpr.cgi)

FIGURE 1 Locations of REDS-Brazil participating blood centers [Color figure can be viewed at wileyonlinelibrary.com]

and Bennett et al,[15] were used to identify transmitted drug-resistant mutations. Mutations listed as causing or contributing to resistance are non-polymorphic in untreated persons and apply to all HIV-1 subtypes according to the World Health Organisation (WHO) report.[3] Sequences from this study were submitted to GenBank under the numbers JQ237931–JQ238236, and KC834581–KC834601.

2.3.2 | HIV drug resistance definition

HIV drug resistance was defined as the presence of ≥1 surveillance drug resistance mutations (SDRM)[15] and classified for one or more of the following drugs: nevirapine, efavirenz, any nucleoside reverse transcriptase inhibitors (NRTI), atazanavir, darunavir or lopinavir. NNRTI resistance was defined as resistance to nevirapine or efavirenz; NRTI resistance was defined as resistance to any nucleoside reverse transcriptase inhibitor drug; and protease inhibitor resistance was defined as resistance to atazanavir, darunavir or lopinavir.[5,16]

2.4 | Statistical analysis

Variables with continuous distributions were reported as median (interquartile range) or means (SDs), depending on the distribution, while categorical variables were summarised as absolute numbers and percentages. When appropriate, 95% confidence intervals were calculated. Differences between patients with and without PDR were assessed using chi-square, or Fisher's exact test for categorical variables and *t*-test or Mann–Whitney *U* test, as appropriate, for continuous variables. All statistical analysis was performed using SAS 9.4, with a 2-sided *p* value of <0.05 considered to be significant. Missing data are shown in the tables for informative purposes but were excluded from the statistical comparisons. Statistical comparisons with HIV risk factors and PDR included all patients who reported each behaviour risk. We did not classify behaviour risk ordinarily, such as higher or lower risk exposures or calculate counts of total reported risks. HIV subtypes were treated as an outcome in the analysis alongside PDR.

2.5 | Ethical issues

Study protocols were approved by the Federal Committee on Human Subjects of the Ministry of Health in Brazil as part of the REDS-II/III International Program, local ethics committees from each of the participating blood centers, IRB of record for Vitalant Research Institute and from the Research Triangle Institute. Written informed consent for all the procedures was obtained from each participant after recall to perform confirmatory HIV test and before the sample collection. As the finding can directly impact clinical care, all laboratory results were returned to the participants.

3 | RESULTS

A total of 716 HIV infected blood donors were enrolled in the study: 341 from REDS-II (2007–2012) and 375 from REDS-III (2012–2017). Of these, 212 HIV donors were excluded from the analysis; 124 (17.3%) for reporting prior whatever ART use; 11 (1.5%) due to lack of information on ART use, and in 77 (10.7%) the HIV sequences were not successfully amplified due to low or undetectable viral load.

TABLE 1 Demographic characteristics, sexual orientation and marital status among 504 blood donors from 2007 to 2017

Demographic characteristics, sexual orientation, and marital status	REDS (2007–2017) HIV infected blood donors *n* = 504 *n* (%)	HIV infected donors with resistance *n* = 64 *n* (%)	*p* value
Site			
Hemope	181 (35.9)	15 (23.4)	0.1
Hemominas	68 (13.5)	11 (17.2)	
FPS	95 (18.8)	17 (26.6)	
Hemorio	160 (31.7)	21(32.8)	
Donor status[a]			
First time	220 (43.7)	26 (40.6)	0.7
Repeat	260 (51.6)	33 (51.6)	
Unknown	24 (4.8)	5 (7.8)	
Donation type[a]			
Community	303 (60.1)	39 (60.9)	0.5
Replacement	176 (34.9)	20 (31.3)	
Unknown	25 (5.0)	5 (7.8)	
Gender			
Male	408 (81.0)	53 (82.8)	0.7
Female	96 (19.0)	11 (17.2)	
Age (years)			
18–25	130 (25.8)	19 (29.7)	0.3
26–30	108 (21.4)	14 (21.9)	
31–39	149 (29.6)	22 (34.4)	
40+	117 (23.2)	9 (14.1)	
Education level			
Never been to school	3 (0.6)	1 (1.6)	0.3
Elementary school	154 (30.6)	18 (28.1)	
High/technical school	248 (49.2)	29 (45.3)	
College or more	97 (19.2)	16 (25.0)	
Do not know/refused	2 (0.4)	0 (0.0)	
Education level B			
≤Elementary school	157 (31.2)	19 (29.7)	0.8
>Elementary school	345 (68.5)	45 (70.3)	
Do not know/refused	2 (0.4)	0 (0.0)	
Sexual orientation[a]			
Heterosexual	286 (56.7)	32 (50.0)	0.3
Bisexual	96 (19.0)	14 (21.9)	
Homosexual	107 (21.2)	17 (26.6)	
Refused/do not know	15 (3.00)	1 (1.5)	
Marital status			
Single, never married	276 (54.8)	39 (60.9)	0.4
Living together, not married	105 (20.8)	14 (21.9)	
Married	74 (14.7)	5 (7.8)	
Divorced/widowed/separated	49 (9.7)	6 (9.4)	

[a]Self-reported.

TABLE 2 General exposure, sexual and behaviour risks among blood donors with evidence of HIV-1 primary antiretroviral resistance

HIV Reported risks	Total *n*	Primary HIV resistance[a] *n* = 64 *n* (%)	*p* value[b]
Occupational exposure			
Exposure to needle or body fluids, ever	67	6 (9.0)	0.4
Non occupational exposure			
Body piercing, ever	111	14 (12.6)	0.9
Tattoos, ever	125	19 (15.2)	0.3
Acunpuncture, ever	32	4 (12.5)	0.9
Surgery procedure, ever	328	44 (13.4)	0.4
Endoscopic/colonoscopic procedure, ever	103	13 (12.6)	0.9
PWID[c] ever (injected any drugs or non-prescription substances)	79	11 (13.9)	0.7
Blood transfusion, ever	19	3 (15.8)	0.7
Inmate, ever	11	0 (0.0)	0.4
Sexual exposure			
SEX with			
Inmate, ever	31	4 (12.9)	0.8
Blood transfusion recipient, ever	24	2 (8.3)	0.8
HIV positive partner, ever	42	4 (9.5)	0.6
HIV positive partner taking ART, ever	42	4 (9.5)	0.6
PWID ever	29	4 (13.8)	0.7
Person with potential job exposure, ever	51	7 (13.7)	0.8
Sexual partner of MSM[d]—males[b]	162	27 (16.7)	0.1
Sexual partner of MSM[d]—females[b]	3	0 (0.0)	0.9
MSM, ever	195	26 (13.3)	0.8
Lifetime number of sex partners			
0	5	1 (20.0)	0.6
1	21	1 (4.8)	
2–3	53	6 (11.3)	
4–6	104	14 (13.5)	
7–10	71	11 (15.5)	
11–20	80	15 (18.8)	
21+	69	6 (8.7)	
Number of sex partners, in the last 12 months			
0	20	4 (20.0)	0.2
1	182	17 (9.3)	
2–3	168	26 (15.5)	
4–6	61	9 (14.8)	
7–10	23	2 (8.7)	
11+	16	0 (0.0)	

[a]From the sequenced samples *n* = 275 (71.2%).
[b]Last 12 months.
[c]Person who injected drugs or non-prescription substance (PWID).
[d]Men who have sex with men (MSM).
[e]Totals must be less due to missing values.

Thus, 500 and 400 HIV infected donors (70.5% of enrolled cases) were included in the study, 304 from REDS II and 200 from REDS III. HIV case participation varied across the blood centers; 181 (35.9%) were from HEMOPE-Recife; 160 (31.7%) from HEMORIO-Rio de Janeiro; 95 (18.8%) from Fundação Pró-Sangue-São Paulo and 68 (13.5%) from HEMOMINAS-Belo Horizonte. There was a predominance of men 408 (81%), and most of the participants had more than 31 years old, 266 (52.8%).

3.1 | Primary drug resistance

Overall, 64 (12.7%) of the 504 HIV infected blood donors had PDR. The PDR prevalence varied across the blood centers. The highest PDR prevalence (17.9%) was observed at São Paulo, followed by Belo Horizonte (16.2%), Rio de Janeiro (13.1%), and the lowest (8.3%) was found in Recife. No demographic and regional differences were observed between HIV-positive participants with or without PDR (Table 1).

TABLE 3 Virologic HIV-1 characteristics by period of donation

Characteristics	REDS-II (2007–2011)		REDS-III (2012–2017)		Total	
	n (%)	95% CI	*n* (%)	95% CI	*n*	*p* value[a]
Genotypes						
B	229 (75.3)	(70.5–80.2)	140 (70.0)	(63.6–76.4)	369	0.13
Non-B	63 (20.7)	(16.2–25.3)	44 (22.0)	(16.3–27.7)	107	
A	1 (0.3)	(0.0–1.0)	-	-		
C	14 (4.6)	(2.3–7.0)	8 (4.0)	(1.3–6.7)		
D	1 (0.3)	(0.0–1.0)	4 (2.0)	(0.06–3.9)		
F/F1	47 (15.5)	(11.4–19.5)	30 (15.0)	(10.0–19.9)		
G	-	-	2 (1.0)	(0–2.4)		
Recombinants	12 (4.0)	(1.8–6.1)	16 (8.0)	(4.2–11.8)	28	
Resistance						
To NRTI[b]	15 (4.9)	-	11(5.5)	-	26	0.8
To NNRTI[c]	23 (7.6)	-	16 (8.0)	-	39	0.8
To PI[d]	18 (5.9)	-	6 (3.0)	-	24	0.1
Any antiretroviral mutation						
SDRMs[e]	37	12.2%	27	13.5%	64	0.6

[a]*p* Value for genotype B, non-B, recombinant by REDS-II/III.
[b]Nucleoside reverse transcriptase inhibitor.
[c]Non-nucleoside reverse transcriptase inhibitor.
[d]Protease inhibitors.
[e]Surveillance drug resistance mutations.

3.2 | Primary drug resistance and HIV behaviour risk factors

3.2.1 | PDR and sexual risk exposures

PDR frequency rates varied according to sexual exposure. Of 408 men, 195 (47.8%) reported sex with other men over the course of a lifetime; of these, 26 (13.3%) presented PDR. One hundred sixty-two (39.7%) men reported sex with other men in the last 12 months; of these, 27 (16.7%) had PDR.

Sex with a person who injects drugs or non-prescription substances (PWID) in the last 12 months was reported by 29 (5.8%) study participants; of these, four (13.8%) had PDR. Also, PDR was found in seven out of 51 (13.7%) blood donors that reported sex with a person under potential biological risk due to job exposure throughout the lifetime.

No significant statistical association was observed between reported sexual risk exposures, the number of sexual partners' lifetime, nor in the last 12 months, and PDR.

3.2.2 | PDR and occupational and non-occupational exposures

Overall, 67 (13.3%) of the HIV infected blood donors reported occupational exposure to needle or body fluid throughout a lifetime; of these, six (9.0%) had PDR. For non-occupational exposures, the highest PDR frequency was found among those reporting blood transfusions throughout a lifetime in which, out of 19 (3.8%) study participants, three (15.8%) presented PDR. Besides, 125 (24.8%) study participants reported tattoos, and of these, 19 (15.2%) had PDR. PWID ever was reported by 79 (15.7%); of these, 11 (13.9%) had PDR.

The remaining risk non-occupational exposures, such as body piercing, acupuncture, surgery, and endoscopic/colonoscopic corresponded to 12.6%, 12.5%, 13.4%, and 12.6% case of PDR, respectively. No subjects who had been an inmate, throughout a lifetime presented PDR. The prevalence of PDR by disclosed risk factors is represented in Table 2.

3.3 | Virologic HIV-1 characteristics during the period of donation

During the 10-year study period, HIV subtype B was the most prevalent genotype, 369 (73.2%), followed by non-B sub-types 107 (21.2%), and 28 (5.6%) of recombinants. Also, small increases in subtype D (from 0.3% to 2%) and recombinant subtypes (from 4% to 8%) were observed. Subtype A was identified among one (0.3%) participant during REDS-II and was not detected during REDS-III. In the opposite direction, subtype G was not present among participants from REDS-II; however, it was detected in two (1%) participants during REDS-III. HIV subtype differences were not significant throughout the period studied. Table 3 shows the HIV genotype characteristic during the donation period.

A decrease in the frequency of drug resistance mutation for protease inhibitor from 5.9% to 3%, followed by a modest increase for NRTI (4.9%–5.5%) and NNRTI (7.6%–8%) was observed. Considering drug class mutations, frequencies for protease inhibitors, NRTI, and NNRTI did not change between the different analysed periods

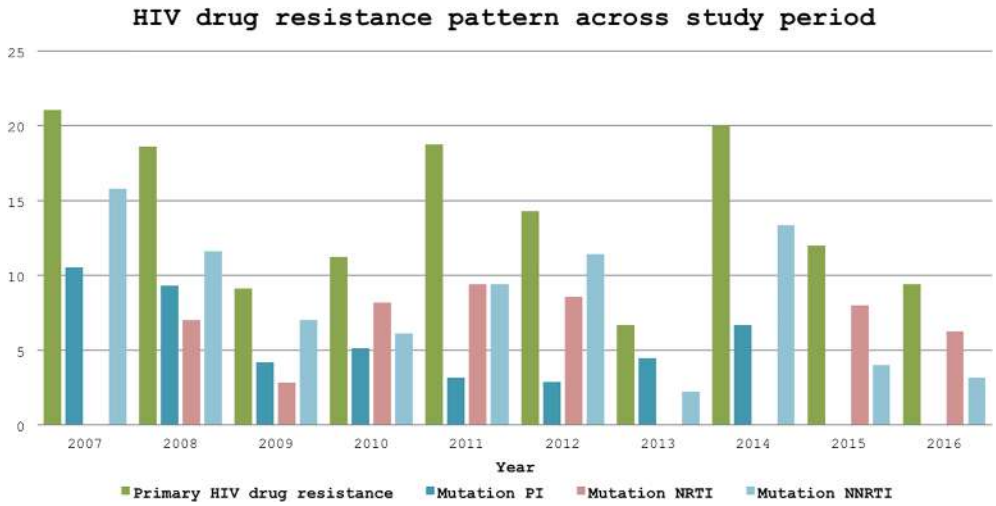

FIGURE 2 Histograms of drug classes resistance by year [Color figure can be viewed at wileyonlinelibrary.com]

(Figure 2). NNRTI resistance was observed in 39 (7.7%) donors, while NRTI was found in 26 (5.1%) and protease inhibitors, 24 (4.7%) (Figure S2). Figure S1 shows different drug classes mutations by study year and Figure S2 depicts the distribution of mutations according to the REDS study phase.

