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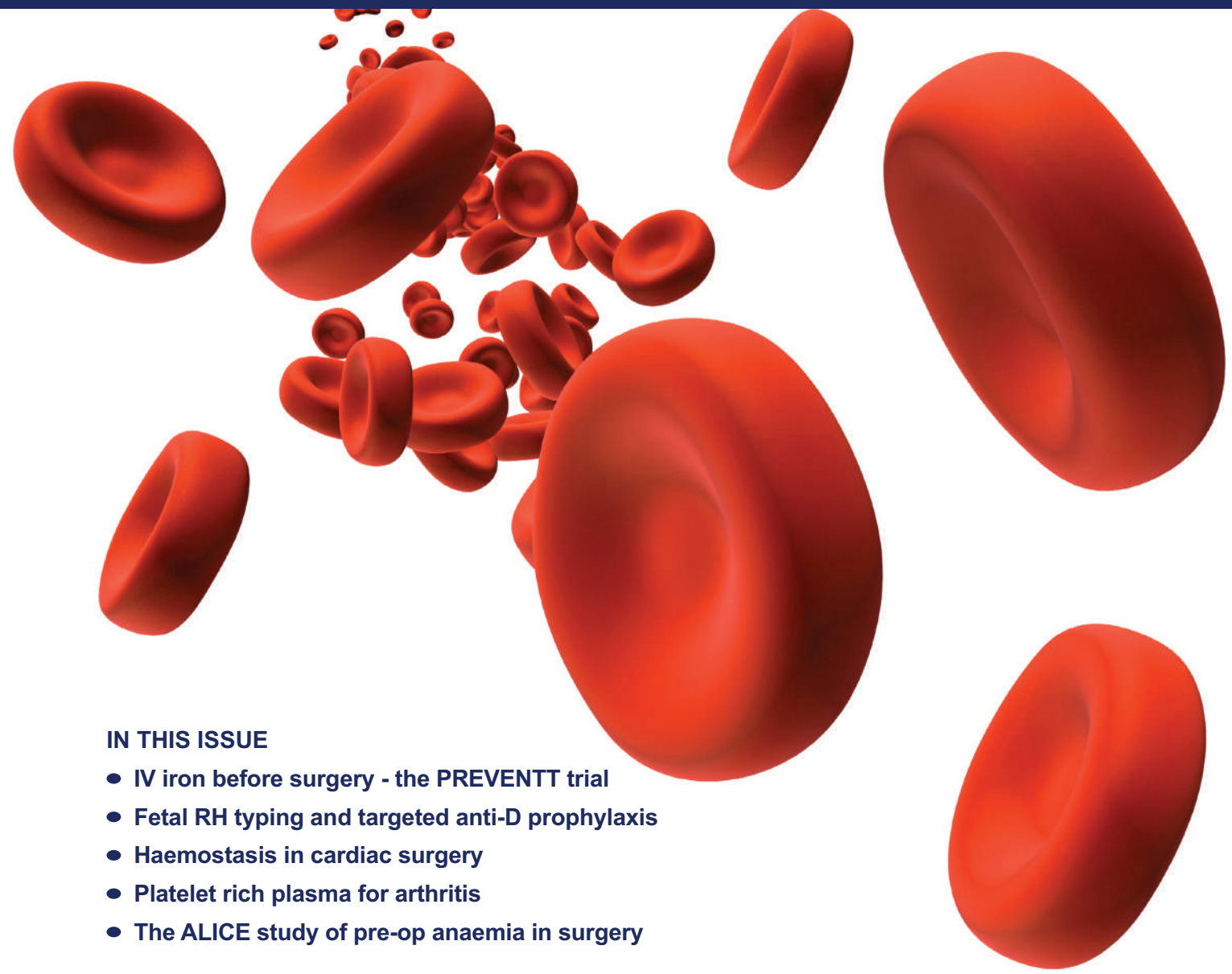

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Transfusion Medicine will publish articles on transfusion medicine in its widest context, including blood transfusion practice (blood procurement, pharmaceutical, clinical, scientific, computing and documentary aspects), immunohaematology, immunogenetics, histocompatibility, medico-legal applications, and related molecular biology and biotechnology.
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EVIDENCE CORNER

TRANSFUSION MEDICINE WILEY

Evidence cornered: Preoperative intravenous iron to treat anaemia before major abdominal surgery (PREVENTT trial)

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Abstract

Clinical question: Does intravenous iron given to anaemic patients (haemoglobin [Hb] <130 g/L for men and 120 g/L for women) before major elective open abdominal surgery reduce the need for blood transfusions or death within 30 days of surgery?

Evidence from trial: There were no significant differences in the rates of blood transfusion or death at 30 days after the index operation between anaemic patients who received intravenous iron preoperatively and those who did not.

KEYWORDS
anaemia, intravenous iron, major surgery, transfusion

International guidelines[1] recommend that patients undergoing major surgery with an expected blood loss of greater than 500 ml or more should be screened for anaemia at least 6–8 weeks before surgery and if less than 4 weeks, intravenous iron therapy should be offered as first-line therapy for patients with iron deficiency anaemia. These guidelines are based on low-quality evidence largely from small randomised controlled trials (RCTs) and observational studies. A Cochrane meta-analysis[2] published in December 2019 concluded that preoperative intravenous iron did not show a clinically significant reduction in allogeneic blood transfusions (risk ratio [RR] 1.21; 95% confidence interval (CI): 0.87–1.70; four RCTs; 200 participants, moderate certainty of evidence). The timing of administration of intravenous iron in the included studies ranged from between 48 h to up to 3 weeks prior to surgery. PREVENTT aimed to increase the certainty of the evidence and is the first, large, double-blind RCT that has evaluated the clinical effectiveness of preoperative intravenous iron.[3] This Transfusion Evidence Synopsis summarises the paper describing this RCT in The Lancet (Box 1).

BOX 1: Evidence box

- **Study design:** Double-blind, parallel-group randomised controlled trial
- **Study dates:** 6 January 2014, to 28 September 2018
- **Location:** United Kingdom, 46 tertiary care centres
- **Setting:** Elective, major open abdominal surgery
- **No. Of patients:** 487 randomised (474 intention to treat analysis, 388 per-protocol analysis)
- **Demographics**
  - Median age 66 years; Female 267/487 (54.8%); predominantly American Society of Anesthesiologists grade II (288/472 [61.0%]) and III ((121/472 [25.6%])
- **Inclusion criteria**
  - Age >18 years, Anaemia defined as haemoglobin <130 g/L for men and <120 g/L for women, major surgery lasting >1 h coded major, major plus or complex major, no specific requirement for preoperative iron deficiency

- **Exclusion criteria**
  - Laparoscopic surgery, bodyweight <50 kg, chronic liver disease, concurrent infection, other cause for anaemia, acquired iron overload, family history haemochromatosis or thalassaemia, transferrin saturation >50%
- **Comparison:** Single 1000 mg dose of ferric carboxymaltose in 100 ml 0.9% saline versus blinded placebo (100 ml 0.9% saline).
- **Co-primary outcomes:**
  - Risk of the composite endpoint of blood transfusion or death, and number of blood transfusion episodes (≥1 red blood cell unit or any other blood component in a 24 h period) from randomisation until 30 days after the index operation
- **Secondary outcomes:**
  - total number blood components transfused at 30 days and 6 months after surgery, change in haemoglobin concentration from randomisation to day of the index operation and at 8 weeks and 6 months after surgery, postoperative complications, intensive care unit and total hospital length of stay, days alive and out of the hospital at 30 days after the index operation, hospital readmission at 8 weeks and 6 months postoperatively, and health-related quality of life

1 | SUMMARY OF THE RESULTS OF THE STUDY

1.1 | Primary outcome

Preoperative intravenous iron did not result in a reduction in the risk of the composite endpoint of blood transfusion, death or the number of blood transfusion episodes between randomisation to 30 days after index operation (Table 1). There was also no evidence of an effect on any of the prespecified subgroup analyses, including patients with iron deficiency defined as ferritin <100 ng/ml and/or transferrin saturation <20%.[1] This finding supports evidence from previous small trials that preoperative iron therapy does not reduce perioperative transfusion requirements.

1.2 | Secondary outcomes

There were no significant differences between the groups for the total number of blood components transfused at 30 days; postoperative complications until 6 months; intensive care unit stay and total hospital length of stay. There was also no difference between groups for days alive and out of hospital at 30 days after index operation or any of the health-related quality of life outcomes.

Preoperative intravenous iron did result in a statistically significant increase in haemoglobin concentration from randomisation to index operation at 8 weeks (mean difference 10.7 g/L; 95% CI: 7.8–13.7) and 6 months (mean difference 7.3 g/L; 95% CI: 3.6–11.1) when compared with placebo. There was a significant reduction in hospital readmissions at 8 weeks following the index operation in the intravenous iron group when compared with placebo. This is likely to be a chance finding; but could also reflect the actual time period required to elicit any effects from intravenous iron, including effects independent of erythropoiesis such as improved cardiopulmonary function, exercise capacity and immunity.[4,5] These findings should inform future hypothesis-testing studies. There were no significant differences in any of the prespecified safety endpoints.

TABLE 1 Outcomes from the PREVENTT trial

Outcomes	Placebo	Intravenous iron	Relative risk (95% CI)
Blood transfusion or death at 30 days	67/237 (28%)	69/237 (29%)	1.03 (0.78–1.37)
Transfusion	67/237 (28%)	68/237 (92%)	
Death	2/237 (1%)	2/237 (1%)	
Anaemia correction at time of surgery	21/243 (10%)	42/244 (21%)	2.06 (1.27–3.35)
Postoperative complications	24/227 (11%)	22/233 (9%)	0.89 (0.52–1.55)
ICU length of stay (median days)	1 (0–3)	2 (0–3)	-
Hospital length of stay (median days, IQR)	9 (5–14)	9 (7–14)	-
Days alive and out of hospital within 30 days (mean, SD)	19.8 (7.5)	19.7 (7.0)	−0.1 (−1.5–1.2)
Any hospital readmission from discharge to:			
8 weeks	51/234 (22%)	31/234 (13%)	0.61 (0.40–0.91)
6 months	73/223 (32%)	58/227 (26%)	0.78 (0.58–1.04)
Safety outcome: adverse reaction to trial therapy	5/240 (2%)	11/240 (5%)	2.20 (0.78–6.24)

Abbreviations: ICU, intensive care unit; IQR, interquartile range.

1.3 | Limitations of the trial

The blood transfusion rate observed in the placebo arm of the trial was 29%, which is less than the 40% used in the original sample size calculation. Therefore, it is possible that the study may have been underpowered. The observed reduction in transfusion rates could be attributed to the widespread adoption of patient blood management principles[4,6] which occurred during the time period of this study. Approximately 1 in 5 patients deviated from the study protocol, which can be expected for interventions being tested in complex perioperative pathways. Patients did not require a confirmed diagnosis of iron deficiency to enter the study with no clear diagnostic pathway defined. Only 29% in included participants had known iron deficiency. These factors may introduce bias and reduce the magnitude of the observed effect in a study which was already underpowered. The inclusion criteria defined that preassessment should occur a minimum of 10 days before surgery. This may miss some urgent cancer patients for whom surgery is a priority and preassessment is less than 10 days before surgery. These patients, with more severe surgical disease, may have benefitted the most from iron therapy. There was no standardised transfusion protocol for participating hospitals.