3.4 | HIV subtype and association with HIV risk factors and behaviour

The frequency of HIV subtypes varied according to the HIV risk factors and sexual behaviour. For each subtype, more than half had surgery (ever), more than 20% had tattoos (ever), and more than 14% had body piercing (ever). Being a sexual partner of MSM during their lifetime was the more frequent sexual behaviour reported, and was found among 41.5% participants with B subtype, 40.2% with Non-B subtypes, and 32.1% with recombinant subtypes. The majority of participants (69.5%) reported having had one to three sexual partners in the last 12 months independent of the subtype. There was no statistically significant difference among risk exposures, sexual behaviours, and HIV subtypes (Table S1).

4 | DISCUSSION

We evaluated more than 500 HIV-positive blood donors at four large Brazilian blood centers across a 10-year timeframe. The highest PDR was found in male donors who reported being sexual partners of MSM during the last 12 months. Our data suggest that the drug resistance mutations remained stable over the study period among the blood donation centers.

The overall PDR prevalence found in our study was consistent with PDR found in previous reports among recently diagnosed ART-naïve people living with HIV in Brazil between 2013 and 2015,[9] and among broader populations of HIV positive blood donors in Brazil, China and Spain.[17,18]

We did not find significant differences in demographics, HIV behaviour risk factors, or HIV genotypes when comparing persons with and without PDR. Additionally, the HIV PDR rate remained stable during the study period, when compared with the previous study report, in which 11.8% of PDR was found.[13]

Blood centers located in the Southeast part of the country had accounted for a twofold higher PDR prevalence compared to the Northeast (Recife). Currently, São Paulo state is responsible for more than 40% of patients receiving antiretroviral treatment in Brazil, and nearly a quarter of the people living with HIV on ART live in this city.[9] The city of São Paulo, the capital of the most populous state in the country, was the first to introduce ART treatment in the 90s.[9] Therefore, due to the sequential use of ART and broader use of unboosted protease inhibitors in the earliest years of the national HIV treatment program, it would be expected that a higher proportion of patients experiencing virological failure, and consequently transmitting HIV drug resistance would be found.[9,19] However, the PDR rate was similar to 19.4% previously reported.[13] Except for the Central-West region where the PDR prevalence in ART-naïve population was found to be lower (6.8%), the PDR rates found in our sample were somewhat similar to the overall 9.5% PDR prevalence observed in a nationwide observational study.[9]

In line with previous Brazilian studies,[9] genotype B was the most prevalent clade, reflecting the foundation of the HIV epidemic in the Brazilian population.[20] We observed an increased frequency among the recombinant clades over the study period that potentially can reflect what is happening in the general population, as a result of lower ART adherence, mainly among young adults.[19] In Brazil, there has been an increase in the genetic diversity of the epidemic when compared with other regions worldwide. In our country, unique recombinants were described in addition to the co-circulation of different HIV-1 clades.[21]

Continuous surveillance of genotypes is needed to corroborate our findings.[19] Overall, mutations leading to resistance appear to be similar among subtypes. Still, certain mutations seem to occur more frequently in non-B subtypes, in particular, consequently influencing first-line ART treatment choices.[22]

Contemporary information on genotyping, drug resistance and the spread of HIVDR strains trends is difficult to obtain, particularly in

low/middle-income countries, and the WHO suggested several monitoring activities to improve surveillance.[2,23] Additionally, preventive measures such as post-exposure prophylaxis (PEP) and pre-exposure prophylaxis (PrEP) may have their efficacy jeopardised by drug resistance. As test-and-start ART strategies are considered, data to support optimal first-line therapy are needed worldwide.[2] Supplementary resistance data extracted from variable samples and analysis of their associated HIV risk factors for PDR are lacking, although necessary to show a reliable picture of the current HIV epidemic status and strict public policies to mitigate HIV epidemics.

This study was completed before PrEP was offered as a Brazilian public health strategy in December 2017 and may serve as a baseline to understand the impact of this policy on drug resistance soon. In Brazil, PrEP is freely available for MSM and transgender women, and there are concerns that it may decrease the capacity of the tests used in the blood centers to identify infected individuals, and HIV breakthrough infection should occur.[24]

We recognise some limitations. First, our convenience sample represents a population that came for blood donation, with an expected low HIV-risk self-perception, not entirely typical of the general population or high-risk key populations. However, given the diversity and relative proportions of the range of self-disclosed risk behaviours, the sample may be, by proxy, characteristic of the HIV infected population in the communities in Brazil where the hemocenters are located. Second, we did not successfully amplify one out of 10 HIV-positives samples, representing a gap in our understanding of subtypes and drug resistance. Unamplifiable samples are likely to have lower viral loads, and we were not able to define the specific reasons why samples where unsuccessfully amplified. We did not run a multi-variate analysis to better approach associations. We considered that our study did not present the right design, sample size, nor power for this purpose. We assessed previous ART exposure by a self-reported questionnaire. If persons stated there were on ART, we did not include their samples in the PDR group, but we could not substantiate the treatment status of any participant. Finally, we did not evaluate mutations of resistance for integrase inhibitor class among these naive HIV infected blood donors. However, this drug class, represented mainly by dolutegravir, was approved for experienced patients since the end of 2015, and as first-line therapy since 2017.

We conclude that ongoing efforts to conduct molecular surveillance on HIV infections in the blood donor population can contribute to an improved understanding of HIV genotype and drug resistance distributions in Brazil. HIV positive blood donors are an informative source of samples for drug resistance monitoring, and the diversity of risks suggests that donor data can be used to gain an impression of what is happening in the broader population as a whole.[25] The role of blood donors for HIV surveillance will vary in different jurisdictions by epidemiology and donor criteria compliance. For public health, donor data merged with other surveillance national-wide databases can be employed for phylogenetic/phylodynamic analyses to understand better epidemics trends, dynamics of drug-resistance spread relative to current primary ART regimens and to provide insights into the success of policies to control HIV epidemics. This may be particularly important with the advent of the widespread availability of PEP and PrEP, and our data serve as a baseline in blood donors before the availability of PrEP. Blood center networks can share information about the national distribution of HIV infection through the different regions of Brazil, which may potentially improve the public health measures on HIV prevention and control.

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CONFLICT OF INTEREST

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

Carlos Henrique Valente Moreira, **Cesar de Almeida-Neto**, **Brian Custer**, **Ester C. Sabino**, and **Thelma T. Gonçalez:** Designed the study. **Tassila Salomon**, **Cecília S. Alencar**, **Paula Loureiro**, **Maria Esther Lopes**, **Carolina Miranda Teixeira**, **Mariana Mundim**, and **Anna Barbara Carneiro-Proietti:** Participated in data collection. **Cecília S. Alencar:** Participated in laboratory procedures. **Liliana Preiss:** Participated in the data analysis. **Carlos Henrique Valente Moreira**, **Tassila Salomon**, **Ester C. Sabino**, **Cesar de Almeida-Neto**, and **Brian Custer:** Wrote the manuscript. All authors revised and approved the final version of the manuscript.

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SUPPORTING INFORMATION

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

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ORIGINAL ARTICLE

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Validation of an apoptosis assay for extracorporeal photopheresis

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Abstract

Objectives: This validation study investigated a flow cytometric apoptosis assay according to good manufacturing practice (GMP).

Background: Extracorporeal photopheresis (ECP) is a treatment for various immunological diseases and cutaneous T-cell lymphomas. It is based on the induction of apoptosis by 8-methoxypsoralene and ultraviolet A light. The quantification of apoptosis is therefore essential for ECP improvements. However, despite numerous publications on apoptosis, validated technical details are lacking.

Methods and materials: Mononuclear cells were collected by apheresis and treated by ECP or camptothecin. Samples taken before and after ECP were cultured for 24, 48 and 72 h and analysed for apoptosis and viability of T cells and monocytes by flow cytometry with Annexin V and 7-AAD staining. Accuracy of the assay, intra- and inter-assay precision and the pre-analytical and analytical stability of the analytes were the investigated parameters.

Results: Our data indicate that the median intra- and inter-assay precision coefficient of variation for T cells was 3.86% and 4.80%, respectively. Pre-analytical stability of T cells and monocytes was ensured during short-term storage for up to 2 h on ice. After staining, analytical stability was limited to 30 min, likely because of ongoing apoptosis and loss of monocytes due to plastic adhesion.

Conclusion: The results of this validation study show that the assay is GMP-compliant and that its reliability, accuracy and precision are acceptable. While pre-analytical stability of the cells was compatible with on-site procedures, our analytical stability data indicate that this assay is not suited for batch mode analysis of ECP products.

KEYWORDS
apoptosis, ECP, extracorporeal photopheresis, validation

1 | INTRODUCTION

Extracorporeal photopheresis (ECP) has been an established therapy for cutaneous T-cell lymphoma, graft-versus-host-disease (GvHD), rejection after solid organ transplantation and various autoimmune diseases for over a decade.[1-4] In this therapeutic procedure, autologous leukocytes are collected by apheresis, treated with 8-methoxypsoralen (8-MOP) and ultraviolet A (UVA) light ex vivo, undergo covalent DNA

strand linkage by 8-MOP and are reinfused back to the patient. This serves to prevent transcription and replication, leading to cell inactivation and apoptosis.[5,6] Assays for apoptosis can therefore be used as a surrogate parameter for pharmacological potency.[7]

Apoptosis is an organised cell death process consisting of different intrinsic and extrinsic apoptosis pathways. Both pathways lead to the activation of proteolytic enzymes (caspases 3, 6, 7) and to DNA fragmentation, DNA budding and chromatin condensation.[8] The mechanism by which apoptotic cells elicit an immunosuppressive effect is very well understood.[9,10] Rapid clearance of apoptotic cells by macrophages and dendritic cells (DCs) results in up-regulation of immunosuppressive factors (e.g., Transforming growth factor beta-β, Interleukine-10) and down-regulation of costimulatory molecules. Such 'tolerized' macrophages and DCs again suppress T-cell effector activity and support regulatory T-cell function.[11,12]

Several techniques are available for the detection of different stages of apoptosis.[13] In particular, flow cytometry is able to discriminate vital from apoptotic cells at the single-cell level in a complex mixture of cells[14,15] Double staining with Annexin V, which binds phosphatidylserine on the surface of apoptotic cells, and 7-AAD as a membrane-impermeable dye for the DNA of dead or damaged cells offers the possibility to simultaneously detect apoptotic and dead cells. Despite its widespread established use,[16,17] data on the robustness and limits of operation of this assay are lacking. We therefore designed this study to characterise the validity of this assay according to the guideline of good manufacturing practice of the European Union (GMP), which ensures quality standards for drug production.[18]

2 | MATERIAL AND METHODS

2.1 | Patients

Data were obtained in 2017/2018 according to the EU Guidelines for GMP.[18] All participants gave their informed consent and received offline ECP as medically indicated. The study included a total of nine patients (eight male, one female), five (56%) with acute GvHD and one each with chronic GvHD, Sézary syndrome, cutaneous T-cell lymphoma and cellular rejection. This study protocol was approved by the local ethics committee (16-101-0046).

2.2 | Photopheresis

Apheresis was carried out with a Spectra Optia (Terumo BCT) apheresis system in cMNC mode. The ECP procedures were set up to yield leukocyte and plasma volumes of 90 and 110 ml, respectively, after cell harvesting. The resulting 200-ml cell suspension was sterilely transferred to a UVA-PIT (PIT Medical Systems GmbH) bag system, consisting of a recirculation bag and a UV-permeable ethylene vinyl acetate irradiation bag. Next, 2 ml of 8-MOP 20 mg/L (Uvadex, Therakos, West Chester, PA) was injected into the bag (final 8-MOP concentration before irradiation: 200 ng/ml). Subsequently, UVA radiation (2 J/cm^2) was delivered through the UVA-PIT system. The treated cell suspension (product) was then immediately administered to the patient.

2.3 | Sample preparation

ECP product samples were taken before the addition of 8-MOP (pre) and after irradiation (post).

Control samples were obtained from healthy blood donors. Mononuclear cells (MNCs) were separated by gradient centrifugation (Biocoll separating solution, Merck) and washed with 2 ml of dulbecco's phosphate-buffered saline (DPBS) buffer (Sigma Aldrich) at 300*g*, 5 min.

Apoptosis testing was carried out 24, 48 and 72 h after incubation in a TexMACS GMP medium (Miltenyi, Bergisch-Gladbach, Germany) with 1% Glutamax (Gibco) at 37°C and 5% CO_2. Cells incubated with camptothecin (Sigma; 200 μM, 4–6 h, 37°C, 5% CO_2) served as positive controls for gate adjusting.[19]

2.4 | Analytics

2.4.1 | Cell count

Cell concentrations were measured undiluted on an XN-550 Automated Hematology Analyzer (Sysmex, Kobe, Japan).