1.4 | Evidence in context

This is the first large RCT of preoperative intravenous iron therapy to treat anaemia before major elective abdominal surgery. The findings support evidence from previous small trials[2] that routine preoperative iron therapy does not reduce perioperative transfusion requirements in this cohort of patients.

1.5 | Implications for research

Preoperative intravenous iron resulted in a sustained improvement in haemoglobin that lasted up to 6 months postoperatively. There was also a reduction in hospital readmissions in the same timeframe. These require further study including the potential role of postoperative iron therapy. Future studies should aim to include patients with diagnosed iron deficiency and evaluate patients who may have other indications for intravenous iron, other than reducing transfusion requirements, such as cardiopulmonary disease. Whether other surgical groups, such as those undergoing orthopaedic surgery, emergency surgery, or older surgical patients, may benefit from intravenous iron is the subject of current research.

1.6 | Implications for practice

Routine use of preoperative intravenous iron to treat anaemia in patients scheduled to undergo elective open major abdominal surgery, without laboratory confirmed iron deficiency, should not be recommended. Current clinical guidelines should be updated accordingly.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

The authors have no competing interests.

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TRANSFUSION PRACTICE

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The Norwegian experience with nationwide implementation of fetal *RHD* genotyping and targeted routine antenatal anti-D prophylaxis

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Abstract

Objectives: To reduce the risk of RhD alloimmunization during the last trimester of pregnancy, a targeted routine antenatal anti-D prophylaxis (RAADP) programme was implemented in Norway in 2016. Here, we present and discuss our experience with the nationwide implementation of the programme, and report sample uptake and preliminary data of de novo anti-D in pregnancy.

Background: The targeted RAADP was advised by the academic community and evaluated by the health authorities. A National Working Group has conducted the implementation in the transfusion services and contributed to organise the administration of the antenatal anti-D prophylaxis. Fetal RhD type is determined by non-invasive prenatal testing at gestational week 24, and anti-D prophylaxis is administrated at gestational week 28 only to women with RhD positive fetuses.

Methods: We describe the implementation process of targeted RAADP in Norway. The sample uptake is calculated by comparing the number of fetal *RHD* screens with the expected number of samples.

Results: The sample uptake shows regional variations: 88%–100% after 3 years. Promising decrease in de novo anti-D detected during pregnancy is observed.

Conclusions: Nationwide targeted RAADP is implemented and included in the Norwegian maternity care programme. Compliance to sample uptake should further improve in some regions. A remaining issue to fulfil is the documentation of the accuracy of the fetal *RHD*-typing at all sites. Post-natal prophylaxis will then be guided by the fetal *RHD* result. Dedicated registries will ensure data to evaluate the expected reduction in pregnancy-related RhD immunisations, which is the final success criterion of the programme.

1 | INTRODUCTION

In Norway, targeted routine antenatal anti-D prophylaxis (RAADP) was implemented nationwide in September 2016. RAADP entails fetal *RHD* genotyping and red cell antibody screening performed at gestational week (GW) 24 in non-immunised RhD negative pregnant women and a single dose of 1500 IU (300 μg) anti-D immunoglobulin (Ig) at GW 28 for those who carry an RhD positive fetus.[1] The routine

follow-up until September 2016 was ABO/RhD typing and red cell antibody screening at the first consultation at about GW 12–16, followed by repeat screening at GWs 32 and 36 in RhD-negative women, given that the first screening was negative.[2] The only anti-D prophylaxis was post-natal, when the newborn was serologically typed to be RhD positive. Post-natal anti-D prophylaxis reduces RhD immunisations significantly but does not prevent immunisations in pregnancy.[3] Therefore, specialists in obstetrics and transfusion medicine initiated the process for implementation of RAADP in maternity care aiming to further reduce the alloimmunization during the last trimester, and accordingly decrease the number of anti-D-associated hemolytic disease of the fetus and newborn (HDFN). The Norwegian Directorate of Health* advised the analysis to be performed as recommended by the Norwegian national guidelines for obstetrics at GW 24.[4]

Our aim is to describe the work and priorities we made before and during the nationwide implementation, and also to present data for adherence to sampling at GW 24 and the preliminary indications of reduction in de novo anti-D in pregnancy.

2 | THE PROCESS OF IMPLEMENTATION OF TARGETED RAADP

In the timeline (Figure 1), the process of implementation of targeted RAADP is illustrated. The method was approved and implementation of RAADP was advised in March 2015. Accordingly, nationwide start-up was on 1 September 2016.

Prenatal diagnostics, including use of cell-free fetal DNA (cffDNA) is strictly regulated by the Norwegian Biotechnology Act.† The ethical and social consequences of any prenatal test must be assessed by The Norwegian Biotechnology Advisory Board‡ and approved by the Ministry of Health and Care Services§ before implementation.[5] In addition, a new technology has to be evaluated in the National System for Managed Introduction of New Health Technologies within the Specialist Health Service,¶ prior to implementation.[6] As a part of the process, the National Working Group for implementation of fetal *RHD* genotyping and antenatal anti-D prophylaxis (NWG) was established in January 2015.

2.1 | The National Working Group for implementation of fetal *RHD* genotyping and antenatal anti-D prophylaxis

The NWG was constituted after an initiative from The Norwegian National Advisory Unit on Immunohematology** (hereafter referred to as Advisory Unit) at Department of Immunology and Transfusion Medicine, Oslo University Hospital, Ullevaal. The group consists of six specialists in immunology and transfusion medicine and two biomedical laboratory scientists with representatives from all the health regions (Figure 2). The primary focus of the NWG has been to achieve consensus in all the practical matters in management of the new RAADP routine.

Already, in March 2015, the Advisory Unit invited representatives from the Directorate of Health, obstetricians from all health regions, leaders of associations of general practitioners (GPs) and midwives to discuss approaches including logistics and information channels. Since this first meeting, the Advisory Unit has arranged and chaired three annual seminars with international experts as lecturers. Representatives from the health authorities were also invited to present their view and work on the topic. Staff from the transfusion services, in addition to GPs, midwives and obstetricians attended the seminars. The last two seminars targeted the transfusion service staff and focused on sharing experiences, evaluating the logistics, as well as discussing challenges and potential for improvement.

Many meetings, lectures and presentations for the target groups were held in the initial phase. Written information to health professionals in the maternity care was distributed, in addition to information on the hospitals' websites. The NWG prepared an information leaflet for RhD negative pregnant women. The leaflet is available on the web and is distributed to the GPs along with the results of the ABO/RhD testing and red cell antibody screening. Representatives from the Advisory Unit informed the general public in the media, including television. Articles regarding the new routine were published in journals for midwives and nurses. The NWG is still active, monitoring the challenges and potential improvements of the routine.

2.2 | Maternity care in Norway

Medical care during pregnancy is free of charge, and the follow-up of uncomplicated pregnancies with nine scheduled appointments takes place in the primary healthcare.

When RAADP was implemented in 2016, 59 119 deliveries were registered.[7] However, birth rate has decreased to 54 403 deliveries in 2019.[7] Assuming 15% RhD negative in the population, we initially estimated 9000 pregnant women would be tested annually to determine the fetal *RHD* type, the estimated number was 8160 in 2019. However, due to immigration, about 23% of the 15–49-year-old women are non-ethnic Norwegians; of these, 35% are of Asian origin and 13% are of African origin.[8] Thus, the

*The Norwegian Directorate of Health is an executive agency and professional authority under the Ministry of Health and Care Services. The Directorate has the role as an executive agency, as a regulatory authority and as an implementing authority in areas of health policy (https://www.helsedirektoratet.no/english/about-the-norwegian-directorate-of-health).

†The Norwegian Biotechnology Act governs areas such as assisted fertilisation, embryonic diagnostics, pre-implantation genetic diagnosis and genetic examinations (https://www.regjeringen.no/en/topics/health-and-care/innsikt/biotechnology/id11717/).

‡The Norwegian Biotechnology Advisory Board is an independent and consultative body appointed by the Norwegian government. The main tasks of the Board are to evaluate the social and ethical consequences of modern biotechnology (https://www.bioteknologiradet.no/english/).

§Ministry of Health and Care Services directs services by means of a comprehensive legislation, annual budgetary allocations and through various governmental institutions (https://www.regjeringen.no/en/dep/hod/id421/).

¶The National System for Managed Introduction of New Health Technologies within the Specialist Health Service is owned by the Ministry of Health and Care Services and has been established to be applied to New Health Technologies entering the specialist healthcare. The main ambition is systematic use of health technology assessments to inform decision-making (https://nyemetoder.no/english).

**The Norwegian National Advisory Unit on Immunohematology is a part of Section for Immunohematology, Department of Immunology and Transfusion Medicine, Oslo University Hospital, Ullevaal. The Advisory Unit functions as a national reference laboratory.