2.4.2 | Flow cytometry

Briefly, 1×10^6 MNCs/tube were stained after treatment with 10 μl of FcR Blocking Reagent (Miltenyi) in 90-μl Cell Staining Buffer (Beckman Coulter). Immunophenotyping of leukocytes was performed with commercially available antibodies from Beckman Coulter (BC): CD45-KrOrange, CD33-PE-Cy7 and CD3-ECD; CD56-APC-AF750, CD20-APC-AF750, CD19-APC-AF750 and CD66b-APC-AF750 were used for lineage exclusion. All primary antibodies were added to the cell suspensions and incubated for 20 min at 4–8°C in the dark. Subsequently, cells were washed with 2 ml of DPBS (300 g, 5 min). The Annexin V/7-AAD kit from BC was used for apoptosis detection. Therefore, cells were suspended in 100 μl of Binding-Buffer, and 10 μl of Annexin V and 20 μl of 7-AAD were added. After an incubation time of 15 min at 4–8°C (dark), 300 μl of Binding-Buffer were added. The following BC antibodies were used as isotype controls: mouse IgG1-ECD for CD3 and mouse IgG1-PC7 for CD33. All antibodies were titrated to obtain an optimal concentration.

Flow cytometric analyses were performed with the Navios Ex and Navios flow cytometers running Cytometry List Mode Data Acquisition Software, versions 2.0 and 1.3, respectively, and Kaluza Analysis Software from Beckman Coulter, version 2.1. The cells were gated as illustrated in Figure 1. T cells (CD3) and monocytes (CD33) were

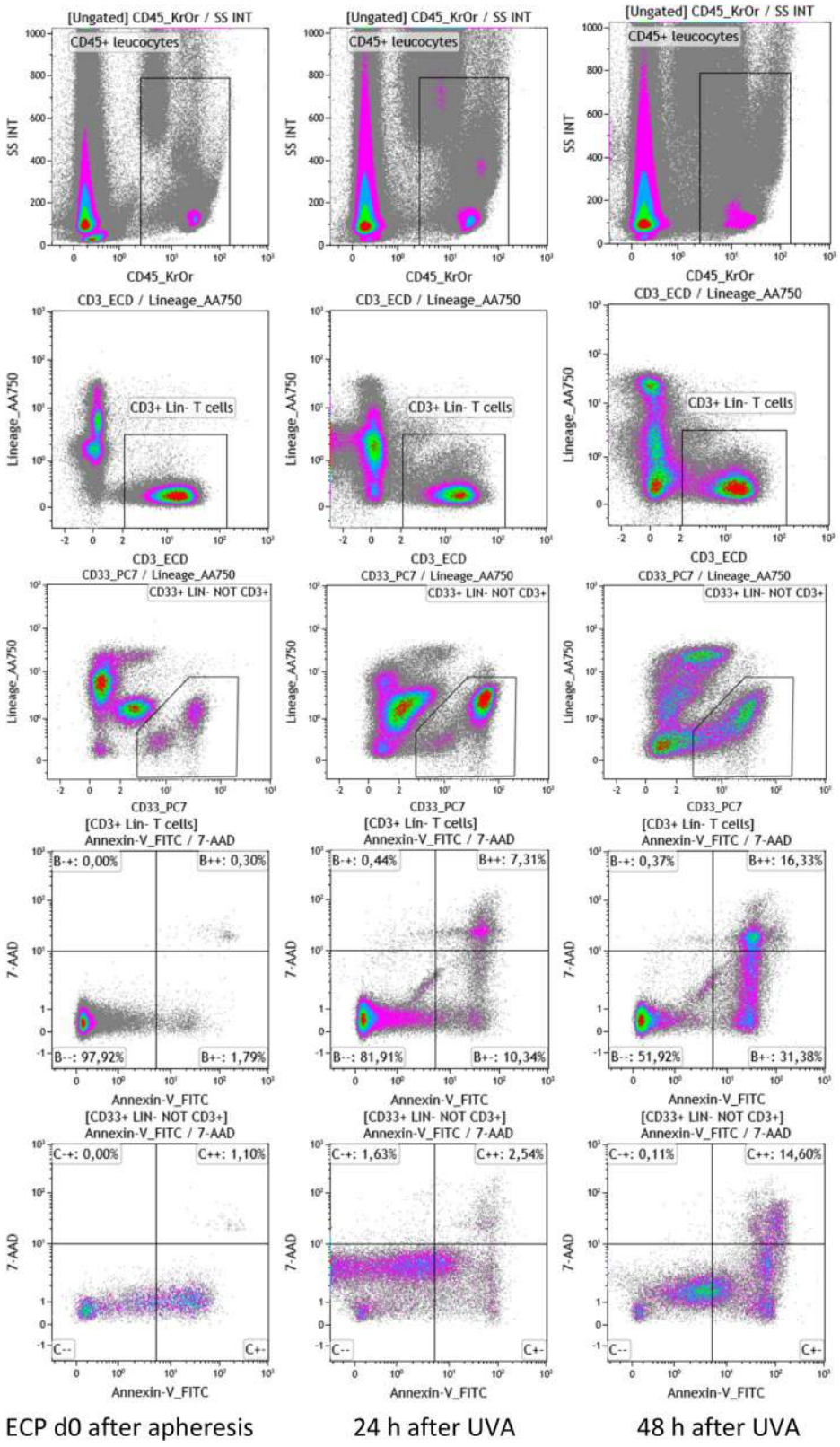

FIGURE 1 Gating strategy in fresh (left column) and incubated extracorporeal photopheresis samples (middle and right column). First, leukocytes were selected (first line), and T cells (CD3) and monocytes (CD33) were analysed by excluding granulocytes, natural killer cells and B cells (second and third lines). Annexin V^+ and 7-$AAD^{+/-}$ cells were sub-gated from T cells (fourth line) and $CD33^+$ monocytes (fifth line). Annexin V exhibited non-apoptosis binding of $CD33^+$ cells; therefore, analysis was limited to Annexin V^+ 7-AAD^+ monocytes [Color figure can be viewed at wileyonlinelibrary.com]

analysed by excluding granulocytes (CD66b), natural killer cells (CD56) and B cells (CD19, CD20). Annexin V^+ and 7-$AAD^{+/-}$ cells were sub-gated from T cells and $CD33^+$ monocytes. Annexin V exhibited non-apoptosis binding of $CD33^+$ cells; therefore, analysis was limited to Annexin V^+ 7-AAD^+ monocytes. Gates were set with isotype controls and Fluorescence Minus One (FMO) control using fresh samples. Aged cells and debris displayed some degree of autofluorescence in channels 3 and 5. We did not exclude these signals in order to capture all cellular material and because results were reported as the difference between treated and untreated samples.

Specific acceptance criteria for specificity, precision and robustness were set. The cut-offs were set such that the unspecificity threshold was ≤5%.

2.5 | Statistical analysis

Microsoft Excel 2010, R and IBM SPSS Statistics 25 were used to collect data; generate figures; and to determine the median, mean and standard deviation. Correlation was determined using the Pearson test. *p*-values below 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Accuracy

First, gating boundaries were verified by using isotype controls or FMOs. We achieved unspecificity values (n = 4) of 0.20%–4.11% for CD3, 0.00%–0.10% for $CD14^+CD16^-$ and 0.49%–4.25% for $CD33^+$. Regarding apoptotic (Annexin V^+/7-AAD^-) or dead cells (Annexin V^+/7-AAD^+), the specification requirement of ≤5% unspecific binding for T cells and monocytes was met; values range from 0.01% to 2.67% (n = 8).

3.2 | Robustness

When analysing a batch of samples (n = ~~6~~10) consecutively by flow cytometry, we observed a trend: T-cell fractions slightly increased, whereas monocyte fractions decreased continuously within less than 20 min (Figure 2). In this time, the tubes were situated in the carousel of the cytometer at room temperature. As a consequence of this, we investigated the analytical stability of the samples after staining. Samples were analysed immediately after staining and after up to 52 min of storage at room temperature and on ice (Figure 3, data combined for both temperatures, n = 14). The reduction of monocytes was clearly visible during storage at room temperature within as little as 30 min (36%), and the decrease was greater at room temperature (48%–63%) than on ice (33%–50%). The content of T cells was almost constant at both temperatures, with a maximum 3.4% increase after 52 min.

In addition to the analytical stability of stained samples, we analysed the pre-analytical stability of analytes stored at room temperature (n = 6) or on ice (n = 8). Clear changes in MNC vitality were observed during storage (Figure 4). Apoptotic and dead T-cell fractions increased by 19% and 81%, respectively, after 4 h of storage on ice. The change was even more pronounced at room temperature (median: 88% and 263%, respectively). In contrast, apoptosis of monocytes was not observed in samples stored for up to 4 h at room temperature or on ice.

Regarding changes in monocyte content during storage, there was no significant reduction of cells stored for up to 4 h. Even incubating the cells from the ECP product for up to 72 h at 37°C in polystyrene tubes resulted in no relevant decrease in monocyte content (data not shown). This seems to be in contrast to our previous analytical stability tests of stained samples, where an approximately 60% decrease in monocytes was detected following storage for up to 52 min after staining. In pre-analytical stability testing of samples stored for up to 4 h before the apoptosis assay, there was no obvious decrease in monocyte content, even at room temperature.

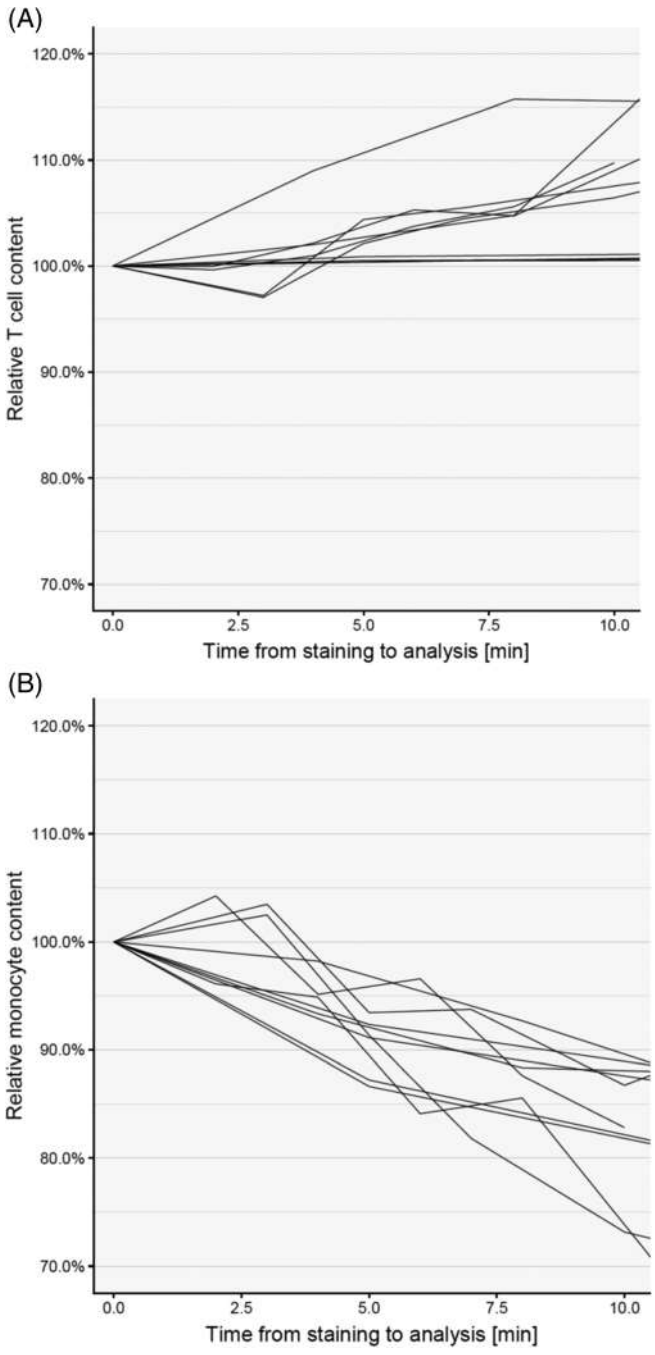

FIGURE 2 Analytical stability influences content of T cells (A) and monocytes (B) at room temperature. The varying content of T cells and monocytes in relation to leukocytes in stained photopheresis samples is regarded over a period of 10 min

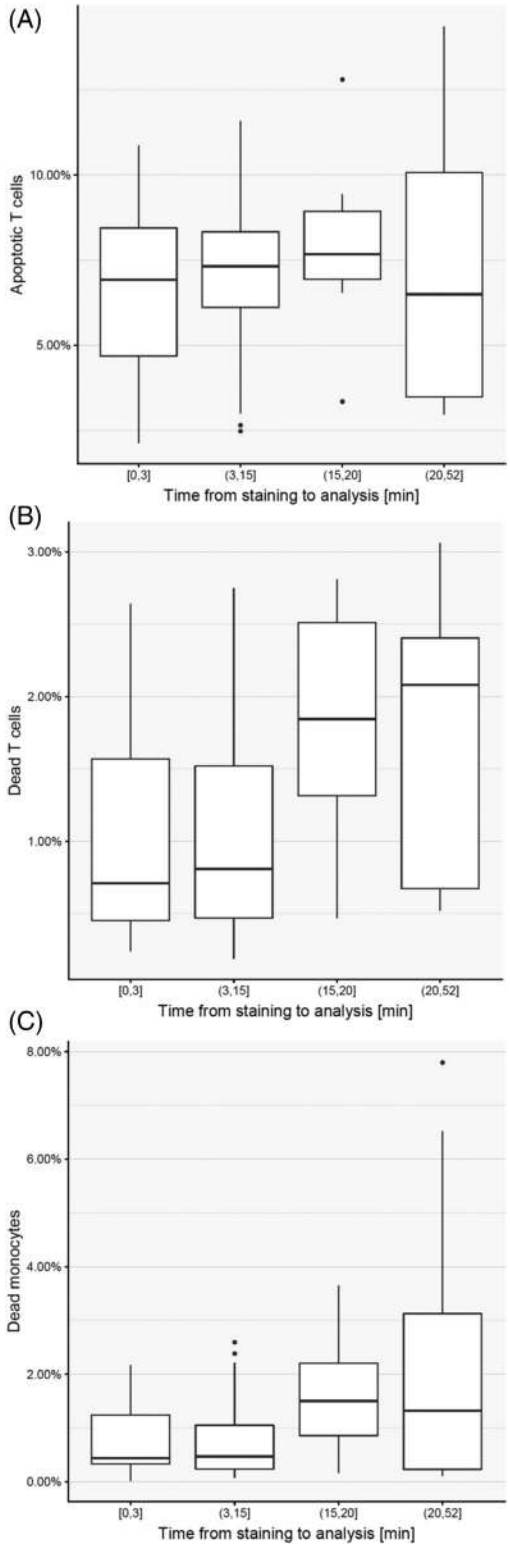

FIGURE 3 Time from staining to analysis (min) in the flow cytometer is shown as analytical stability of apoptosis in T cells (A, B) and monocytes (C). After 0–3 min (n = 22), there were 6.93% apoptotic T cells, 0.71% dead T cells and 0.44% dead monocytes. After 3–15 min (n = 37), 7.32% apoptotic T cells, 0.81% dead T cells and 0.47% dead monocytes were detected. After 15–20 min (n = 12), there were 7.67% apoptotic T cells, 1.85% dead T cells and 1.50% dead monocytes. After 20–52 min (n = 26), 6.50% apoptotic T cells, 2.08% dead T cells and 1.30% dead monocytes were analysed (median values)

However, the intended apoptosis of MNCs was obtained by incubating the cells for up to 72 h at 37°C and 5% CO_2. Apoptosis (in post/pre samples, each n = 3) was induced after 48 h (rates: 47%–163% for $CD3^+$ Annexin V^+ 7-AAD^+, 69%–336% for $CD3^+$ Annexin V^+ 7-AAD^- and 68%–115% for $CD33^+$ Annexin V^+ 7-AAD^+) and continued for up to 72 h.