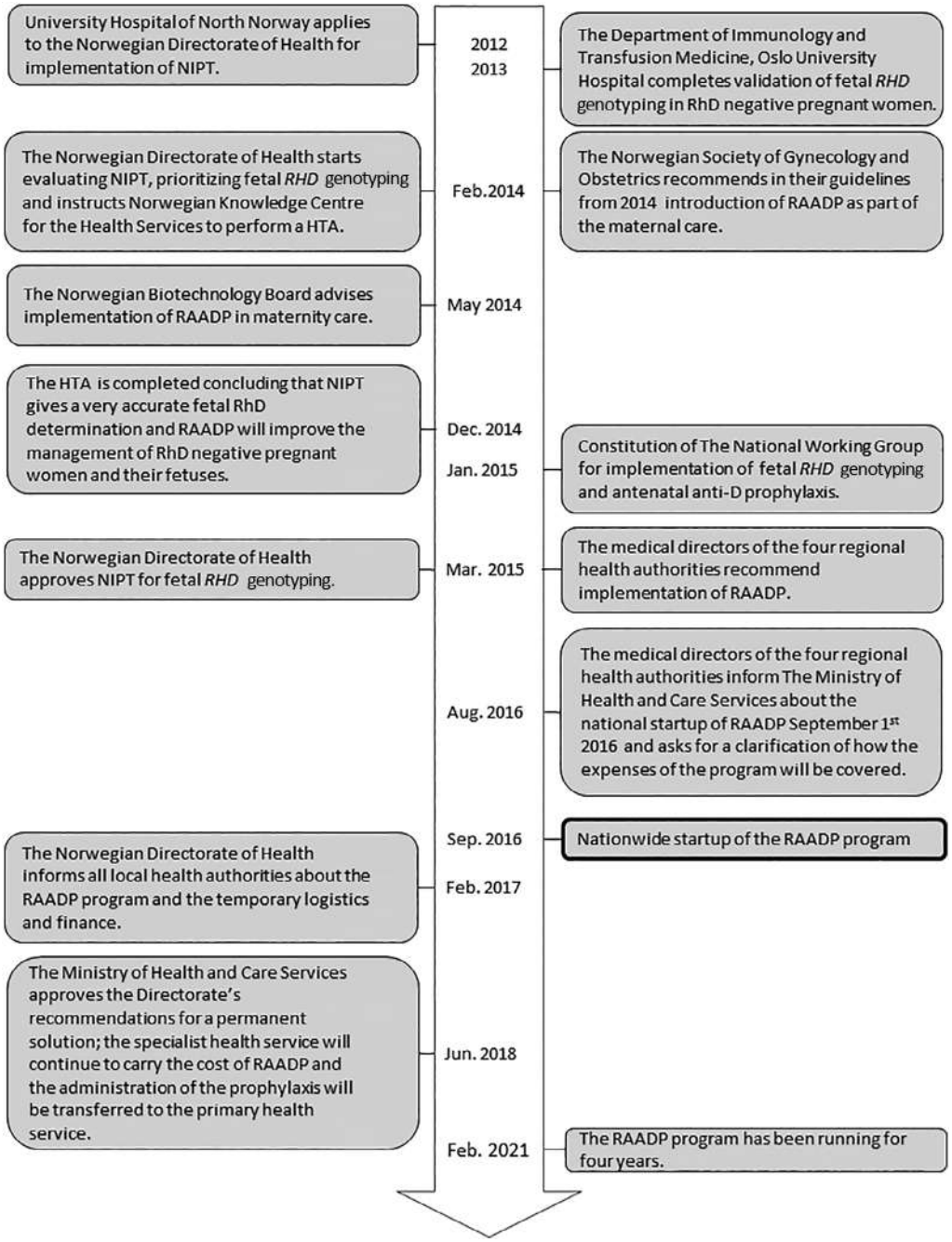

FIGURE 1 The timeline shows the process from application, evaluation and approval to final implementation of the target RAADP programme in Norway. HTA, health technology assessment; NIPT, non-invasive prenatal test; RAADP: routine antenatal anti-D prophylaxis

prevalence of RhD-negative women of childbearing age might in fact be less than 15% owing to lower frequency of RhD negatives in Asians and Africans.

The NWG contributed to update the "Maternal Health Card" specifying the GWs when fetal *RHD* typing and antenatal anti-D prophylaxis should be performed and administered, respectively. The Maternal Health Card is an important tool in pregnancy follow-up; the woman keeps this document with pregnancy-relevant information filled out by caretakers. In addition, all referral forms for antenatal testing were revised to include fetal *RHD* genotyping at GW 24.

2.3 | Targeted RAADP—logistics

The academic community, as well as the medical directors of the four regional health authorities[††] (Figure 2), agreed that administration of RAADP was a task for the primary healthcare. However, while waiting

[††]The four regional health authorities (Figure 1) are owned by the Ministry of Health and Care Services and have responsibility for the specialist health services in their respective regions. This responsibility includes patient treatment, education of medical staff, research and training of patients and relatives (https://www.regjeringen.no/en/dep/hod/organisation-and-management-of-the-ministry-of-health-and-care-services/Departments/the-department-of-hospital-ownership/id1413/).

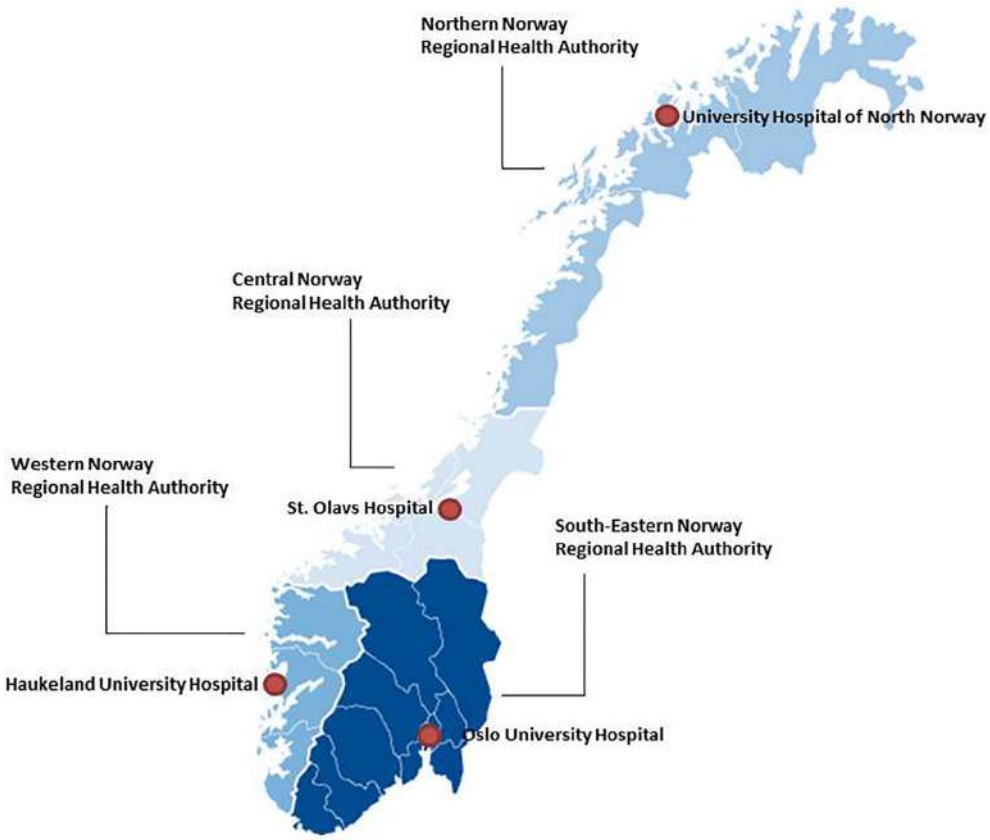

FIGURE 2 Overview of the four regional health authorities and the hospitals performing fetal *RHD* genotyping [Color figure can be viewed at wileyonlinelibrary.com]

for the Directorate of Health's guidelines and instructions, the medical directors instructed the hospitals to cover the costs and administer the prophylaxis. In close cooperation with the transfusion services, logistics at the maternity outpatient clinics were established. The transfusion laboratories send the test results to the woman's GP or midwife. In all health regions except for the Health Region of Northern Norway, the result is also sent to the maternity outpatient clinic that schedules an appointment for women at GW 28 if the fetus is predicted to be RhD positive. When given, RAADP is documented in the Maternal Health Card, medical record and also reported back to the GP or midwife. By this means, a uniform and adequate management of these women is achieved.

In North Norway, large areas are sparsely populated, and a maternity outpatient clinic may be hours away. Therefore, when anti-D prophylaxis is indicated, the GP sends an electronic prescription to one of the hospital pharmacies in the region and receives a vial of anti-D Ig that is administered by the GP or midwife at GW 28.

In June 2018, the Directorate of Health presented a proposition of permanent solution recommending the RAADP to be administrated in the primary healthcare. The proposition was approved by the Ministry that also recommended the specialist healthcare to continue to carry the expenses of the antenatal prophylaxis, since they initiated and advised the implementation of targeted RAADP, administers the post-natal prophylaxis, and will eventually benefit from reduction of RhD immunisations. Finding nationwide logistics for distribution of the anti-D Ig to the GPs and midwives is now delegated to the regional health authorities. The permanent solution is still not implemented, but experiences from the Health Region of Northern Norway will be valuable in the process.

2.4 | Fetal *RHD* genotyping

Fetal *RHD* genotyping in Finland and the Netherlands is centralised at one laboratory, while the Norwegian system is decentralised with one laboratory in each health region (Figure 2) like the Danish.[9] In order to achieve nationwide coverage from the beginning, the Advisory Unit analysed the samples from the Health Regions of Western and Northern Norway until their laboratories were ready in January and in May 2018, respectively.

While ABO/RhD typing and the antibody screening in samples from pregnant women are performed at 18 local laboratories, the samples for fetal *RHD* genotyping are forwarded to the regional test laboratory. Samples are shipped by post or courier, and result reports are sent electronically or by post. Decentralisation is primarily a political decision, but we have also geographical challenges that benefit from decentralisation, as long distances delay shipments. A low test volume at some sites may encounter the quality and increase the overall costs, as we lose the economics of scale. However, all four laboratories participate in a national and an international external quality control scheme. The NWG with representatives from all four sites has established the routine together and maintains close cooperation.

Several precautions in the assay set-up avoid primarily false-negative results. Firstly, the fetal *RHD* screening is performed at GW 24 when low level of cffDNA is not an issue.[9,10] Secondly, cell-free

DNA is isolated with automated extraction from 1 to 2 ml of EDTA-plasma, no more than 7 days after sampling. The plasma equivalent per PCR is 0.08–0.25 ml. Except for the set-up at University Hospital of North Norway where the assay targets exons 5, 7 and 10, the assays are identical, targeting exons 7 and 10.[1] Finally, the samples are run in triplicates and a housekeeping gene is amplified as an extraction control. *RHD* positive and negative controls are included in all runs; the concentration (10–25 pg/well) of the lowest positive control (DNA from leucocytes from an RhD [hemizygote] positive donor) is close to the detection limit of the assay. No false-negative results have been confirmed so far. Fetal *RHD* genotyping at GWs 24–29 demonstrates high performance and reported sensitivity at ~99.9%.[9,10] A false-negative result will mean no antenatal anti-D prophylaxis to a woman at risk of alloimmunization. False positive or inconclusive results due to serologically RhD negative *RHD* variants are unavoidable. Due to immigration from Asia and Africa, we expect increased prevalence of DEL, *RHD**pseudogene and partial D variants among RhD-negative women.[9] In women with *RHD* variants, amplification of the maternal *RHD* will preclude determination of the fetal *RHD* type. Antenatal prophylaxis is recommended to these women unless they have weak D type 1, 2 or 3, since individuals with these variants do not make alloanti-D.[11]

2.5 | *RHD* genotyping to reveal weak D types 1, 2 and 3

Primo 2019, the Advisory Unit initiated a national 2-year study to identify weak D types 1, 2 and 3 mainly in pregnant women but also in female patients and blood donors ≤50 years. Individuals with weak D types 1, 2 or 3 are not at risk for RhD alloimmunization. They can be managed as RhD positive regarding transfusion and pregnancy, and unnecessary administration of anti-D prophylaxis can be avoided.[11] We perform extended *RHD* genotyping when serologic RhD typing is weak (≤2+) and when maternal *RHD* is detected in fetal *RHD* screening using RBC-FluoGene Dweak/variant and RBC-FluoGene CDE according to the manufacturer's instructions (inno-train, Kronberg, Germany). We recommend antenatal prophylaxis to women with a D variant other than weak D types 1, 2 or 3. Thus, identification of the D variants helps to manage these women correctly.