3.3 | Precision

For repeatability assessment, samples (n = 6) were stained and analysed sixfold by the same person. The median coefficient of variation (CV) for $CD3^+$, $CD33^+$ and $CD3^+$ Annexin V^+ 7-AAD^- cells was 3.86%, 6.9% and 7.08%, respectively. Cell populations with small (<5%) dead T-cell and $CD33^+$ cell fractions had larger CV values.

As T cells exhibited high analytical stability over the analytical period, this cell population was suitable for comparative analysis of the two cytometers. Inter-operator variability was detected on both cytometers (n = 6): median CVs for T cells were 4.8% (0.01%–13.27%), 6.02% (5.86%–6.18%) and 1.01% (0.12%–8.68%), and deviation was <20%.

Comparative analysis revealed that the size of the $CD3^+$ fraction was 25.7%–55.7% (pre) and 24.1%–56.0% (post) according to Navios and 28.3%–48.5% (pre) and 26.5%–45.9% (post) according to Navios Ex. The maximum CV for T cells was 4.79% with Navios Ex and 10.77% with Navios. Accordingly, the overall CV was 4.8% (median), which indicated high comparability between operators on both cytometers. Regarding $CD33^+$ cells, the post samples had a CV of 16.75%, which confirmed the analytical instability of the monocytes, as was already described.

Obviously, cell counts determined using Navios Ex showed lower variation than those obtained with Navios (Figure 5). The new and more precise cuvette design of the flow chamber of the Navios Ex could be the reason for this. Both systems met the specification requirement of CV ≤15%. The condition for comparability of Navios Ex and Navios was met, as demonstrated by the correlation coefficient (r) of r = 0.974 for $CD3^+$ values and r = 0.778 for $CD33^+$ values (p < 0.01).

4 | DISCUSSION

ECP could be performed with the so-called "in-line" technique where apheresis and irradiation are performed by the same device or "offline" with separate machines for apheresis and irradiation. Offline photopheresates are medicinal products[20] that require quality control. This may include cellular composition, haematocrit and residual 8-MOP and should comprise a functional assay that relates to ECP's potency. As the apoptosis of lymphocytes is a well-known mechanism of photopheresis,[17] we designed the present study to evaluate the validity and GMP compliance of an apoptosis assay for MNCs based on flow cytometry with Annexin V and 7-AAD staining.

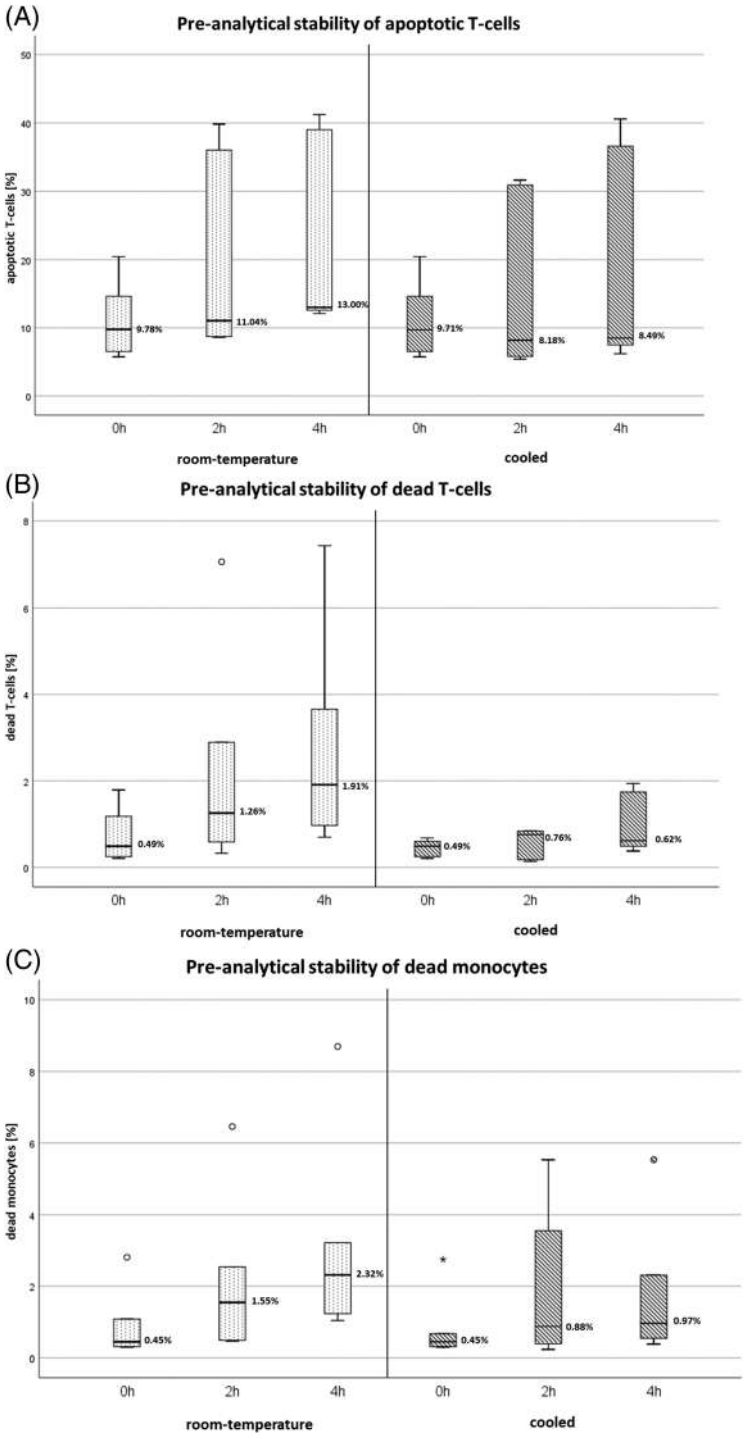

FIGURE 4 Pre-analytical stability of T cells (A, B) and monocytes (C) (%) is shown at room temperature (n = 6) and cooled on ice (n = 8). Stability of photopheresis samples was analysed at two temperatures before staining. There was no significant difference between both temperatures (p > 0.05)

A panel of antibodies developed for this purpose was used to analyse the apoptosis of T cells and monocytes. Isotype controls have been used traditionally as gating controls, and FMO controls are used when gating real positive events.[21,22] Gating boundaries for this assay were set up with fresh samples. This excludes autofluorescence signals with fresh cells. However, increased autofluorescence signals of aged cells were partially included in T-cell and monocyte gating. This strategy, designed to prevent loss of dead cell material, could have resulted in false high apoptosis signals. However, because we reported the results as the difference between treated and untreated cells, confounding as a result of this should have been marginal. In addition, Annexin V exhibited unspecific binding of myeloid cells, possibly because of platelets sticking to monocytes with a high affinity to Annexin V.[23-25] Thus, we limited the analysis to Annexin V^+ 7-AAD^+ monocytes.

In the course of validation testing, we determined that the analytical stability of the photopheresis samples was a crucial parameter. After analysing a batch of stained samples, we noticed that changes in cell content had occurred. A loss of monocytes was observed over the

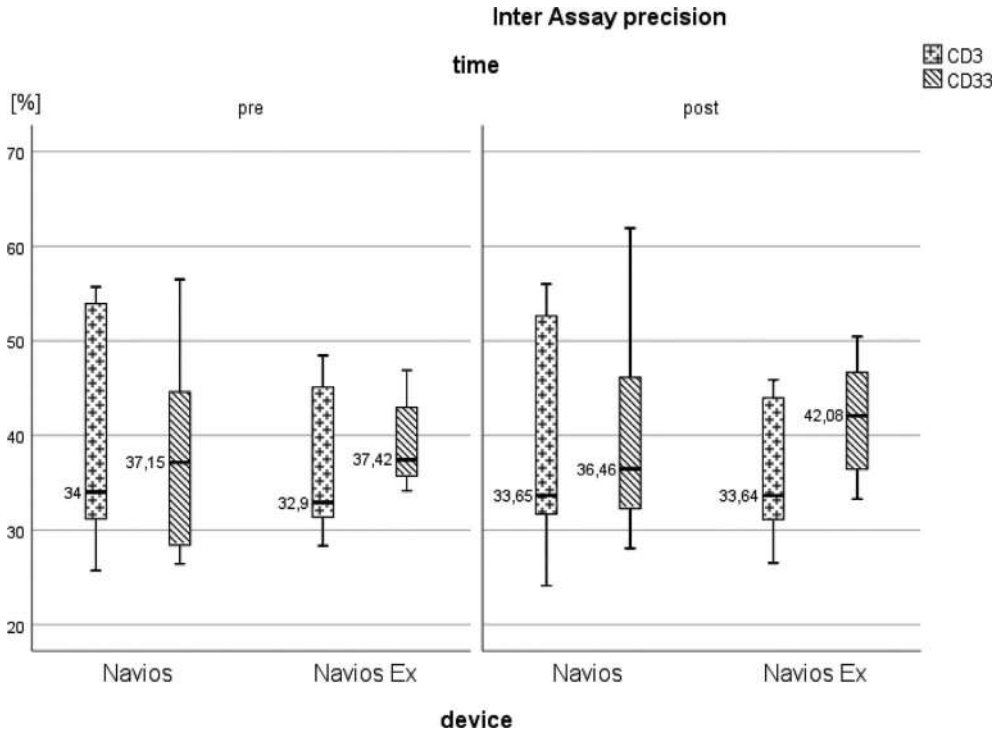

FIGURE 5 Comparison of CD3 and CD33 values (%) of fresh photopheresis samples (pre and post) determined using the Navios versus Navios Ex flow cytometers

analytical period of up to 52 min, including stand-by time in the flow cytometer. This loss was likely a result of monocyte adhesion to plastic material. Furthermore, stained cells might be susceptible to activation by agonistic antibodies, making them less robust during storage. Hence, stained samples should be analysed immediately after staining, and stand-by time in the machine should be taken into account. Considering the stained ECP samples prior to isotype controls in the flow cytometer could avoid cell changes and be appropriate for routine analysis. Regarding pre-analytical stability, proceeding apoptosis was found. Thus, staining and analysis should be performed immediately, and samples should be stored on ice for limited periods only.

For precision testing, $CD3^+$ cells were preferentially used because of their stability over the analytical period. Regarding intra-assay precision, the CV for CD3 cells was 3.86%, which meets the internal specification of <15%. Comparison of the two flow cytometers showed that Navios Ex achieves lower variability. This is possibly due to the improved cuvette design of the flow chamber of the Navios Ex compared with that of the Navios. Considering differences in personnel, flow cytometers and pre- and post-ECP samples, our validation study results demonstrated that both systems achieve a CV of <15% and a deviation of <20% and confirmed their high-intermediate precision.

The onset of apoptosis following ECP could be detected after 48 h of incubation. Cultivation of cells for more than 48 h resulted in no relevant increase in apoptosis. This is in accordance with the findings of other groups[16] that considered an apoptosis induction of >15% at an incubation time of 24–48 h to be a successful ECP.

In this assay, we considered both T-cell and monocyte apoptosis. The latter is considered controversial as monocytes seem to be less sensitive for apoptosis induction. It is believed that irradiated monocytes may differentiate into DCs. Monocytes could therefore be detracted from apoptosis calculation.

In summary, flow cytometry with Annexin V and 7-AAD staining detected the vitality of MNCs at the GMP level and yielded valid results as determined in this validation study considering the pre-analytical and analytical stability of samples kept under different storage conditions for different times. For routine quality control, we focussed on $CD3^+$ T-cell apoptosis after 24–48 h as a convenient potency marker.

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CONFLICT OF INTEREST

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

Viola Hähnel, Frauke Dormann and Norbert Ahrens conceptualized the study; Viola Hähnel, Frauke Dormann, Katharina Kronenberg and James A Hutchinson analysed the data; Viola Hähnel, Ralph Burkhardt and Norbert Ahrens wrote the manuscript; and all authors edited the manuscript.

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ORIGINAL ARTICLE

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Detection and analysis of blood donors seropositive for syphilis

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Abstract

Background: The increasing incidence of syphilis worldwide has called attention to the risk of transmission by transfusion.

Aims: To determine the prevalence of active syphilis in blood donors and characterise the serological profile of syphilis-positive donors.

Methods: Samples positive for *Treponema pallidum* using the chemiluminescent microparticle immunoassay (CMIA) during blood donor screening from 2017 to 2018 were tested by the Venereal Disease Research Laboratory (VDRL) non-treponemal test and for anti-*T. pallidum* IgM by ELISA (Immunoassay Enzyme test for detection of IgM antibodies). The INNO-LIA Syphilis test (Line Immuno Assay solid test for confirmation antibodies to *Treponema pallidum*) was performed as a confirmatory test on samples that were positive on ELISA-IgM but negative on VDRL. ELISA-IgM (+) samples were also tested for *T. pallidum* DNA in sera by real-time polymerase chain reaction (PCR).