2.6 | Anti-D immunoglobulin: dose and timing

Antenatal anti-D Ig has in a number of clinical studies proved to reduce the incidence of RhD alloimmunization significantly.[12,13] Either a single dose of 1250–1500 IU anti-D Ig at GWs 28–30[14-20] or two doses of 500–625 IU at GW 28 and GW 34 are used in different countries.[14,17,19] The Norwegian guidelines recommend a single dose of 1500 IU at GW 28.[4] This approach is consistent with the majority of the above-mentioned guidelines. A two-dose regimen is less cost-effective and has shown lower compliance.[14]

Alloimmunization that may occur between GW 24 and GW 28 is of concern, as we do not perform antibody screen beyond GW 24. The seroconversion rate before GW 28 is reported to be low (0.099%) with minimal risk for the fetus during the index pregnancy.[21] Extrapolation to the Norwegian population will mean eight RhD alloimmunizations per year. Severe HDFN after seroconversion, also in primigravidae, is reported as well.[22] Although the interval between GW 24 and GW 28 is at present necessary to perform the fetal *RHD* genotyping, reporting the result and scheduling prophylaxis administration, a fully electronic reporting system would shorten the interval. Antibody screening can then be performed even closer to GW 28. However, our routine is in concordance with the other guidelines that recommend antibody screening at GW 24–28.[14,17,19]

Our second concern is ensuring adequate and protective anti-D Ig levels during the whole last trimester, especially in the last weeks of pregnancy. No current guidelines recommend a second antenatal dose, when the routine prophylaxis is given no earlier than GW 28.[14,17,19] Post-term pregnancy is a risk factor for RhD alloimmunization, probably due to insufficient levels of anti-D Ig.[23] However, despite low anti-D Ig levels at birth, RAADP is shown to reduce the number of alloimmunizations and HDFN.[13,20] Additional studies are needed in order to determine whether a repeat dose of anti-D Ig should be given in the last weeks of pregnancy or in case the woman has not given birth within 12 weeks after antenatal prophylaxis.

2.7 | Costs

Targeted RAADP avoids unnecessary administration of anti-D Ig to 40% of RhD-negative pregnant women with RhD-negative fetuses, corresponding to more than 3000 RhD-negative women annually in the Norwegian setting. Anti-D Ig is a limited resource and the use of a product of human origin should be limited to women who will benefit from it.

As part of the HTA (Figure 1), a cost-effectiveness analysis (CEA) was performed to estimate the health economic consequences of introducing RAADP.[6] Post-natal prophylaxis only (the current routine back in 2014) was compared with universal antenatal anti-D prophylaxis and NIPT-guided targeted antenatal prophylaxis. The evaluated health effect was the incidence of RhD immunisation. The CEA was based on a Swedish cohort study that demonstrated a relative risk of 0.55 (95% confidence interval, 0.32–0.87) for RhD immunisation with NIPT-guided prophylaxis versus only post-natal prophylaxis.[12] Screening of all pregnant RhD-negative women with NIPT and targeted anti-D prophylaxis gives an additional annual cost of ~4.5 million NOK[1], while administration of prophylaxis to all RhD-negative women without NIPT gives an additional annual cost of ~4 million NOK,[‡‡] corresponding to EUR 450000/USD 540000 and EUR 400000/USD 480000, respectively.[6] Since the difference is acceptable, the RAADP programme is considered to be cost-effective. The estimated cost per avoided RhD immunisation (Incremental Cost-Effectiveness Ratio [ICER]) is approximately 122 000 NOK[1] (EUR 12000/USD 15000). Additionally, the sensitivity of the test and compliance to the

[‡‡]The amounts from the HTA are indexed from 2014-monetary value to 2020-monetary value, using the retail price index (RPI) (Statistics Norway).

programme will have an impact on the total cost of the programme; the higher the sensitivity and compliance, the lower the costs will be. A strategic choice we made to reduce costly elements has been collecting samples for fetal *RHD* genotyping and administration of anti-D Ig at routine appointments at GWs 24 and 28.

We plan of terminating newborn RhD testing like in Denmark and the Netherlands, when we are confident of the accuracy of the test results at all sites.[9] Routines are simplified when post-natal prophylaxis can be guided by the fetal *RHD* result and eliminates the cost of serologic RhD typing of the newborns.

2.8 | Quality registry

A national quality registry to monitor the outcomes of the targeted RAADP programme, including the effect on the number of RhD alloimmunizations, is currently not established mainly due to privacy policy regulations. Therefore, all local transfusion services and especially those performing antenatal *RHD* genotyping have been strongly encouraged to establish local registries. To ensure accuracy and high sensitivity of fetal *RHD* determination, the predicted fetal RhD type should be compared with the newborn's RhD type during the initial phase of the targeted RAADP. All maternity wards and local transfusion services have been instructed to report discrepancies. The samples from the mother and the newborn are extendedly investigated at the Advisory Unit to elucidate the reason of the discrepancy.

2.9 | The Medical Birth Registry of Norway

The Medical Birth Registry of Norway collects data about all pregnancies and births for research and analysis purposes. During 2020, additional information about RhD negative pregnancies was incorporated. Even though less detailed than the local registries, the result of antibody screen, predicted fetal RhD type, newborn's RhD type and whether antenatal prophylaxis is administered when indicated have been included. These data will give a national overview to evaluate whether the RAADP programme reduces the number of pregnancy-related RhD alloimmunizations.

2.10 | External quality assessment of fetal *RHD* genotyping

The Advisory Unit has initiated a national external quality assessment workshop for the four laboratories performing the fetal *RHD* genotyping. The aim is to assure the quality of the testing, in addition to correct and uniform recommendations about anti-D prophylaxis. All four laboratories also participate in the quality assessment organised by Clausen et al[24] and have correctly reported results and recommendations.

2.11 | Sample uptake

The aim of the RAADP programme is to reach all RhD negative pregnant women. Table 1 shows the number of RhD negative pregnant

TABLE 1 The number of women with expected date of delivery and a fetal *RHD* screen performed in years 2017–2019 and the calculated sample uptake (compliance) in all four health regions

Estimated date of delivery (year)	Number of fetal *RHD* typings	Number of deliveries[7]	Estimated number of RhD-negative pregnant women[b]	Compliance (%)
Health Region of South-Eastern Norway				
2017	3456	31 256	4688	74
2018	3964	30 727	4609	86
2019	4021	30 584	4343	88
Health Region of Northern Norway				
2017	n.a.			
2018	407	3243[a]	405	100
2019	541	4433	554	98
Health Region of Western Norway				
2017	n.a.			
2018	1229	8645[a]	1297	95
2019	1692	11 941	1791	95
Health Region of Central Norway				
2017	754	7561	1134	67
2018	1004	7283	1092	92
2019	972	7201	1080	90

Abbreviation: n.a. not assessed.

[a]This number is adjusted to 8.5 months, due to registration of fetal *RHD* typing started on 1 January 2018.

[b]To calculate the number of RhD negative pregnant women, a frequency of 15% RhD negative is used, except for Health Region of Northern Norway where the frequency is 12.5%, as shown in the Blood Bank data.

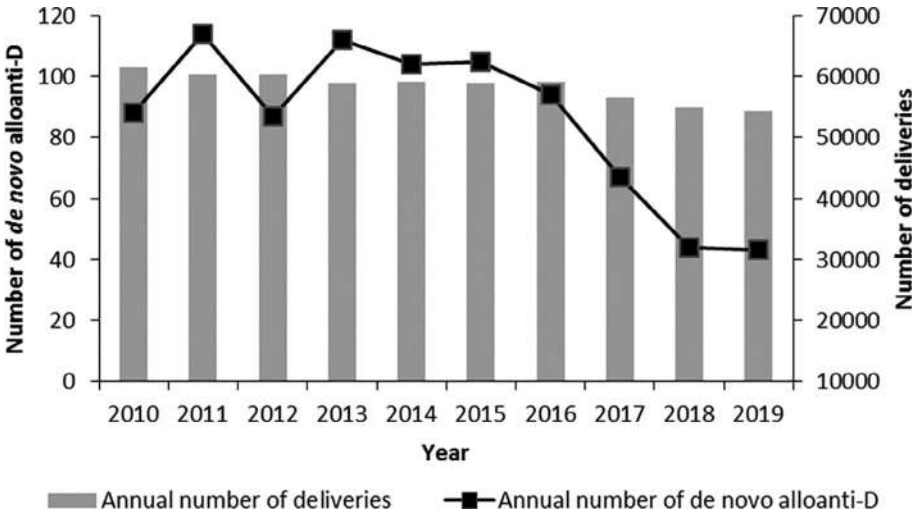

FIGURE 3 The number of de novo alloanti-D detected during pregnancy[25] and the total number of deliveries 2010–2019[7]

women who were screened for fetal *RHD* genotype in different health regions and who had an expected date of delivery in years 2017, 2018 and 2019. The number of RhD negative women screened was compared to the estimated number of RhD negative pregnant women based on the number of deliveries.[7] The sample uptake (Table 1) in the Health Regions of South Eastern and Central Norway is similar to the Danish (84%–90%) shortly after launch, while Health Regions of Western and Northern Norway rapidly achieved results closer to what was obtained in the Netherlands, Finland and Sweden (>98%).[9] A possible explanation may be better cooperation and improved information flow in these regions. Electronic referral forms and reports are implemented at the test sites in West and North Norway, while the Central region uses electronic reporting to a great extent. The test site in South East uses paper-based referral forms and reports.

2.12 | The number of de novo anti-D detected during pregnancy

All transfusion laboratories in Norway annually report the number of de novo alloanti-D detected during pregnancy anonymously.[25] The numbers should be interpreted with caution regarding possible double registration of the same patient from different hospitals and probable reporting of anti-D due to prophylaxis as alloanti-D. However, a substantial decrease in the number of de novo alloanti-D is observed since 2018 (Figure 3). Whether the RAADP programme has had an impact here is too early to conclude, but it is promising.

3 | CONCLUSION

The implementation of targeted RAADP has been effectively conducted in the maternity care and the transfusion services. Administration of anti-D Ig guided by fetal *RHD* genotyping avoids administration of unnecessary prophylaxis to about 40% RhD negative pregnant women with RhD negative fetuses. Compliance with the programme is essential for the outcome; from uptake of samples to administration of the prophylaxis and should be as close as possible to 100%. Therefore, the lower sample uptake observed in two of the health regions will be followed closely.

The National Working Group and the transfusion services have played an important role during the implementation of the RAADP programme and will continue the surveillance of the programme and will be involved in potential improvements. The evaluation of the screening accuracy in all health regions has to be fulfilled to discontinue routine newborn RhD typing. Finally, data from dedicated registries will show the success of the targeted RAADP programme; expectedly fewer RhD immunisations and decreased number of HDFN. Promising tendency with reduced number of de novo anti-D detected during pregnancy is already observed.

CONFLICT OF INTEREST

None of the authors have conflict of interest related to the manuscript.

AUTHOR CONTRIBUTIONS

Kirsten Sørensen, Geir Tomter, Abid Hussain Llohn, Kristin Gjerde Hagen, Aurora Espinosa, Mirajana Grujic Arsenovic, Aud Norunn Ulvahaug, Tatjana Sundic and Cigdem Akalin Akkøk designed the study. Kirsten Sørensen, Mette Bævre, Geir Tomter, Abid Hussain Llohn, Ingvild Hausberg Sørvoll, Aud Norunn Ulvahaug and Barbora Jacobsen contributed with essential data. Kirsten Sørensen, Abid Hussain Llohn and Ingvild Hausberg Sørvoll analysed the data. Kirsten Sørensen and Cigdem Akalin Akkøk wrote the manuscript. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

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An assessment of the management of anaemia in acute care settings in the United Kingdom: The value of a collaborative approach

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Abstract

Objectives: Patients presenting to acute care settings with anaemia are at risk of inadequate investigation and inappropriate blood transfusion. In collaboration with Haematology Specialty Trainee Audit and Research (HaemSTAR), this study set out to assess current red blood cell (RBC) transfusion practice and anaemia management in acute care settings across the United Kingdom.

Methods and Results: Fifteen different hospitals participated in the study over a period of a month beginning 01 January 2020. Eight-hundred and twenty-eight eligible patients presenting to acute care settings with anaemia received RBC transfusions during this period. Of these, 159 (19.2%) received inappropriate transfusions according to National Institute for Health and Care Excellence guidelines, and 257 (31%) could have been treated with alternatives to transfusion. One-hundred and fifty-four (18.6%) did not have a cause for their anaemia identified by the time they were discharged from hospital, and in over 50% of these cases that was because of inadequate investigation with blood tests, specialist investigation or referral, or both.

Conclusion: This study found that the appropriateness of transfusion and investigation of anaemia in acute care settings warrant improvement and also demonstrates the value of HaemSTAR in facilitating time-efficient collection of high-quality data.

KEYWORDS
acute care, anaemia, HaemSTAR, iron deficiency, red blood cells, transfusion

1 | INTRODUCTION

Red blood cell (RBC) transfusion is one of the most common procedures performed in hospitals and can be lifesaving. Despite significant improvements over the past decades in transfusion safety, transfusion is still associated with appreciable risks of adverse events.[1] Evidence-based guidelines from the National Institute for Health and Care Excellence (NICE) aim to avoid unnecessary blood transfusions and their associated risks and make best use of the blood supply.[2] However, studies show that in both emergency department (ED) and adult inpatient settings, iron deficiency anaemia (IDA) remains under recognised, inappropriately transfused, and inadequately treated with iron therapy.[3]

Inadequate investigation of the cause of anaemia and the avoidance of unnecessary blood transfusions are issues particularly pertinent to acute care settings, where there is often great pressure to treat patients quickly so that they can continue their journey through the hospital or be discharged. These patients are at risk of important diagnoses being missed and of exposure to the risks associated with blood transfusion.

Alexandros Rampotas and Catherine F. Prodger are joint first authors.

Khadadah et al[3] showed that implementing quality improvement interventions significantly improved the appropriateness of RBC transfusions in an ED setting, and a pilot study in Oxford suggested that similar such interventions could be beneficial in the United Kingdom.[4] In collaboration with HaemSTAR, a UK-wide network of haematology trainees that co-ordinates non-malignant haematology research, this study set out to assess current RBC transfusion practice and anaemia management in acute care settings in hospitals across the United Kingdom.

2 | METHODS

2.1 | Study design

This was a nationwide observational study designed with the aims of assessing the appropriateness of RBC transfusions administered in acute care settings, assessing the adequacy of investigation of anaemia in patients transfused with RBCs in acute care settings, and quantifying the number of patients with incompletely investigated anaemia who received transfusions on more than one occasion within 6 months on either side of the index transfusion encounter.

2.2 | Eligibility

Patients were eligible for inclusion in this study if they were aged 18 or older and had received an RBC transfusion in an acute care setting between I and 31 January 2019. The following were considered acute care areas: EDs, acute medical units, ambulatory assessment units, surgical emergency units, and short stay units.

2.3 | Categorisation of eligible patients

The blood transfusion laboratory information systems of participating hospitals were interrogated to identify patients who had received RBC transfusions during the relevant time period. Analysis of electronic patient records allowed eligible patients to be categorised into one of the following five groups: *substantial bleed*; *non-substantial bleed*; *pre-existing diagnosis* that explained their anaemia, such as haematology patients and oncology patients receiving chemotherapy; *cause for anaemia identified* during admission; and *no known cause for anaemia* at the time of discharge from hospital.

For the purpose of this study, a substantial bleed was defined as a history of bleeding requiring four or more units of red cells over 72 h. A history of bleeding but requiring less than four units of red cells over 72 h was considered a non-substantial bleed.

Patients could not be placed in more than one group. Bleeding was excluded as a cause for the third and fourth group.

2.4 | Assessment of anaemia

A list of the investigations expected for a patient presenting with anaemia requiring RBC transfusion in order for them to be considered adequately investigated was compiled using NICE recommendations[2,4,5] and guidelines from the Northern Ireland Transfusion Commitee[6] (Table). In addition to further blood tests, specialist referral or investigation was also required if appropriate. For example, if further blood tests confirmed IDA, referral to gastroenterology, endoscopy, or other relevant clinical specialty was required to determine its cause, or alternatively documentation was required about why further investigation was not carried out. Specialist blood tests, such as investigation for haemolysis, were not included, as they would be part of investigations carried out after referral to a haematologist.

Once a diagnosis of the cause of the anaemia had been made, it was considered reasonable to stop investigations, so a patient did not have to have had all the blood tests and specialist referral or investigations performed in order to be considered adequately investigated.

An assessment was also made as to whether or not each blood transfusion had been administered appropriately, with appropriateness defined as an indication provided in NICE guidelines[2]; acute bleeding, regardless of whether substantial or non-substantial; haemoglobin (Hb) <70 g/L; Hb <80 g/L in patients with acute coronary syndrome; and the presence of symptoms in patients with chronic anaemia.

For those patients who had no known cause for their anaemia at the time of discharge from hospital, further analysis of their records was performed to identify any additional RBC transfusions administered in the 6 months before and after this index transfusion encounter. We hypothesised that the repeat transfusion(s) might have been avoided had the cause of anaemia been identified and treated directly at their first transfusion encounter.

2.5 | HaemSTAR

This study was a collaboration with HaemSTAR. It was advertised to its trainee haematologists from each of the seven Health Education England regions, Northern Ireland, Scotland, and Wales through a monthly newsletter and via its website. This study was one of four that HaemSTAR was involved in at the time. Fifteen hospitals contributed data, including both teaching hospitals and district general hospitals.

All patient data were anonymised at source and treated according to the principles of the Declaration of Helsinki and the UK Data Protection Act (1998).

The study received service evaluation approval at each participating site. Anonymised data were collected and analysed retrospectively. Transfusion data were obtained from the hospital blood transfusion laboratories, and administration of blood components was confirmed by manually reviewing electronic or paper records.

TABLE 1 Investigations expected for a patient with anaemia

	Microcytic anaemia	Normocytic anaemia	Macrocytic anaemia
Investigations	Blood film Iron studies, including ferritin and transferrin saturations CRP Urea, creatinine, eGFR	Blood film Iron studies, including ferritin and transferrin saturations CRP Urea, creatinine, eGFR B12 and folate	Blood film Urea, creatinine, eGFR B12 and folate LFTs

Abbreviations: B12, vitamin B12; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate (calculated using the abbreviated MDRD equation); LFTs, liver function tests.

TABLE 2 Use of transfusions and causes of anaemia in all patients

	N = 828
All transfusions	
Age in years (median, IQR)[a]	74 (62–86)
Male	374 (51.6%)
Female	351 (48.4%)
Cause of anaemia	
Substantial bleeding	116 (14%)
Non-substantial bleeding	206 (24.9%)
Other known cause	169 (20.4%)
Cause identified	179 (21.6%)
Unknown cause	154 (18.6%)
Not anaemic	4 (0.5%)
Anaemia characteristics[b]	
Pre-transfusion Hb in g/L (median, IQR)	71 (62–80)
MCV in fl (median, IQR)	89.6 (IQR: 89.6–95.8)
Microcytic	521 (63.1%)
Normocytic	225 (27.3%)
Macrocytic	79 (9.6%)
Appropriate transfusion	
Yes	669 (80.8%)
No	159 (19.2%)
Alternatives to transfusion	
Yes	257 (31%)
No	571 (69%)

Abbreviations: Hb, haemoglobin; IQR, interquartile range; MVC, mean cell volume.
[a]Age and Sex data were not available for 103 patients due to local audit policy restrictions.
[b]The pre-transfusion MCV was unknown in three patients.

3 | RESULTS

A total of 828 patients met the eligibility criteria for the study (Table 2). There were 351 female and 374 male patients, while for 103 patients there were no age and sex data due to local audit policy restrictions on the provision of such data for the study. The patients had a median age of 74 (interquartile range [IQR]: 62–86). The cause of the anaemia was identified in 491 (59.3%) patients: substantial bleeding 116 (14%), non-substantial bleeding 206 (24.9%), other known cause 169 (20.4%). There were 333 (18.6%) patients for whom the cause of the anaemia was identified later during the admission, and 154 (18.6%) where it remained unknown, while 4 (0.5%) patients were transfused without being anaemic. The median Hb at the time of presentation in an acute care setting was 72 g/L (IQR: 60–86) with a median MCV of 89.6 fl (IQR: 89.6–95.8). In 669 of the 828 (80.8%) patients, transfusions were appropriate, but in 159 patients (19.2%) they were inappropriate according to the NICE guidelines.[2] In 257 (31%) patients there were alternatives to transfusion that could have been used. One-hundred and seventy (66%) of these patients were appropriately transfused and so the alternative treatment could have been given alongside RBC transfusion. Eighty-seven (34%) patients were inappropriately transfused and could have avoided RBC transfusion altogether had the alternative treatment been used. Of the 154 patients in whom the cause of anaemia was unknown at the time of discharge, 53 (34%) either received further transfusions in the following 6 months or had been transfused for anaemia that was inadequately investigated in the preceding 6 months.

Out of the 159 patients who had inappropriate transfusions, the cause of the anaemia was already known in 44 (27.7%) from the patient history. In the majority (112/159, 70.4%) of the remaining patients, the cause was unknown at the time of the transfusion (Table 3). It was identified during admission in 63 of these patients but remained unknown in 49 patients. The remaining three patients who received RBCs inappropriately were transfused inappropriately large numbers of units of RBCs given their clinical histories and Hb drops. One of these patients was transfused four units of RBCs for epistaxis which resolved soon after presentation. In 87 of 159 (54.7%) patients, there were alternatives to transfusion that could have been given.

Out of 333 patients where the cause of the anaemia was unknown, only 145 (43.5%) were investigated adequately (Table 4). From the 188 (56.5%) patients who were not adequately investigated, 136 (72.3%) did not have all the recommended blood tests, 117 (62.2%) were not referred to a specialist for investigation, and 65 (34.6%) had neither adequate blood test investigations nor a referral to an appropriate specialist. The median Hb was 69 (IQR 60–77) and the median MCV 89.3 (IQR 78–96.5), with most patients being normocytic 190 (59%), followed by microcytic 105 (31.5%) and macrocytic 38 (11.4%).