Results: Of 248 542 samples screened, 1679 (0.67%) were positive for syphilis by CMIA. Further analysis was performed on 1144 (68.1%) of these samples. Of those tested, 16% were ELISA IgM(+)/VDRL(+), 16.5% were ELISA IgM(–)/VDRL(+), 4.1% were ELISA IgM(+)/VDRL(–), and 63.4% were ELISA IgM (–)/VDRL(–). The INNO-LIA Syphilis test results were 33 (3%) positive, 2 (0.2%) undetermined and 12 (1%) negative. Of the 230 EIA-IgM(+) samples (20.1%), 5 (2.2%) were PCR positive. The prevalence of active syphilis in 2017 and 2018 was 0.1% and 0.07%, respectively, and overall prevalence of serologic markers for syphilis was highest among male, unmarried, 25–34-year-olds with a high school education and who were first-time donors.

Conclusion: There is a risk of transfusion-transmitted syphilis in blood banks that exclusively use the VDRL test for donor screening, as is currently the situation in some Brazilian blood centres, as well as in other blood centres around the world.

KEYWORDS

blood donors, IgM antibody, syphilis, *Treponema pallidum*, VDRL

1 | INTRODUCTION

Transmission of syphilis by blood transfusion has re-emerged in many countries as a threat to public health, especially among vulnerable populations. This likelihood requires a re-evaluation of current diagnostic tools and implementation of enhanced haemovigilance programmes.[1-10]

Since the mid-1980s, the need to continue serological screening for syphilis in blood donors has been debated. Although the American Association of Blood Banks has not required testing for syphilis since 1985, the US Food and Drug Administration (FDA) has not supported this change, and screening for syphilis has remained mandatory. The US Food and Drug Administration's (FDA's) position was to maintain syphilis testing as an indirect test of risk-related behaviour for susceptibility to human immunodeficency virus (HIV) infection rather than for the prevention of transfusion-transmitted syphilis.[11]

Brazil began serological screening for syphilis in blood donors using the VDRL test in 1969, and this test is still widely used.[1,9] Between 2010 and 2016, approximately 230 000 new cases of syphilis were reported, most of them located in the southeast region. According to data from the 2016 Epidemiological Bulletin, between 2014 and 2015, acquired syphilis increased by 32.7%, syphilis in pregnant women by 20.9% and congenital syphilis by 19%. In 2015, the total number of reported cases of syphilis in Brazil was 65 878. In the same period, the detection rate was 42.7 cases per 100 000 inhabitants, mostly among men (136 835, 60.1%). Although the increase in syphilis cases over time was evident, the Ministry of Health only announced that the country faced a syphilis epidemic in 2016.[4]

Transmission of *Treponema pallidum*, the causative agent of syphilis, through blood transfusion, although rare, is possible and is recognised as the third form of syphilis acquisition. Syphilis was the first transfusion-transmitted infection to be systematically investigated in blood donors following the implementation of serological screening in 1938.[5] Prior to the initiation of testing, more than 100 cases of transfusion-related syphilis were reported. After the start of serological screening in blood banks, there was a drastic reduction in the number of cases of transfusion-transmitted syphilis. In the last 40 years, only three cases of transfusion-related syphilis transmission have been reported.[6-8]

Despite this very low incidence, serological screening for syphilis remains mandatory in many countries, including Brazil. The significant reduction in transmission by transfusion was not only due to the introduction of syphilis screening in blood banks and improvement in donor recruitment but also because of its low incidence among blood donors in the 1990s and early 2000s, as well as *T. pallidum*'s inability to survive in refrigerated blood products.[9]

Transfusion transmission became so rare in developed countries in the late 1990s that the need for maintaining mandatory serological screening for syphilis in blood banks began to be questioned. However, cases of transfusion-transmitted syphilis may increase again because of the current resurgence of this infection, relaxation in donor selection criteria due to social pressure and non-compliance in donor screening interview responses. Platelet concentrates, frequently used in the treatment of patients with haemo-oncogenic disorders, although typically stored in pouches with oxygen in which *T. pallidum* cannot survive, may also be stored at ambient temperature (20–24°C) where the organism can remain viable. Thus, further discussion about whether or not mandatory donor screening should be enforced is worthwhile.[3,9]

Regarding recent efforts in transfusion medicine to improve safety conditions for donors, current screening protocols have limitations in the clinical interpretation of serological patterns, especially in asymptomatic blood donors.[1] The present study sought to determine the prevalence of active syphilis in blood donors, evaluate the reliability of the prevalent VDRL test and characterise individuals who were positive for *T. pallidum*.

2 | METHODS

2.1 | Study design

We conducted a retrospective cross-sectional analysis in 2019–2020 of samples from blood donations obtained between January 2017 and December 2018 at Fundação Pró-Sangue (FPS), the blood centre of São Paulo, Brazil, that were seropositive for syphilis. The study was approved by the Ethical Committee of Hospital das Clínicas at the University of São Paulo and the ethical review board of FPS (assent n°. 2.470.318) and was financially supported by FAPESP (2017/23028-9).

2.2 | Laboratory analyses, risk behaviours, donation type and motivational factors

All qualified candidate blood donors are routinely serologically screened for HIV types 1 and 2; human T-cell lymphotropic virus (HTLV) 1 and 2; hepatitis B (hepatitis B surface antigen [HBsAg] and total antibody to hepatitis B core antigen [anti-HBc]) and hepatitis C; syphilis and Chagas disease; and nucleic acid test (NAT) HIV, NAT-HBV (hepatitis B virus) and NAT-HCV (hepatitis C virus). All positive samples are stored in a repository and are available for subsequent in-depth analysis (retrovigilance). Donor samples that were positive for anti-treponema by a chemiluminescent microparticle immunoassay (CMIA) (Abbott Architect) during their initial screening were obtained and tested for IgM antibody to *T. pallidum* by ELISA (Euroimmun) and by the non-treponema-specific VDRL test (ANTIGEN-Omega Diagnostics). All samples positive for EIA-IgM or VDRL were tested by real-time PCR for *T. pallidum* DNA. The INNO-LIA Syphilis-Fujirebio Immunoblot test was also performed on samples that were EIA-IgM positive and VDRL negative (Figure 1).

As a routine in our blood centre, all donors who tested positive in any serologic screening test are recalled to collect a new sample to confirm the original results. They are also subjected to an interview about risk factors for syphilis and other transfusion-transmitted

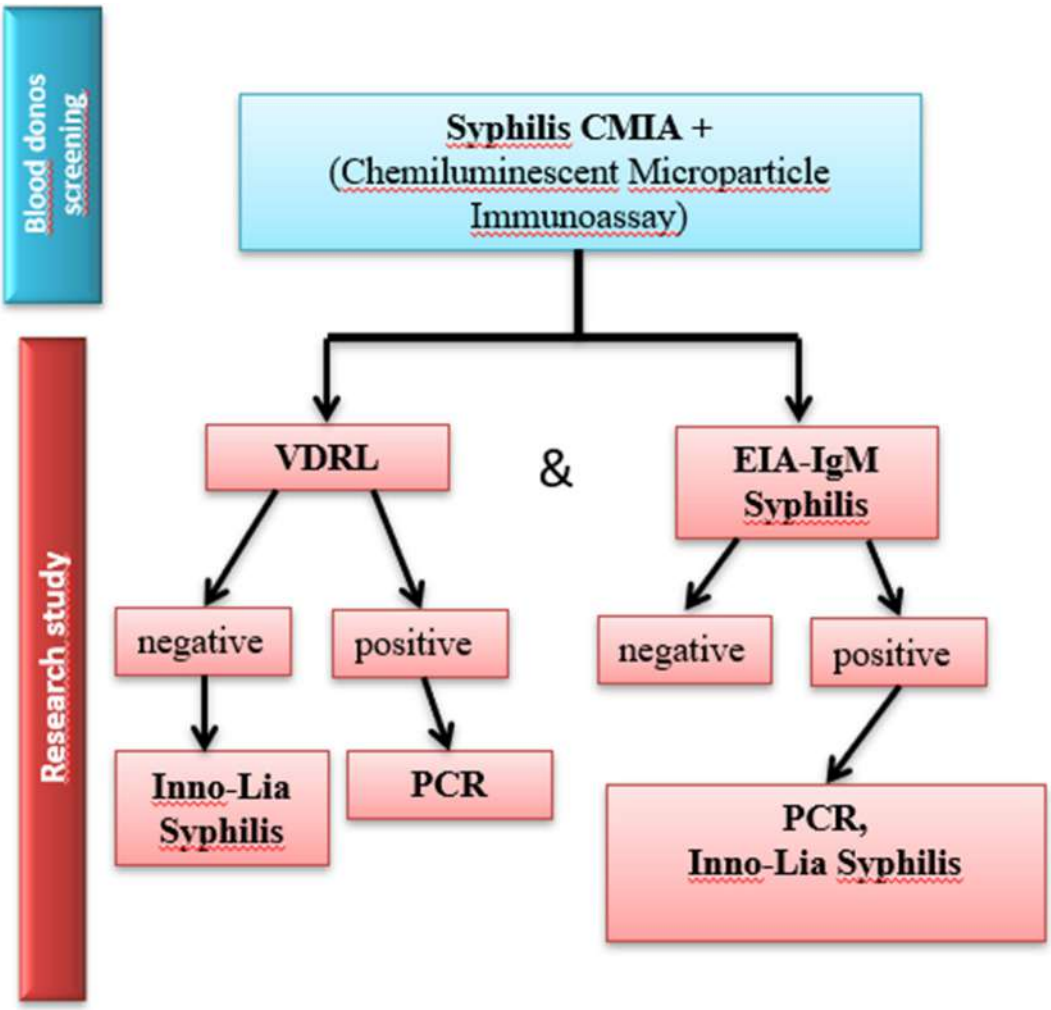

FIGURE 1 Flow chart of the study design. The CMIA test was used in the initial screening of blood donors. Positive samples were tested for VDRL, IgM antibody to *Treponema pallidum* (EIA-IgM), *T. pallidum* DNA by PCR and by the INNO-LIA Syphilis assay [Color figure can be viewed at wileyonlinelibrary.com]

diseases and their motivation to donate blood. The interviews are face to face, in a private room, using a standardised questionnaire and are conducted by trained physicians according to the Standard Operation Procedures of our institution. Donors who tested positive in the CMIA test were requested to provide repeat samples to confirm the results. When they returned for assay results, notification and counselling, the donors filled out a questionnaire to assess their risk factors for becoming infected with syphilis and motivations for blood donation. The questions asked included: "In the past 12 months, with how many different people have you had sex?" "Concerning your steady sexual partners, what was the frequency of condom use when you had sex?" and "Have you ever exchanged (given or received) money or drugs to have sex with someone?" Motivations for blood donation were classified as direct appeal, altruism and self-interest according to a previous publication.[12]

Donation type was classified as (i) first-time donation (a donation from an individual who had never donated in our blood centre), (ii) repeat donation (a donation from a person who donated at least twice in the last 12 months) and (iii) sporadic donation (a donation from someone who donated at least twice within an interval greater than 12 months).[13]

To detect *T. pallidum* DNA, 500 µl of serum were extracted by MagNa Pure Compact Nucleic Acid Isolation—Large Volume kit (Roche, Germany) in an automatized system MagNa Pure Compact (Roche, Germany), according to the manufacturer's protocol, and were subjected to real-time PCR. TaqMan was performed in StepOne Plus TM Real-Time PCR Systems (Life Technologies, Foster City, CA, USA). The primers and probes were designed using the assay of the design programme (Applied BioSystems, Carlsbad, CA, USA) targeting the polA gene of *T. pallidum*.[10]

2.3 | Statistical analyses

We used the SPSS 17 software (SPSS Inc/IBM Chicago, USA) for the statistical analyses. Sociodemographic variables included gender; age group; marital status; educational attainment; and first-time, repeat or sporadic donor status. Comparisons between the frequencies of the

TABLE 1 Demographic characteristics of CMIA-positive and syphilis IgM-positive blood donors

	CMIA+							*EIA-IgM +*						
	2017 (*n* = 617)		2018 (*n* = 527)		Total (*n* = 1144)		*p*-Value	2017 (*n* = 138)		2018 (*n* = 92)		Total (*n* = 230)		*p*-Value
Gender							0.6606							0.915
Male	337	(54.6%)	281	(53.3%)	618	(54.0%)		70	(50.7%)	46	(50.0%)	116	(50.4%)	
Female	280	(45.4%)	246	(46.7%)	526	(46.0%)		68	(49.3%)	46	(50.0%)	114	(49.6%)	
Age (years)							0.196							0.036
17–24	117	(19%)	110	(20.9%)	227	(19.8%)		34	(24.6%)	37	(40.2%)	71	(30.9%)	
25–34	188	(30.5%)	160	(30.4%)	348	(30.4%)		57	(41.3%)	35	(38%)	92	(40%)	
35–44	136	(22%)	88	(16.7%)	224	(19.6%)		32	(23.2%)	10	(10.9%)	42	(18.3%)	
45–54	102	(16.5%)	94	(17.8%)	196	(17.1%)		12	(8.7%)	6	(6.5%)	18	(7.8%)	
≥55	74	(12%)	75	(14.2%)	149	(13%)		3	(2.2%)	4	(4.3%)	7	(3%)	
Educational level							0.2273							0.5796
<Elementary school	43	(6.9%)	39	(7.4%)	82	(7.2%)		3	(2.3%)	3	(3.3%)	6	(2.6%)	
Elementary school	66	(10.7%)	73	(13.9%)	139	(12.2%)		12	(8.9%)	8	(8.8%)	20	(8.7%)	
High School	373	(60.5%)	307	(58.5%)	680	(59.5%)		96	(69.0%)	66	(71.5%)	162	(70.4%)	
College and above	135	(21.9%)	106	(20.2%)	241	(21.1%)		27	(19.8%)	15	(16.4%)	42	(18.3%)	
Marital status							0.02853							0.2795
Single	343	(55.6%)	273	(51.8%)	616	(53.8%)		92	(66.7%)	71	(77.2%)	163	(70.9%)	
Married	209	(33.9%)	173	(32.8%)	382	(33.4%)		35	(25.4%)	14	(15.2%)	49	(21.3%)	
Divorced/Separated	21	(3.4%)	28	(5.3%)	49	(4.3%)		3	(2.1%)	3	(3.3%)	6	(2.6%)	
Other	44	(7.1%)	53	(10.1%)	97	(8.5%)		8	(5.8%)	4	(4.3%)	12	(5.2%)	
Donation type							0.8913							0.6981
First time	604	(97.9%)	517	(98.1%)	1121	(98.0%)		135	(97.8%)	91	(98.9%)	226	(98.3%)	
Repeat	5	(0.8%)	4	(0.8%)	9	(0.8%)		2	(1.5%)	1	(1.1%)	3	(1.3%)	
Sporadic	8	(1.3%)	6	(1.1%)	14	(1.2%)		1	(0.7%)	0	(0.0%)	1	(0.4%)	
Donors who returned for notification and counselling	217	(35.2%)	190	(36.1%)	407	(35.6%)		39	(28.3%)	26	(28.3%)	65	(28.3%)	

TABLE 2 Motivations to donate blood among syphilis-positive blood donors

	Direct appeal		Altruism		Self-interest		
	n	(%)	*n*	(%)	*n*	(%)	*p*-Value
CMIA +	238	(58.5%)	157	(38.6%)	12	(2.9%)	
VDRL	67	(16.5%)	38	(9.3%)	3	(0.7%)	0.68
ELISA IgM +	36	(8.8%)	28	(6.9%)	1	(0.2%)	0.590
INNO-LIA +	73	(17.9%)	41	(10.1%)	3	(0.7%)	0.510
Active syphilis	36	(8.8%)	25	(6.1%)	0	0	0.328

sociodemographic characteristics and the treponemal/non-treponemal assay results were performed using the Pearson Chi-square (χ^2) test. Results were considered statistically significant at $p < 0.05$.

3 | RESULTS

Among 248 542 (123 851 in 2017 and 123 691 in 2018) samples screened, 1679 (0.67%) were positive in the CMIA assay. Of the 1144 (68.1%) positive patients available for inclusion in the study, 16.0% were EIA-IgM(+)/VDRL(+), 16.5% were EIA-IgM(–)/VDRL(+), 4.1% were EIA-IgM(+)/VDRL(–), and 63.4% were EIA-IgM(–)/VDRL(–). The INNO-LIA Syphilis test, performed as a confirmatory test in 47 (4.1%) samples that were EIA-IgM positive and VDRL negative, yielded 33 (3.0%) positive results, 2 (0.2%) that were inconclusive and 12 (1.0%) that were negative. Of the 230 EIA-IgM (+) samples, 5 (2.2%) were positive for *Treponema* DNA by real-time PCR.

In 2017, the prevalence of collected blood that was positive for syphilis screening tests was 0.77%. It was higher among men (54.6%) who were unmarried (55.6%) and between 25 and 34 years old (30.5%) with a high school education (60.5%) and who were first-time donors (97.9%). In 2018, the prevalence of blood units positive for syphilis screening tests was 0.62%. Among the EIA-IgM-positive samples, 10.4% were positive for antibody to HBV anti-Core, 1.1% for antibody to HIV, 1.5% for anti-HTLV-1/2 and 1.1% for NAT-HIV (Table 1).

Among the 1144 donors who returned following notification for counselling, 407 (35.6%) completed the questionnaire, and 33 (2.9%) responded affirmatively to the question, "Have you ever exchanged (given or received) money or drugs to have sex with someone?" Of these, 57.7% were above 45 years of age, 53.3% were married, 53.3% graduated from high school, and 100% were first-time donors.

In Table 2, we show that, among motivation choices, a direct appeal was the most frequent response (58.5%), followed by altruism (38.6%).

Associations with condom usage are shown in Table 3. About a third of female and a quarter of male donors never used condoms during sex. The prevalence rate for syphilis was 55.5% among donors who did not use condoms, 25.3% for those who used condoms sometimes and 16% for those who always used condoms ($p < 0.044$). Among the women positive for syphilis, 15.5% were between 45 and 54 years old, 28.2% were married, 30.2% had a high school education level, and 56.6% were first-time donors ($p < 0.0001$) (Tables 3 and 4).

4 | DISCUSSION

In blood donations provided to Fundação Pró-Sangue Hemocentro de São Paulo, one of the largest blood banks in the city of São Paulo, between 2015 and 2017, there was an apparent increase of 24% in the detection of syphilis-associated markers, from 0.62% in 2015 to 0.73% in 2016 and 0.77% in 2017, followed by a small decline to 0.62% in 2018 ($p < 0.0001$). A similar increase was reported in 2014–2015 in the United States (3). Also paralleling our findings, the donors in their study with the highest rate of a positive test were men, unmarried, between 25 and 34 years old, with a high school education and were first-time blood donors (3).

The prevalence of active syphilis among blood donors seen at our centre between 2017 and 2018 was 0.09%. We identified 35 cases that were negative for syphilis by VDRL but were anti-*T. pallidum* IgM and INNO-LIA positive. These findings are consistent with a study by Moore et al, who showed that non-treponemal tests for primary syphilis infection were negative in 30%–50% of infected individuals.[12-17] They strongly suggest that there remains a risk for transfusion-transmitted syphilis in those blood banks that exclusively use the VDRL test for syphilis donor screening. In addition, the detection of co-infection with HIV, HBV or HTLV-1/2 in 2.2%, 10.4% and 1.5% of syphilis-positive cases, respectively, suggests that the application of treponemal-specific tests are also relevant for the prevention of the transfusion-related transmission of other sexually transmitted infections.[18]

Our demonstration that 2.2% of EIA-IgM-positive donors had *T. pallidum* DNA in their circulation is consistent with a previous investigation by Dow et al and suggests that this organism might still be present in some individuals despite evidence of an antibody response. It must be acknowledged that detection of *T. pallidum* DNA cannot distinguish between the presence of viable or dead organisms.[5,18] Similar results were found in our previous study conducted in 2014, where we detected 2 (1.02%) cases positive for *T. pallidum* DNA from a total of 197 blood samples from donors positive for syphilis.[10] However, the routine use of a nucleic acid amplification test for syphilis is not recommended for all blood donors due to expense, the need for trained personnel and uncertainty about organism viability. In addition, treponema-specific antibody tests appear to be sufficient to identify infected individuals and prevent transfusion transmission.[5,10]

Among our blood donors who were positive for syphilis, it was not surprising that the highest risk of active syphilis infection occurred in those who did use condoms. This observation was also previously described by Hopkins et al in 2004.[15] The preferential screening for

TABLE 3 Associations with condom usage in blood donors

	How often did you use condoms (*n* = 407)						
	Never		Sometimes		Always		*p*-Value
Gender[a]							0.0555
Male	98	52.1%	51	27.1%	39	20.8%	
Female	128	62.1%	52	25.2%	26	12.6%	
Age (years)[a]							<0.0000001
17–24	20	28.2%	29	40.8%	22	31.0%	
25–34	53	48.2%	36	32.7%	21	19.1%	
35–44	44	60.3%	21	28.8%	8	11.0%	
45–54	61	73.5%	10	12.0%	12	14.5%	
≥55	48	84.2%	7	12.3%	2	3.5%	
Education[a]							0.0283
<Elementary school	30	81.1%	7	18.9%	0	0.0%	
Elementary school	24	60.0%	10	25.0%	6	15.0%	
High school	119	52.0%	67	29.3%	43	18.8%	
College and above	51	59.3%	19	22.1%	16	18.6%	
Marital status[a]							<0.0000001
Single	76	39.8%	66	34.6%	49	25.7%	
Married	111	74.5%	28	18.8%	10	6.7%	
Divorced/separated	15	88.2%	1	5.9%	1	55.9%	
Other	24	64.9%	8	21.6%	5	13.5%	
Donation type[a]							0.8443
First time	223	57.3%	101	26.0%	65	16.7%	
Repeat	1	50.0%	1	50.0%	0	0.0%	
Sporadic	2	66.7%	1	33.3%	0	0.0%	

[a]Total may be missing two values.

TABLE 4 The association between detection of syphilis, condom usage and specific behaviours

	CMIA +		ELISA IgM +		
	n	(%)	*n*	(%)	*p*-Value
How often did you use condoms when you had sex?					0.0593
Never	226	(57.4%)	27	(42.9%)	
Sometimes	103	(26.1%)	25	(39.7%)	
Always	65	(16.5%)	11	(17.5%)	
Have you ever exchanged money or drugs to have sex with someone?					0.145
Yes	33	(8.1%)	2	(3.1%)	
No	374	(91.9%)	63	(96.9%)	

sexually transmitted diseases in blood donors who engage in unprotected sexual intercourse is certainly warranted.

A direct appeal for blood was the most frequent motivation for blood donation, followed by altruism, similar to our previous findings.[19] Only 2.9% of donors were motivated by self-interest. More research is needed as to why individuals who are at elevated risk for syphilis and othersexually transmitted diseases (STDs) volunteer to donate blood. The availability of STD testing at the blood centre might be an additional motivating factor that overlaps with more socially accepted responses such as altruism and direct appeal.[19-21]

An advantage of our study was the ability to analyse findings from a large number of individuals, all of whom underwent a similar testing protocol from a single major specialised service. This increases the probability of uniform handling of all specimens. One limitation of our study is that the bacterial load for *T. pallidum* in donated blood is low because donors are typically healthy and asymptomatic individuals. Therefore, we

were unable to further analyse the PCR-positive samples for other treponemal genes. This would have been of value to provide evidence of the possible presence of intact organisms. This limitation was also reported by Ferreira et al in 2014.[12] Other limitations include the absence of data on the length of syphilis infection and mode of acquisition in positive donors.

Since 2016, serological evidence of syphilis has become the most prevalent marker for infectious disease found in blood donors at our institution. Continuous monitoring of the profile of syphilis-infected donors at this time of re-emergence of the infection is useful and relevant not only for blood banks but also as a reflection of the epidemiological status of syphilis in the community. Availability of these data can contribute to the refocusing of health policies and priorities. Our demonstration that 3-% of donors with acute phase syphilis antibodies were negative in the VDRL test strongly suggests that non-treponemal tests are not ideal for screening blood donors. In addition to a lack of sensitivity, results of these assays are subjective and require interpretation by an experienced technician.[10]

In conclusion, we emphasise that, due to the increased incidence of syphilis among blood donors worldwide, it is clearly necessary that new syphilis screening guidelines for blood donors be established to maximise transfusion safety.

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CONFLICT OF INTEREST

The authors have no competing interests.

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SHORT COMMUNICATION

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Plasma pooling in combination with amotosalen/UVA pathogen inactivation to increase standardisation and safety of therapeutic plasma units

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Abstract

Objectives: Assessment of the impact of pooling five single-donor plasma (SDP) units to obtain six pathogen-reduced therapeutic plasma (PTP) units on standardisation and the retention of labile coagulation factors.

Background: SDP shows a high inter-donor variability with potential implications for the clinical treatment outcome. Additionally, there is still an existing risk for window-period transmissions of blood borne pathogens including newly emerging pathogens.

Methods/Materials: Five ABO-identical SDP units were pooled, treated with the INTERCEPT™ Blood System (Cerus Corporation, U.S.A.) and split into six PTP units which were frozen and thawed after 30 days. The variability in volume, labile coagulation factor retention and activity was assessed.

Results: The variability of volumes between the PTP units was reduced by 46% compared to SDP units. The variability in coagulation factor content between the PTP units was reduced by 63% compared to SDP units. Moderate, but significant losses of coagulation factors (except for vWF) were observed in PTPs compared to SDPs.

Conclusion: The pooling of five SDP units to obtain six PTP units significantly increases product standardisation with potential implications for safety, economics as well as transfusion-transmitted pathogen safety, making it an interesting alternative to quarantine SDP (qSDP) and pathogen-reduced SDP.

KEYWORDS
amotosalen/UVA, pathogen inactivation, plasma pooling, plasma standardisation

1 | INTRODUCTION

The total protein profile (including contents of coagulation factors) in individual fresh frozen plasma (FFP) units shows a high variability due to the impact of genetic factors on the human proteome.[1] Additionally, individual levels of coagulation factors depend on the plasma collection methods and procedures[2] as well as on the blood group. In Poland, most therapeutic plasma units are produced from individual whole-blood donations (89% in 2018). Despite the implementation of automation for whole blood separation there are significant differences in volume between plasma units, which also depend on the individual manufacturing methods. High individual variations in coagulation factor content in plasma may affect treatment outcome and render the therapeutic effect of transfusion less predictable.

Plasma pooling to overcome individual coagulation factor variations and volume variations may be a potential solution. However, plasma pooling without any pathogen inactivation procedure also bears an increased risk of pathogen transmission. For that reason, plasma pooling alone was not recommended in Poland. Despite the gradual implementation of safety measures like restrictive donor selection, improved blood collection procedures and the introduction of highly sensitive tests, the highest number of HCV window period infections of blood donors among European countries was noted in Poland since the implementation of HCV NAT screening through 2008.[3] The frequency of HIV infection among Polish blood donors is relatively high as compared to other developed countries. A significant part of HIV positive donors (22.8%) where infected less than 100 days before donation (Fiebig stage I–V). Currently, the residual risk of donations in the diagnostic window period in Poland is higher for HIV than for HBV and HCV (Piotr Grabarczyk, unpublished data). To address concerns regarding the transmission of blood borne viruses, the requirement to conduct either quarantine of SDP until second negative test of the donor (qSDP) or pathogen inactivation-treatment (PI-SDP) was introduced in Poland already in 2008.[4] Currently the riboflavin/UVB light pathogen reduction system (Mirasol™), the methylene blue/visible light (Theraflex™ MB) and amotosalen/UVA light (INTERCEPT™ Blood System) pathogen inactivation systems are used in Polish blood centres. 10.23% of plasma units in Poland were pathogen-reduced in 2018.[4] Additionally, SD-plasma (solvent-detergent, Octaplas™) is available through pharmacies. In the light of newly emerging pathogens (Dengue Virus, West-Nile Virus, Zika Virus) and the current COVID-19 pandemic, the concept of qSDP is becoming questionable. The majority of infected individuals shows no symptoms, despite positive NAT results.[5-8] SARS-CoV-2 (the virus causing COVID-19) genomic RNA has been detected in blood products from asymptomatic donors.[9] Evidence for transmission by blood transfusion has not been shown yet but cannot be excluded.[10] The current situation shows that the qSDP concept only works for specific pathogens if testing is applied, which is not the case for SARS-CoV-2 as well as potentially newly emerging pathogens in the future.