4 | DISCUSSION

Patients presenting to acute care settings with anaemia remain at risk of inappropriate blood transfusion and inadequate investigation of their anaemia despite the availability of practice guidelines and the ready accessibility of inexpensive and safe alternative treatments to blood transfusion such as iron replacement.

Although there are a number of well-established initiatives in place to enforce restrictive transfusion practices and reduce unnecessary blood component usage, this study found that nearly 20% of patients treated with RBC transfusions received them inappropriately and that in the majority of these cases patients had anaemia of unknown cause. We hypothesise that pressure to rapidly manage patients in acute care settings results in inappropriate transfusion, inadequate investigation of anaemia, and underuse of alternatives to transfusion such as iron therapy for IDA. Although Khadadah et al[3] showed that educational presentations and the introduction of an algorithm for IDA management in the ED improved RBC transfusion appropriateness and the use of intravenous iron as an alternative to transfusion, other studies have shown that educational initiatives do not result in sustained changes to practice,[7] perhaps as a result of frequently rotating clinical staff. This emphasises the importance of continuous education of healthcare professionals regarding the appropriate investigation of anaemia and indications for transfusion as a strategy to minimise unnecessary exposure to blood components and their associated risks. Bedside electronic transfusion systems have been successful in reducing transfusion errors.[8] Similarly, electronic clinical decision support algorithms integrated with electronic patient records have been successful in reducing the inappropriate use of blood.[9] A similar IT-supported strategy for alerting clinicians to the appropriate investigation of anaemia could be effective, for example, by suggesting a 'toolkit' of potentially relevant investigations for anaemia from which the user can choose those pertinent to their particular clinical scenario and type of anaemia.

This study also demonstrated that 19% of patients transfused for anaemia in acute care settings did not have the cause of their anaemia identified by the time they were discharged from hospital. One in three of these patients required further potentially avoidable transfusions, while over half of these patients were not fully investigated for the cause of their anaemia. In these incompletely investigated patients, a third had neither all appropriate blood tests nor an

TABLE 3 Transfusions and causes of anaemia in patients who were inappropriately transfused

	***N* = 159**
Inappropriate transfusions	
Age in years (median, IQR)[a]	79 (69–86)
Male[a]	67
Female[a] (6)	86
Cause of anaemia	
Substantial bleeding	0
Non-substantial bleeding	3 (2.5%)
Other known cause	44 (27.7%)
Cause identified	49 (30.8%)
Unknown cause	63 (39.6%)
Not anaemic	0
Anaemia characteristics[b]	Overall (*N* = 828)
Pre-transfusion Hb in g/L (median, IQR)	78 (73–85)
MCV in fl (median, IQR)	89.9 (81–94.2)
Microcytic	106 (66.7%)
Normocytic	44 (27.7%)
Macrocytic	9 (5.6%)
Alternatives to transfusion	
Yes	87 (54.7%)
No	72 (45.2%)

Abbreviations: Hb, haemoglobin; IQR, interquartile range; MVC, mean cell volume.
[a,b]Number of unavailable age, sex andpre-transfusion MCV data is unknown for this subset of results.

TABLE 4 Investigation and characteristics of anaemia of unknown cause

Unknown cause of anaemia or where the cause was identified post transfusion	***N* = 333**	**Further transfusions**	**Investigations missed**
Appropriate investigation of anaemia	145 (43.5%)	Yes 50 (34.5%)	
		No 95 (65.5%)	
Inappropriate investigation of anaemia	188 (56.5%)	Yes 69 (36.7%)	Blood tests 136 (72.3%)
		No 119 (63.3%)	Specialist referral 117 (62.2%)
			Both 65 (34.6%)
Pre-transfusion Hb in g/L (median, IQR)	69 (60–77)		
MCV in fl (median, IQR)	89.3 (78–96.5)		
Microcytic	105 (31.5%)		
Normocytic	190 (59%)		
Macrocytic	38 (11.4%)		

Abbreviations: IQR, interquartile range.

appropriate specialist referral. Incomplete investigation of anaemia risks important and potentially treatable underlying diagnoses, such as gastrointestinal and gynaecological malignancies and peptic ulcer disease, being missed. Late diagnosis of malignancies is associated with poor survival, avoidable deaths, and more complications from treatment.[10] There are a number of possible explanations for these findings. Time pressures and the introduction of the 4-h target in EDs in the United Kingdom may mean that an incidental finding of anaemia is overlooked while more pressing complaints are dealt with. If investigation of anaemia is initiated in the ED, there may not be time to wait for the results of additional blood tests such as haematinics and blood films before the patient breaches the 4-h target, possibly encouraging a tendency towards treating anaemia with red cell transfusion for ease and convenience. In addition, by their very nature as acute care areas, patients tend not to stay in these settings for very long and are either discharged back to the care of their general practitioner or admitted under a specialty team. Robust systems need to be in place to ensure appropriate follow-up of any blood tests taken by ED but not reported by the time the patient leaves hospital or is admitted under the care of a different team.

This project did not collect data on why patients were inappropriately transfused and inadequately investigated, but it is important to understand the reasons when thinking of strategies to improve anaemia management. Some electronic blood transfusion systems require the user to enter an explanation before proceeding with an order that falls outside agreed transfusion guidelines, and analysing these explanations may identify targets to improve transfusion appropriateness. It has also been shown that using electronic blood transfusion systems to identify blood component requests that fall outside of guidelines and provide education directly to the clinical teams placing the order reduces blood component usage.[9]

This study was conducted across the United Kingdom and demonstrates the value of collaborative research. HaemSTAR enabled the original hypothesis, generated from a small study in Oxford, to be tested on a large number of patients from different trusts with different local guidelines. It is therefore a true representation of UK practice. What would have been a logistically challenging and labour-intensive process for one person became small and straightforward when divided among many. The period for data collection can therefore be relatively short, allowing HaemSTAR to undertake a number of such studies each year. There were only minor communication issues, but overall this study highlights the feasibility and value of such a network to conduct a clinical study in a large number of hospitals.

5 | CONCLUSION

This study highlights anaemia management as an area of clinical practice in need of improvement by showing that patients presenting to acute care areas with anaemia are at risk of both inappropriate transfusion and inadequate investigation. Within this population, those patients with anaemia of unknown aetiology are of particular interest as they are most likely to receive blood transfusions unnecessarily and also most at risk of important underlying diagnoses, such as malignancy, being missed or delayed. While this study is limited by its retrospective nature, it does nonetheless contribute valuable data and may be helpful in guiding further studies in this field.

CONFLICTS OF INTEREST

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

This study was designed by Prodger and Murphy. Collection of data was coordinated by Rampotas and facilitated by HaemSTAR. Rampotas analysed the data and the manuscript was written by Prodger, Rampotas, and Murphy.

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APPENDIX

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ORIGINAL ARTICLE

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The effect of non-point-of-care haemostasis management protocol implementation in cardiac surgery: A systematic review

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Abstract

Objectives: This systematic review aims to outline the evidence on the implementation of a non-point-of-care (non-point-of-care [POC]) haemostasis management protocol compared to experience-based practice in adult cardiac surgery.

Background: Management of coagulopathy in cardiac surgery is complex and remains highly variable among centres and physicians. Although various guidelines recommend the implementation of a transfusion protocol, the literature on this topic has never been systematically reviewed.

Methods: PubMed, Embase, Cochrane Library, and Web of Science were searched from January 2000 till May 2020.

Results: A total of seven studies (one randomised controlled trial [RCT], one prospective cohort study, and five retrospective studies) met the inclusion criteria. Among the six non-randomised, controlled studies, the risk of bias was determined to be serious to critical, and the one RCT was determined to have a high risk of bias. Five studies showed a significant reduction in red blood cells, fresh frozen plasma, and/or platelet transfusion after the implementation of a structural non-POC algorithm, ranging from 2% to 28%, 2% to 19.5%, and 7% to17%, respectively. One study found that fewer patients required transfusion of any blood component in the protocol group. Another study had reported a significantly increased transfusion rate of platelet concentrate in the haemostasis algorithm group.

Conclusion: Owing to the high heterogeneity and a substantial risk of bias of the included studies, no conclusion can be drawn on the additive value of the implementation of a cardiac-surgery-specific non-POC transfusion and haemostasis management algorithm compared to experience-based practice. To define the exact impact of a transfusion protocol on blood product transfusion, bleeding, and adverse events, well-designed prospective clinical trials are required.

KEYWORDS
cardiac surgery, haemostasis, protocol, transfusion

1 | INTRODUCTION

Cardiac surgery is associated with major blood loss and the subsequent need for allogeneic blood transfusion. The origin of coagulopathy is multifactorial, owing to the invasiveness of the procedure, use of anticoagulants, and exposure to the extracorporeal bypass circuit.[1] This makes management of coagulopathy complex in this setting. Although patient blood management has greatly improved over the last decades,[2] there still remains wide variation in transfusion rates among different centres.[3–5] An explanation might be the differences in transfusion practices among institutions and physicians.

To overcome this heterogeneity in practice, various guidelines support the use of a haemostasis algorithm for the management of non-surgical (i.e., coagulopathic) bleeding, aiming to improve outcome.[6–8] This algorithmic approach can be guided by point-of-care (POC) haemostasis monitoring (e.g., TEG®, ROTEM®, Multiplate®, or VerifyNow®) to identify the underlying cause of bleeding. In the last decades, much emphasis has been placed on the use of these devices in cardiac surgery. While the first studies showed impressive results, more recent data indicate reduced benefit from the implementation of POC coagulation management.[9–11] In many studies, these devices were implemented in combination with a structural haemostasis management protocol, leading to the investigation of two interventions in the study group, which might bias the results.[12–16]

We hypothesised that the implementation of a structural non-POC haemostasis management protocol by itself would reduce bleeding and transfusion compared to experience-based practice. Therefore, we performed a systematic review of the literature to investigate the effect of the implementation of a non-POC-based haemostasis management protocol on blood components transfusion in adult cardiac surgery.

2 | METHOD

The systematic review was performed in accordance with the recommendation for systematic reviews[17] and the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) method. PubMed, Embase, Wiley/Cochrane Library, and Clarivate Analytics/Web of Science Core Collection were searched from inception until 6 May 2020 (R. B., J. C. F. K., and M. M.). Search strategies were developed specifically for each database. The following question was the fundamental for the literature search: 'Does the implementation of a non-POC guided haemostasis management protocol lead to a reduction in transfusion in cardiac surgery?'[18]

Participants undergoing cardiothoracic surgical procedures with or without cardiopulmonary bypass were considered eligible. Randomised controlled trials (RCTs), retrospective cohort studies, and matched case–control studies were included when evaluating the effect of transfusion requirements after the implementation of a non-POC guided haemostasis management protocol compared to the clinician's judgement with or without the guidance of conventional coagulation tests. Conventional coagulation tests included the following: prothrombin time (PT), activated partial thromboplastin time, activated clotting time, fibrinogen, and thrombocyte count. In line with the current European guideline on haemostasis and transfusion in cardiac surgery,[7] only studies published from 2000 onwards were considered eligible, as patient blood management strategies, surgical techniques, and cardiopulmonary bypass practice before 2000 differ greatly from current practice. We excluded case reports, non-English language, animal studies, use of (POC) haemostasis monitoring (e.g., TEG®, ROTEM®, Multiplate®, or VerifyNow®), and studies including patients below 18 years of age. PubMed, Embase, Wiley/Cochrane Library, and Clarivate Analytics/Web of Science Core Collection were searched from inception until 6 May 2020 (R. B., J. C. F. K., and M. M.), using thesaurus for cardiothoracic surgery, algorithm/protocol, and bleeding/transfusion. Data S1 shows the full search strategy per database.