Amotosalen/UVA (AS) pathogen inactivation technology (INTERCEPT™ Blood System, Cerus Corporation), a targeted photochemical reaction irreversibly crosslinking nucleic acids,[11] allows the treatment of pools of five plasma units and splitting into six standardised therapeutic units.[12] The key objectives for this production method are the prevention of transmission of infectious agents and the preservation of the haemostatic capacity as well as the clinical effectiveness. AS-treatment of plasma effectively inactivates a broad spectrum of pathogens, including newly emerging pathogens.[13,14] In vitro studies revealed a moderate loss of coagulation factors post PI-treatment, with an overall retention meeting the regulatory criteria for therapeutic use.[15,16] Also, plasma treated with the INTERCEPT Blood System has been analysed in a series of clinical studies including patients with acquired and inherited coagulopathies as well as patients requiring therapeutic plasma exchange. In all these studies, the plasma showed a high level of tolerability and a safety profile comparable to conventional plasma.[17,18]

Our Institute, the Lodz Regional Blood Transfusion Center, is a Polish mid-size blood centre with almost 61.000 whole blood donations in 2018. We produced almost 61.000 plasma units (30% of them for clinical use, 6% of those pathogen-reduced) in 2018. In the current study, we intended to evaluate the impact of pooling five SDP units to obtain six PTP units and AS pathogen inactivation treatment on plasma standardisation and the content of labile coagulation factors as a potential alternative to qSDP and PI-SDP to potentially improve the clinical safety of our plasma products.

2 | MATERIAL AND METHODS

2.1 | Whole blood collection and plasma preparation

Whole blood units (450 ± 50 ml) were collected from voluntary donors at the Regional Blood Transfusion Center (RBTC) in Lodz using Compoflow™ containers (Fresenius Kabi, Germany) with citrate/phosphate/dextrose solution (CPD) as anticoagulant. The whole blood units were stored for 2 h at room temperature (RT) followed by centrifugation at 2699×*g* for 11 min at 22° and subsequent separation into red cells, buffy coat and plasma with a CompoMat™ G5 Automated Blood Component Separator (Fresenius Kabi). All units of whole blood were tested negatively for anti-HIV1/2, anti-HCV, HBsAg, HIV-RNA, HCV-RNA, HBV-DNA and *Treponoma Pallidum* antibodies according to Polish guidelines.

2.2 | Plasma pooling and pathogen inactivation

Five fresh ABO-identical SDP units were pooled with an Optipool DONOpack™ Plasma Pooling Set (Cerus Corporation) according to the manufacturer's instructions at RT <4 h post collection. After mixing, the pool was divided into two equal weight minipools. Each minipool was treated with the INTERCEPT™ Processing Set for Plasma (Cerus Corporation) using the INTERCEPT™ Illuminator INT 100 (Cerus Corporation) according to the manufacturer's instructions. Residual amotosalen and photoproducts were removed with the built-in compound adsorption device. Each minipool was subsequently divided into three storage bags resulting in six PTP units. The plasma units were frozen using a shock freezer (MABAG, Germany) within 8 h post collection as FFP and stored at ≤−25°C according to Polish guidelines.

2.3 | Sampling and analytical testing

Samples were collected from the SDPs before pooling, from the pools directly after pooling and mixing (pre-inactivation) and from the PTP units immediately after pathogen inactivation, into Eppendorf®Safe-Lock microcentrifuge tubes (volume 1.5 ml). Samples were frozen at −30°C and stored for 30 days. All samples were thawed simultaneously at 37°C in a water bath and analysed at the same time for each assay

with an ACL TOP 500 Haemostasis Testing System (Werfen, Spain). Prothrombin Time (PT) was analysed with a HemosIL RecombiPlasTin 2G kit (Werfen). Activated Partial Thromboplastin Time (APTT) was analysed with an APTT-SP (liquid) kit (Werfen). Fibrinogen activity was analysed with a HemosIL Q.F.A Thrombin (Bovine) kit (Werfen), based on the Clauss method. Factor VIII (FVIII) activity was analysed with the HemosIL Factor VIII deficient plasma kit (Werfen), a one-stage activity assay. Factor IX (FIX) activity was analysed with the HemosIL Factor IX deficient plasma kit (Werfen), a one-stage activity assay. Von Willebrand Factor antigen (vWF ag) was analysed with the HemosIL von Willebrand Factor Antigen kit (Werfen), an automated latex enhanced immunoassay. Von Willebrand Factor activity (vWF a) was analysed with the HemosIL von Willebrand Factor Ristocetin Cofactor Activity kit (Werfen), an automated latex enhanced immunoassay. All tests were conducted according to the manufacturer's instructions.

2.4 | Data analysis

Comparison of plasma pre and post pathogen-inactivation treatment was performed using the two-sample paired *t* test. Two-tailed *p* values of <0.01 are considered statistically significant.

3 | RESULTS

3.1 | The impact of plasma pooling on plasma volume standardisation

Twenty-five SDP units have been collected to generate five pools of five ABO-identical SDP units respectively. Three pools were blood group B, one blood group A and one blood group O. The total volume loss during processing (pooling, pathogen inactivation, splitting) was 5.5 ± 1.9%. However, since six PTP units were produced from originally five SDP units, the total volume reduction per plasma unit was 21.1 ± 3.5%, an additional volume reduction of 15.6% (Table 1). The difference between the highest and lowest volume was reduced from 40 ml between SDPs to 22 ml between PTPs, a reduction of 46.5%.

3.2 | The impact of plasma pooling on fibrinogen and labile coagulation factors standardisation

The median fibrinogen content was 219 mg/dl (155–266) in SDPs and 177 mg/dl (168–179) in PTPs, a reduction of the spectrum (span between lowest and highest value) of 90.1%. The median FVIII content was 85 IU/dl (64–142) in SDPs and 70 IU/dl (49–87) in PTPs, a reduction of the spectrum of 51.3%. The median FIX content was 113 IU/dl (80–141) in SDPs and 86 IU/dl (70–96) in PTPs, a reduction of the spectrum of 57.4%. Median vWF activity was 75 IU/dl (47–120) in SDPs and 84 IU/dl (56–89) in PTPs, a reduction of the spectrum of 54.8% (Table 2). In total, the distribution of values found for the contents of fibrinogen and labile coagulation factors was reduced 63.4% in PTPs compared to SDPs.

3.3 | The impact of plasma pooling and pathogen-inactivation treatment on the content of fibrinogen, labile coagulation factors and coagulation time

The average fibrinogen retention was 78.7% with respect to the concentration (Table 3). However, the average total fibrinogen content per PTP unit was reduced 37.2 ± 2.5% compared to the SDP units, 24.6% due to processing and 12.6% due to volume reduction by splitting five units into six. The average FVIII retention was 67.8% with respect to the concentration. However, the average total FVIII content per PTP unit was reduced 42.1 ± 11.3% compared to the SDP units, 30.5% due to processing and 11.6% due to volume reduction by splitting five units into six. The average FIX retention was 74.8% with respect to the concentration. The average total FIX content per PTP unit was reduced 40.9 ± 2.6% compared to the SDP units, 29.1% due to processing and 11.8% due to volume reduction by splitting five units into six. Since the average vWF activity was not significantly different pre- and post-treatment (*p* value 0.014) we also analysed the vWF antigen content, which was also not significantly different (*p* value 0.377). The average PT was prolonged for 5.2%, the average APTT for 14.5% (Table 3). The lowest FVIII, FIX and vWF concentrations were measured in the blood group O minipools as expected.

4 | DISCUSSION

High inter-donor variability in plasma factor content, coagulation time and variability in volumes of SDP doses could impact the clinical effectiveness of a plasma transfusion, hence the predictability of the treatment outcome. In the study presented here, the difference between the highest and lowest volume found was 46% less within the PTP units compared to the SDP units, the difference between highest and lowest concentration of fibrinogen and labile coagulation factors was reduced by 63%. A total reduction of variability of 55% (volume and coagulation factor concentration) between the units respectively was observed when the plasma units were produced by the novel approach of pooling five donor plasma units to gain six therapeutic plasma units in combination with AS pathogen inactivation. Moderate, but significant losses of coagulation factors (except for vWF) were observed post thawing of PTPs compared to fresh SDPs, findings which are in line with previously published data using amotosalen/UVA PI[12,19,20] and in compliance with European standards.[21] Also, a moderate prolongation of the coagulation time was observed, which is considered likely not being clinically relevant.

4.1 | Potential clinical benefits of reduced inter-donor variability

Better standardisation of therapeutic plasma units has major benefits in clinical practice. The treating physician ordering a unit for the

TABLE 1 Volumes (ml) of the initially collected single donor plasma units (SDP), the total volume of the SDPs before pooling, the final pathogen-reduced therapeutic plasma units (PTP), the total volume of the originally pooled units post processing (PP) and the total volume loss during processing (pooling, PI-treatment, splitting) as well as the blood groups (BG) of each ABO-identical pool

SDP number	Volume SDP (ml)	Pool number	Total volume (ml)	Volume PTP (ml)	Total volume PP (ml)	Volume loss PP (ml)	BG
1	258	1	1246	195	1170	76	O
2	259						
3	246						
4	234						
5	249						
6	250	2	1202	184	1104	98	A
7	232						
8	240						
9	232						
10	248						
11	268	3	1282	207	1242	40	B
12	248						
13	264						
14	263						
15	239						
16	250	4	1212	194	1164	52	B
17	232						
18	237						
19	243						
20	250						
21	261	5	1229	193	1158	71	B
22	251						
23	255						
24	233						
25	229						
Mean	247 ± 12		1234 ± 32	194 ± 8	1168 ± 49	69 ± 21	
Median	248		1229	194	1164	71	
Min	228		1202	184	1104	40	
Max	268		1282	207	1242	98	

Note: Mean values are expressed ± SD.

TABLE 2 Variability of the content of labile coagulation factors and fibrinogen in single donor plasma units (SDPs) and pathogen-reduced therapeutic plasma units (PTPs)

	SDP unit		PTP unit	
Factor	Content	Variability[a]	Content	Variability[a]
Fibrinogen (mg/dl)	219 (155–266)	111	177 (168–179)	11
Factor VIII (IU/dl)	85 (64–142)	78	70 (49–87)	38
Factor IX (IU/dl)	113 (80–141)	61	86 (70–96)	26
vWF a (IU/dl)	75 (47–120)	73	84 (56–89)	33

Note: The content columns are showing the median, minimum and maximum value.
Abbreviation: vWF a, von Willebrand factor activity.
[a]Difference between unit with the lowest and the unit with the highest value.

treatment of a patient can rely much better on the therapeutic effect of the unit as he can expect that unit to contain comparable amounts of coagulation factors needed. A recent study reported significant inter-donor variability in therapeutic plasma units in vitro, thus potentially impacting the protective effects of plasma-based resuscitation during treatment of haemorrhagic shock in vivo, with potential

TABLE 3 Comparison of the fibrinogen and labile coagulation factor content as well as the coagulation time pre- and post PI-treatment

Test	Pre-PI	Post-PI	Retention[a]	*p* value[b]
Fibrinogen (mg/dl)	221.4 ± 18.6	174.5 ± 4.8	78.7	<0.001
Factor VIII (IU/dl)	103.6 ± 16.1	70.2 ± 15.3	67.8	<0.001
Factor IX (IU/dl)	110.4 ± 13.4	82.6 ± 10.3	74.8	<0.001
vWF a (IU/dl)	83.6 ± 10.0	75.8 ± 15.0	90.7	0.014
vWF ag (IU/dl)	106.0 ± 13.4	90.0 ± 14.7	84.9	0.377
PT (s)	11.6 ± 0.2	12.2 ± 0.3	N/A	<0.001
APTT (s)	29.6 ± 2.1	33.9 ± 2.4	N/A	<0.001

Note: The values are showing the mean ± SD (n = 5).
Abbreviation: APTT, activated partial thromboplastin time; NA, not applicable; PT, prothrombin time; vWF a, von Willebrand factor activity; vWF ag, von Willebrand factor antigen.
[a]Expressed in %.
[b]Two-tailed p value.

implications for patient clinical outcome.[22] But also, beyond the "standard" factors a standardised product has advantages. The authors of the above-mentioned study furthermore concluded that also the concentration of certain chemokines in plasma has an impact on the mitigation of endothelial cell permeability. Higher levels of monocyte chemotactic protein 1 (MCP-1), interleukin-1 receptor agonist (IL-1 Ra) and other chemokines were significantly increased in plasma units having a protective effect in haemorrhagic shock treatment (means reducing endothelial permeability). These findings show that inter-donor variability may cause an uncertainty in treatment outcome which could potentially be mitigated by an increased standardisation of plasma units. Another example for inter-donor variability impacting the clinical outcome is the presence of human leucocyte antigen (HLA) and human neutrophil antigen (HNA) antibodies in the plasma of single donors, causing transfusion-related acute lung injury (TRALI), the leading cause of transfusion-related mortality. Despite the introduction of protective measures, in particular deferral of high-risk donors, there is still a remaining risk which could be mitigated (besides other measures) by plasma pooling, diluting the concentration of potentially harmful antibodies.[23] However, a more predictable treatment outcome and increased safety by plasma pooling and PI does not necessarily translate into a better treatment outcome.