Two reviewers (R. B. and R. G.) independently screened the titles retrieved from the search for potential eligibility. The selected titles were merged, duplicates were removed, and the subsequent results were further screened by abstract. This was repeated after the abstract selection, leading to the full text selection. The subsequent papers were read and, when relevant, included in the final selection. The references of all included papers were also screened for possible eligibility.

Two authors (R. B. and C. B.) independently assessed the risk of bias using the Cochrane Collaborations Risk of Bias Tool for Randomised Trials[19] and Risk of Bias in Non-randomised Studies of Interventions for prospective and retrospective studies.[20] If additional information was required for the systematic review, the authors of the included studies were requested to provide this information. A standardised form was used to extract data from the included studies for assessment of study quality and evidence synthesis. Extracted information included the following variables: year of publication, study design, sample size, type of surgery, blood component transfusion rate, in-hospital mortality, chest tube drainage, rethoracotomy, and information for assessment of the risk of bias. Data extraction forms were completed by one author (R. B.) and checked by another (M. M.).

The primary outcome included the proportion of patients transfused with allogeneic blood, including red blood cell (RBC), fresh frozen plasma (FFP), and platelet (PLT) concentrates. The secondary outcomes were adverse events, including in-hospital mortality, chest tube drainage, and rethoracotomy. Data collection included author, publication date, study design, participants, type of operation, blood product transfusion rate, in-hospital mortality, chest tube drainage, and rethoracotomy.

3 | RESULTS

3.1 | Patient characteristics

After the selection process, seven publications were identified investigating a non-POC-guided haemostasis management protocol in cardiac surgery compared to experience-based practice

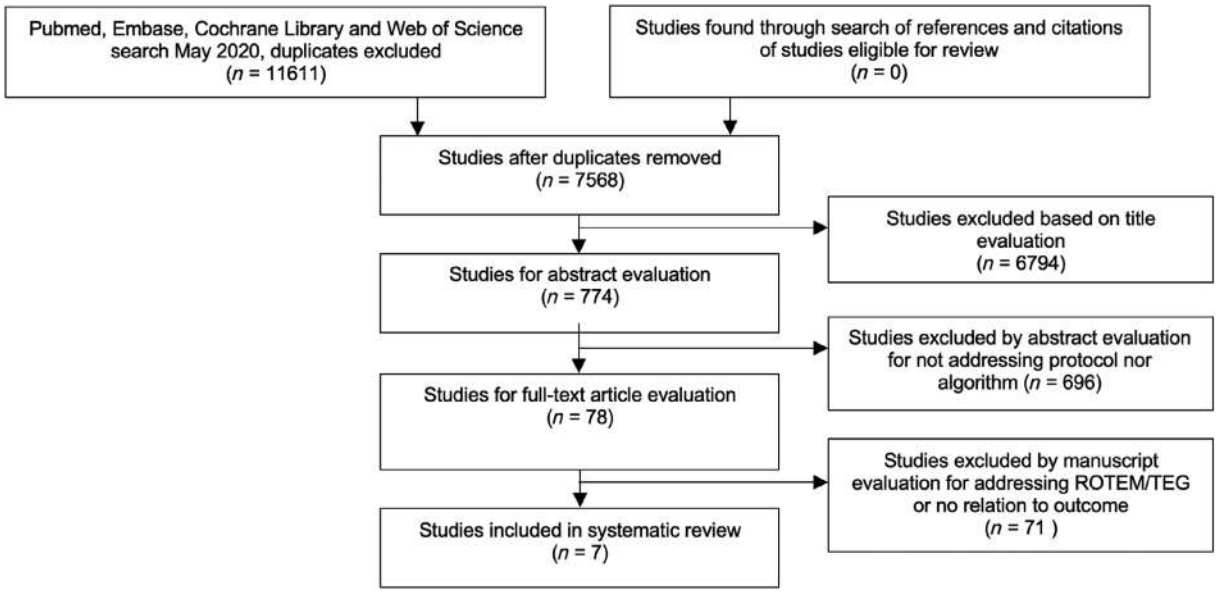

FIGURE 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses flow diagram

TABLE 1 Details of the randomised studies

				Transfusion and haemostasis management	
Study	**Design**	***n***	**Population**	**Control group**	**Intervention group**
Capraro et al.[21]	RCT	58	Mixed elective cardiac surgery, bleeding >1.5 ml/kg 15 min after first mediastinal drains emptying	Conventional coagulation tests were prohibited and only ACT after heparin neutralisation was performed	Conventional coagulation tests: thrombocyte count, PT, aPTT, ACT
				Transfusion based on clinical discretion No transfusion triggers reported	Transfusion according to an algorithm with sequential order of treatment modalities during the immediate recovery period (1 h after surgery): Step 1: Hb <90 g/L: 1 unit of RBC and new haemoglobin measurement before each RBC unit Step 2: Thrombo <100 × 10^9/L: 1 unit of PLT/10 kg, round up to the nearest full 4 units Step 3: aPTT or PT 1.5× normal value: FFP 10 ml/kg Step 4: ACT >10 s than preoperative ACT: protamine 0.5 mg/kg Step 5: Bleeding time > 12 min: DDAVP 0.3 μg/kg Step 6: Normal values in all previous tests: tranexamic acid 10 mg/kg

Abbreviations: ACT, activated clotting time; aPTT, activated partial thromboplastin time; DDAVP, desmopressin; FFP, fresh frozen plasma; Hb, haemoglobin; PLT, platelet; PT, prothrombin time; RBC, red blood cells; RCT, randomised controlled trial.

(Figure 1).[21–27] In total, 8555 patients were included in this systematic review. The study populations included mixed cardiac surgery,[22,23,26] isolated coronary artery bypass graft (CABG) surgery,[27] pulmonary endarterectomy,[25] and cardiac surgery patients with excessive blood loss.[21,24] Details concerning the transfusion and haemostasis management practice in the control group and intervention group of the included studies are reported in Tables 1 and 2.

3.2 | Study characteristics

The final selection included one RCT, one prospective cohort study, and five retrospective cohort studies.[21–27] The one RCT was determined to have a high risk of bias. Among the six non-randomised controlled studies, the risk of bias was determined to be serious to critical, as shown in Table 3. A detailed assessment of the risk of bias is available in Data S2.

TABLE 2 Details of the non-randomised studies included in the systematic review

Study	Design	*n*	Population	Transfusion and haemostasis management	
				Control group	**Intervention group**
Bilecen et al.[22]	RC	5219	Mixed cardiac surgery	Conventional coagulation test: thrombocyte count Transfusion based on the discretion of the anesthesiologist Transfusion triggers reported: • RBC transfusion: Hb < 4.0 mmol/L in healthy normovolemic patients, blood loss from one locus (age 60 year) Hb < 5.0 mmol/L in healthy normovolemic patients, blood loss form one locus (age > 60 years) or multiple loci (age < 60 years) Hb < 6.0 mmol/L patients with severe heart or lung disease (age not relevant) • PLT transfusion: thrombocyte count <100 × 10^9 • FFP transfusion trigger was not clear	Conventional coagulation tests: thrombocyte count, aPTT, PT, fibrinogen, ACT, Ca^{2+} Implementing cell saver blood in the decision to transfuse blood products Transfusion according to an algorithm with sequential order of treatment modalities: Step 1: Start surgery: Heparin initial dose (4 mg/kg) and tranexamic acid 2 g Step 2: Pre-end CPB: • Hb < 5.0 mmoL/L, Hct < 0.23: 1 unit of RBC • Thrombo <80 × 10^9/L: 1 unit of PLT • Plasma loss >1 L: 2 units of FFP • Plasma loss >2 L: 4 units of FFP • Loss >50% circ. vol.: 4 units of FFP • Fibrinogen <1.2 g/L: 4 units of FFP • DDAVP 0.3 μg/kg, if ≥1 factor present: anti-PLT therapy, Ao stenosis surgery, CPB time >180 min or • urgent/emergent procedure Step 3: Post-CPB • Tranexamic acid 1 g • Microvascular bleeding and ACT >140 s: protamine antagonise 1:1 • Microvacsulair bleeding, if yes: Hb <5.3 mmol/L or Hct < 0.25: 1× unit of RBC; Thrombo <80 × 10^9/L: 1× unit of PLT; Plasma loss >1 L: 2 units of FFP; Plasma loss >2 L, >50% circ. vol. or fibrinogen <1.2 g/L: 4 units of FFP Step 4: Post-CPB • Microvascular bleeding: 1 unit PLT or 2 units of FFP; if not given previously • ACT >140 s: protamine antagonise • Laboratory: Ca^{2+}, blood gas analysis, thrombocyten count, fibrinogen, aPTT/PT Step 5: Post-CPB: Microvacsulair bleeding and ACT <140 s, if yes: Thrombo <80 × 10^9/L: 1× unit of PLT; Plasma loss >1 L and thrombo >80 × 10^9/L: 2 units of FFP; Plasma loss >2 L, >50% circ. vol. or fibrinogen <1.2 g/L: 4 units of FFP Step 6: Microvascular bleeding persists: all patients 2 g fibrinogen. If fibrinogen <1 g/L: 2 g extra fibrinogen
Ereth et al.[23]	RC	975	Mixed cardiac surgery	Conventional coagulation tests not mentioned Transfusion timing based on clinical discretion No transfusion triggers reported	Coagulation and haemostatic test: details not mentioned (abstract information) Transfusion according to an algorithm with pre-set coagulation and haemostatic test values guide transfusion (no further information available)
Karkouti et al.[24]	RC	1875	Mixed cardiac surgery with excessive blood loss and received ≥4 RBC within the first day of surgery	Conventional coagulation tests not mentioned Transfusion timing based on informal clinical guidelines No transfusion triggers reported	Conventional coagulation tests: thrombocyte count, aPTT/PT, fibrinogen, ACT, ionised calcium Transfusion according to an algorithm with sequential order of treatment modalities: Step 1: • Top-up antifibrinolytics/protamine: If early bleed and aprotinin used, continue at 50 000 KIU/h; if tranexamic acid used or late bleed, consider tranexamic acid bolus 50 mg/kg. Protamine: Target ACT within 10% of baseline or until there is no response to additional protamine administration. • Consider DDAVP (16–20 mcg) • Laboratory: blood gas analysis, Hct, Lytes, Ca^{2+}, complete blood count (heamoglobin, platelet count), aPTT/PT, fibrinogen

(Continues)

TABLE 2 (Continued)