4.2 | Plasma pooling and safety concerns

In many countries, SDP units are the standard of care. Historically the concerns regarding pooling have been the contamination of human plasma units with pathogens, for plasma particularly viruses, and to a certain degree, parasites. Current European guidelines allow the pooling of up to 12 human plasma units,[21] reflecting the advancements in diagnostic screening in the last decades. Pathogen inactivation technology poses an additional layer of safety to reduce the risk of pathogen transmission. AS pathogen inactivation, which we evaluated in our study, efficiently inactivates blood borne viruses and other pathogens of concern in human plasma,[13] also HIV, HBV and HCV (viruses which could be transmitted despite the usage of state of the art screening tests during a window period).

4.3 | Economical and operational considerations

The pooling of five SDP units resulting in six PTPs concept allows for the production of a more standardised product with potentially increased predictability of the clinical treatment outcome (which needs to be evaluated further in clinical studies), with a potential to reduce overall treatment costs by reducing inefficient treatment and adverse reactions. Due to the pathogen inactivation treatment of pools, only two pathogen inactivation processing sets are used to manufacture six PTPs. The additional cost of those two sets and the pooling set could be partially mitigated by the generation of six PTP units from five SDP units, allowing additional economic benefits. The current COVID-19 pandemic demonstrated how quickly a new pathogen could spread, and how quickly borders close and the exchange of products could become a challenge. Independence of the blood transfusion centres in generating pathogen-reduced pooled plasma from their donors locally poses an important feature of preparedness to guarantee an independent blood supply in case of future pandemics or other adverse reactions. In the Polish guidelines updated in 2020 the procedure of plasma pooling and inactivation is approved.

4.4 | Conclusion

The pooling of five SDP units to obtain six PTP units significantly increases plasma standardisation with potential implications for safety of the transfusion recipient, making it an interesting alternative to qSDP and pathogen-reduced SDP, especially in the light of pandemic preparedness.

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CONFLICT OF INTEREST

Marcus Picard-Maureau is employee of Cerus Europe B.V., the manufacturer of the INTERCEPT Blood System. All other authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Michal Bubinski, **Pawel Szykula** and **Kamila Kluska:** Performed the research study. **Michal Bubinski**, **Agnieszka Gronowska**, **Marcus Picard-Maureau** and **Elzbieta Lachert:** Designed the research study. **Ilona Kuleta** and **Elzbieta Lachert:** Contributed to essential reagents and tools. **Michal Bubinski**, **Agnieszka Gronowska** and **Marcus Picard-Maureau:** Analysed the data. **Michal Bubinski**, **Elzbieta Lachert** and **Marcus Picard-Maureau:** Wrote the manuscript. All authors reviewed and approved the final draft.

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TRANSFUSION MEDICINE WILEY

LETTER TO THE EDITOR

Use of recombinant Von Willebrand factor during transcatheter aortic valve replacement in a patient with acquired von Willebrand syndrome

To the Editor,

Acquired von Willebrand syndrome (aVWS) is a clinical and biological entity similar to congenital von Willebrand disease (VWD). Although aVWS is rare, its true incidence is most likely underestimated due to the diagnostic complexity. Many underlying diseases are associated with aVWS, such as autoimmune, cardiovascular, lymphoproliferative, myeloproliferative or malignant disorders. Cardiovascular disorders especially include congenital heart defects, aortic stenosis, and the use of left ventricular assist devices.[1,2] The therapeutic strategy consists in identifying the underlying pathology in order to treat the underlying condition. In cases where such remedies are not possible, only symptomatic treatment is available. The management of surgeries in patients with aVWS can be complicated in particular, due to the often reduced half-life of von Willebrand factor (VWF). We report on the use of vonicog alfa, the first recombinant VWF, during transcatheter aortic valve replacement (TAVR) in a patient with aVWS.

This case concerns a 72-year-old male patient with hypertension who presented with VWD diagnosed 10 years ago, with lower gastrointestinal (GI) bleeding. Factor VIII (FVIII), Willebrand antigen (VWF:Ag) and ristocetin cofactor activity (VWF:Rco) levels were respectively at 40% (50%–150%), 20% (50%–150%) and <10% (50%–150%), associated with loss of high molecular weight multimers (HMWM). An acquired aetiology was evoked, as there was no personal or family history of bleeding. The ratio between VWF propeptide and antigen was found to be 3.95 (<2.4), thus further supporting this hypothesis. A complete clinical and biological assessment led to the diagnosis of GI angiodysplasia, calcified aortic stenosis (AS), and monoclonal gammopathy of undetermined significance (MGUS) with IgM kappa. The latter seemed to be the leading aetiology of aVWS.

Recently, the patient's coronary syndrome and calcified AS worsened, and it was decided to perform a TAVR. First, on 19 August 2020, a coronary angioplasty with placement of two active stents on the proximal circumflex, was performed under a single injection of 40 IU/kg of vonicog alfa and 40 IU/kg of octocog alfa. The recovery of vonicog alfa was calculated during the coronary angioplasty at 0.75 IU/dl per IU/kg with a half-life of 12 h (Table 1). The TAVR procedure was performed via the left common carotid 1 month later, with implantation of one valve. The TAVR was preceded by a bolus of 91 IU/kg of vonicog alfa and 40 IU/kg of octocog alfa. The recovery during the procedure was 1 IU/dl per IU/kg. The operation lasted 3 h and no abnormal bleeding was observed. Moreover, given the existence of conduction disorders before TAVR and an episode of sinus node dysfunction post-procedure, placement of a pacemaker was indicated. A cardiac pacemaker was implanted 48 h after the TAVR, under a bolus of 91 IU/kg of vonicog alfa.

Following this double procedure, 64 IU/kg of vonicog alfa were administered daily for 4 days, and this brought the residual VWF:Rco level to between 40% and 50% (Figure 1). The haemoglobin level remained stable throughout the patient's stay (between 11 and 12 g/dl), with nevertheless some non-serious haematomas at the incisions. The patient left the cardiology department after 10 days, including seven in intensive care. Upon discussion with colleagues, Clopidogrel 75 was introduced for 1 month. The antiplatelet therapy was nevertheless stopped on the seventh day after the patient's discharge due to an episode of epistaxis which required nasal packing and the injection of 65 IU/kg of vonicog alfa. The patient was seen again 1 month after the procedure. His clinical condition was satisfactory from a cardiological point of view, but he reports experiencing epistaxis on a daily basis.

The management of surgery in patients with aVWS is often complicated. From a therapeutic point of view, there are several options: desmopressin when there is no contraindication, intravenous immunoglobulins (IVIG), plasma VWF concentrates with or without FVIII, FVIII alone or, if these fail, recombinant activated factor VII. IVIG are especially useful in cases associated with MGUS, but are usually ineffective in IgM-MGUS.[2] Because the half-life of endogenous or exogenous VWF can be very short, a pharmacokinetic (PK) study before any major surgery is justified. The VWF dose needed depends on the clinical situation and can range from 30 to 100 IU/kg.[3] There are no consistent guidelines for the treatment of aVWS, likely due to its rarity.

In this context, we report here the case of a polypathological patient with aVWS in connection with a MGUS who underwent a TAVR under vonicog alfa. Vonicog alfa is the first recombinant VWF product and contains no FVIII. It is manufactured in the absence of animal or other human plasma proteins, which eliminates all risks of pathogenic transmissions.[4] PK assessments performed as part of prospective phase studies in patients with type 3 or severe type 1 VWD have demonstrated that the terminal half-life of vonicog alfa is longer than that of plasma-derived VWF (pd-VWF). The peak plasma vonicog alfa levels are observed less than 60 min after injection and its half-life is 21.9 h.[5] The efficacy of vonicog alfa in surgery was studied in a phase 3 study that included 15 surgeries performed in patients with congenital VWD (10 major and 5 minor).[6]

TABLE 1 Comparative pharmacokinetics between pd-VWF (Wilfactin®) and vonicog alpha

	VIII:C (%)		VWF:Ag (%)		VWF:Rco (%)	
	40 IU/kg octocog alpha 40 IU/kg Wilfactin®	40 IU/kg octocog alpha 40 IU/kg vonicog alpha	40 IU/kg octocog alpha 40 IU/kg Wilfactin®	40 IU/kg octocog alpha 40 IU/kg vonicog alpha	40 IU/kg octocog alpha 40 IU/kg Wilfactin®	40 IU/kg octocog alpha 40 IU/kg vonicog alpha
T0	29	31	21	41	<10	<10
T1h	93	85	103	89	30	36
T4h	80	98	84	87	<10	33
T8h	73	91	67	84	<10	25
T12h	60	89	62	74	<10	21

Abbreviations: Rco, ristocetin cofactor activity; VWF, Willebrand factor.

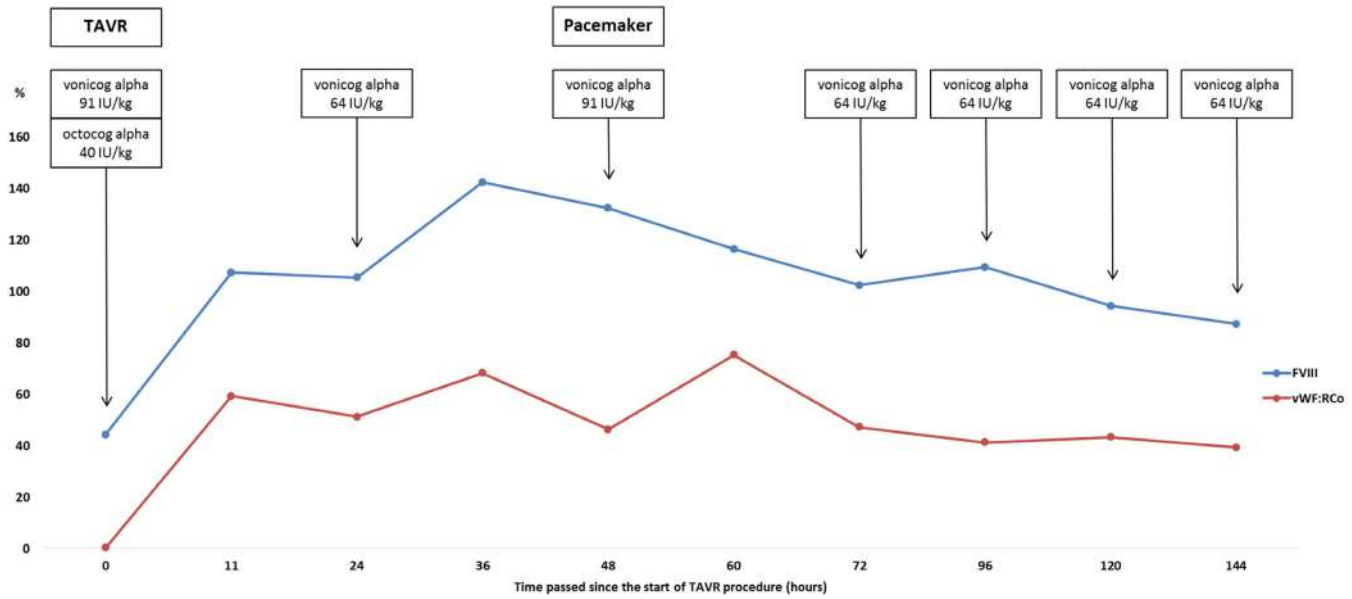

FIGURE 1 Evolution of Willebrand factor (VWF):ristocetin cofactor activity (Rco) and Factor VIII (FVIII) levels during and after the procedures [Color figure can be viewed at wileyonlinelibrary.com]

Regarding our patient, a PK analysis was conducted both with pd-VWF (Wilfactin®) and with vonicog alfa. Blood samples were collected at baseline (T0) and respectively 1 h (T1h), 4 h (T4h), 8 h (T8h) and 12 h (T12h) after infusion (Table 1). PK parameters with vonicog alfa (half-life of 12 h and recovery of 0.75 IU/dl per IU/kg) seemed to be better than those obtained 3 weeks ago with pd-VWF, which prompted us to prescribe vonicog alfa for the TAVR. Though TAVR is not a major surgery, it is still a complex procedure associated with a real risk of perioperative bleeding.[7]

In the case reported here, the efficacy of vonicog alfa was deemed to be excellent. However, the total quantity of vonicog alfa administered (502 IU/kg over 6 days) was greater than the overall median surgical dose of vonicog alfa (220.4 IU/kg, range 63.8–648.4 IU/kg) used in the phase 3 study.[6] Aside from the preoperative bolus of FVIII, no further injection of FVIII was necessary, which attests to the endogenous stabilisation of FVIII by vonicog alfa.

Vonicog alfa could represent an interesting alternative for surgery in patients with aVWS, who often have greatly reduced HMWM. During the production process, vonicog alfa has no exposure to ADAMTS 13 and therefore contains intact HMWM with significant haemostatic power and probably a better capacity to stabilise FVIII and the clot. Indeed HMWM play a crucial role in primary haemostasis because of their binding capacity for collagen and platelet receptors, thus facilitating platelet aggregation under shear stress.[8]

To date, there have been few reports in the literature of experience with vonicog alfa in the perioperative management of patients with aVWS. Weyand et al reported the use of vonicog alfa in a 2-year-old child with moderate aVWS during a valve replacement.[9] Furthermore, Tran et al reported in their review a clinical case similar to ours, of a patient with aVWS associated with myeloma who underwent a valve replacement, in whom vonicog alfa was ultimately not used in due to the high concomitant need for FVIII.[10]

While global conclusions cannot be drawn from the case we present here, the role of vonicog alfa in the management of surgery in patients with aVWS should be studied in greater detail, particularly in the current context of IGIV shortage.

CONFLICT OF INTEREST

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

Dominique Desprez: Designed the study. **Laurent Sattler** and **Dominique Desprez:** Analysed the data and wrote the paper. **Laurent Sattler**, **Olivier Feugeas**, **Ulun Crimizade**, **Sébastien Hess**, **Lélia Grunebaum** and **Dominique Desprez:** Critically reviewed the manuscript. All authors approved the final version.

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