Study	Design	*n*	Population	Transfusion and haemostasis management	
				Control group	Intervention group
					Step 2: • Rule out surgical source: prolonged (>2 h) exploration post-CPB during original surgery or return to operation room for re-exploration • Avoid/correct anaemia: RBC transfusion to keep Hct > 24% • Correct (potential) coagulopathy: Thrombo <80× 10^9/L: 5 units of platelets; INR > 1.5: 2–4 units of FFP; Fib <1.0 g/L: 8 units of cryoprecipitate Step 3: Consider rFVIIa (2.4–4.8 mg up to two doses) if: • ≥2 L blood loss • ≥4 units of RBC/≥5 units of platelets/≥4 units of FFP • Hct >24%/thrombo >80 × 10^9/L/INR < 1.5/Fibrinogen >1 g/L
McRae et al.[25]	RC	25	Elective PEA for CTEPH	Conventional coagulation tests: thrombocyte count, INR, ACT, and fibrinogen	Conventional coagulation tests: thrombocyte count, INR, ACT, and fibrinogen
				Transfusion based on the discretion of the anesthesiologist Transfusion triggers reported: • RBC transfusion: Hb < 80–90 g/L • PLT transfusion: thrombocyte count 50-100 × 10^9 • FFP transfusion: INR > 2	Transfusion according to an algorithm with sequential order of treatment modalities: Step 1: Start surgery • Autologous blood predonation in patients with a preoperative Hb >130 g/L • Standardised use of cell-saver technique • Use antifibrinolytics (aprotinin [08/2005–10/2007] and tranexamic acid [10/2007–03/2009] was standardised) Step 2: Pre-end CPB: Autologous blood reinfused before heparin reversal with protamine. Step 3: Post-CPB • Ongoing bleeding associated with an abnormal INR: ○ FFP transfusion (10–15 ml/kg) ○ PLT transfusion, if: persistent bleeding despite administration of FFP or patient had known underlying platelet disorder ○ RBC transfusion, if: Hb < 80 g/L or Hct <25% • INR >2 and absence of ongoing clinical bleeding: no treatment, allowed to drift down spontaneously and intravenous infusion of unfractioned heparin was started 4–6 h post-operatively or INR <2
Rosenthal et al.[26]	PC	152	Mixed elective cardiac surgery	Conventional coagulation tests: not mentioned	Conventional coagulation tests not mentioned (abstract information)
				Transfusion based on clinical discretion No transfusion triggers reported	Transfusion according to a guideline-based standard operating procedure for transfusion triggers (no further information available)
Silva et al.[27]	RC	251	Elective and emergency CABG. No use of CPB: control group 94% vs. intervention group 91%	Conventional coagulation tests: not mentioned	Conventional coagulation tests: thrombocyte count, INR
				Transfusion based on clinical discretion No transfusion triggers reported	Epsilon-aminocaproic acid use was standardised Protocol with criteria to perform transfusion for: • RBC transfusion: ○ Hb <11 g/dl in patients with unstable coronary disease ○ Hb <10 g/dl for patients in clinical situations with risk more elevated for bleeding or low intraoperative tissue perfusion, falciform anaemia, thalassemia, age over 65 years old, etc.

TABLE 2 (Continued)

Study	Design	*n*	Population	Transfusion and haemostasis management	
				Control group	Intervention group
					○ Hb < 10 g/dl and symptomatic anaemia without specific treatment ○ Hb 7–10 g/dl in patients with risk to cardiac ischemia in preoperative period ○ Hb <7 g/dl in asymptomatic patient in the perioperative period; ○ Acute anaemia caused by bleeding with clinical criteria of low tissue perfusion such as tachycardia, hypotension, late capillary refill, tachypnea, low urinary output, altered mental status. • PLT transfusion: ○ Active bleeding with thrombo <50 ml/mm^3 ○ Platelet dysfunction with active bleeding ○ Thrombo <20 000 associated with chemotherapy, tumour invasion, leukaemia or bone marrow aplasia. • FFP transfusion: ○ Active bleeding followed by multiple coagulation factor deficiency; ○ Hepatopathy patients with ISI >1.5 and with signals of active bleeding or in preoperatory period.

Abbreviations: ACT, activated clotting time; aPTT, activated partial thromboplastin time; Ca^{2+}, ionised calcium; CABG, coronary artery bypass graft surgery; CPB, cardiopulmonary bypass; CTEPH, chronic thromboembolic pulmonary hypertension; DDAVP, desmopressin; FFP, fresh frozen plasma; Hb, haemoglobin; Hct, haemotocrit; INR, International Normalised Ratio; ISI, International Standard Index; PC, prospective cohort; PEA, pulmonary endarterectomy; PLT, platelet; PT, prothrombin time; RBC, red blood cells; RC, retrospective cohort; rFVIIa, Recombinant factor VIIa.

TABLE 3 Risk of bias of the included studies

Cochrane Collaborations Risk of Bias Tool for Randomised Trials		Risk of bias in non-randomised studies of interventions						
Bias domain	Capraro et al. 2001	Bias domain	Bilecen et al. 2014	Ereth et al. 2012	Karkouti et al. 2006	McRae et al. 2011	Rosenthal et al. 2013	Silva et al. 2013
Random sequence generation (selection bias)	High risk	Due to confounding	Serious risk	Critical risk	Critical risk	Serious risk	Critical risk	Critical risk
Allocation concealment (selection bias)	High risk	Selection of participants	Low risk	No information	Moderate risk	Low risk	Low risk	Low risk
Blinding of participants and personnel (performance bias)	High risk	Classification of intervention	Low risk	No information	Low risk	Low risk	No information	Low risk
Blinding of outcome assessment (detection bias)	High risk	Deviations from intended interventions	Moderate risk	No information	Serious risk	No information	No information	Low risk
Incomplete outcome data (attrition bias)	Low risk	Missing data	Low risk	No information	No information	Low risk	No information	No information
Selective reporting (reporting bias)	Unclear	Measurement of outcomes	Low risk	No information	Low risk	Low risk	No information	Low risk
Other bias	Unclear risk	Selection of reported results	Low risk	No information	Low risk	Low risk	No information	Low risk
Overall bias	High risk	Overall bias	Serious risk	Critical risk	Critical risk	Serious risk	Critical risk	Critical risk

3.3 | Primary outcome

Five studies showed a significant reduction of transfused RBC, FFP, and/or PLT after the implementation of a structural non-POC transfusion and haemostasis protocol in mixed cardiac surgery and CABG surgery. These five studies combined form the vast majority of the included patients (8472 out of the total 8555 patients). In these studies, the reduction in RBC, FFP, and PLT transfusion ranged from 2% to 28%, 2% to19.5%, and 7% to 17%, respectively (Table 4).[22–24,26,27]

In the study of Bilecen et al., patients undergoing mixed cardiac surgery accounted for more than 60% of the patients included in this systematic review. The study showed no difference between the control and intervention group regarding the mean amount of transfusion of RBC, FFP, and PLTs. Regarding the proportion of patients transfused, significantly fewer patients were transfused with RBC (29% vs. 27%; $p < 0.05$) and FFP (11% vs. 9%; $p < 0.05$) after the implementation of a non-POC transfusion algorithm.[22]

Karkouti et al. implemented a haemostasis protocol among patients with excessive blood loss, defined as transfusion of ≥4 RBC units within the first day of surgery. The number of RBC transfusions remained unchanged despite the higher transfusion trigger for RBC transfusion after the implementation of a haemostasis algorithm (haematocrit trigger increase from 18% to 20%). Still, the percentage of patients requiring FFP and PLT transfusion decreased. Interestingly, in categorical analysis, an increase in massive FFP transfusion (>11 units) was found in the haemostasis protocol group (9% vs. 14%), which might be explained by an increased incidence of complex surgery in the haemostasis algorithm group.[24]

Silva et al. implemented a transfusion protocol in patients undergoing isolated CABG surgery of which the majority used cardiopulmonary bypass (94% vs. 91%, *p*-value: not significant). This resulted in a decreased amount of RBC, FFP, and PLT transfusion of 28%, 13%, and 11%, respectively.[27]

The study of Ereth et al. successfully implemented a transfusion protocol in mixed cardiac surgery. The study showed a reduction in blood component exposure of RBC, FFP, and also PLT (16.2%, 19.5%, and 17%, respectively).[23] The study of Rosenthal et al. showed a decrease in the number of transfused patients (40.5% vs. 18.2%) and RBC transfusion requirements (26.2% vs. 10.9%) in elective cardiac surgery patients.[26]

The small retrospective study of McRae et al. introduced a transfusion algorithm in patients with chronic thromboembolic pulmonary hypertension undergoing elective pulmonary endarterectomy. The percentage of patients requiring transfusion of any blood components reduced significantly after the implementation of a transfusion algorithm (89% vs. 44%).[25]

Capraro et al. conducted a randomised controlled trial, with a small sample size, in patients undergoing elective mixed cardiac surgery with an increased bleeding tendency after heparin neutralisation (i.e., bleeding >1.5 ml/kg in 15 min after the mediastinal drains were emptied for the first time). This was the only study reporting an increased rate of PLT transfusion (10% vs. 50%; $p = 0.001$) after the implementation of a haemostasis management algorithm during the first post-operative hour. The other rates of blood component transfusion were similar among the groups.[21]

3.4 | Secondary outcome

None of the studies found a difference in rethoracotomies or in-hospital mortality among the groups (Table 5).[21–24,27] Three studies reported on post-operative blood loss, of which one study found a significant difference in post-operative chest tube drainage in favour of the introduction of a non-POC haemostasis protocol compared to standard therapy.[21,23,25]

4 | DISCUSSION

Bleeding after cardiac surgery is common and frequently due to haemostatic disturbances with a multifactorial origin.[1] To date, treatment of post-operative coagulopathy remains highly variable among centres and physicians.[3–5] In order to guide and make uniform haemostasis treatment, many institutions have developed haemostasis treatment protocols. Although several guidelines recommend the use of these transfusion algorithms, its evidence has not been well outlined.[6,7,28] This systematic review included several studies which concluded that the implementation of a cardiac surgery-specific haemostasis management protocol (not based on POC monitoring) might contribute to a reduction in blood component transfusion compared to experience-based transfusion practice. However, all studies had a serious to critical risk of bias, which hampered the deduction of the additive value of these transfusion protocols. Therefore, the principal finding of this systematic review is that no conclusions can be drawn on the additive value of a non-POC haemostasis protocol compared to experience-based practice in cardiac surgery.

The quality of the evidence was low because of several factors. In terms of heterogeneity, in various included trials the intervention group differed in several important aspects from the control group. In some studies, only the intervention group routinely received tranexamic acid.[21,22,24] As tranexamic acid has been shown to reduce bleeding and transfusion, this might have influenced the results.[29] Moreover, some studies introduced the use of a cell saver technique to reduce RBC transfusion with the implementation of a structural transfusion algorithm.[22,25] A recently published meta-analysis showed that cell salvage tends to decrease the rate of RBC transfusion in cardiac surgery, possibly biasing the results.[30] Furthermore, McRae et al. implemented autologous blood predonation in patients with preoperative haemoglobin >130 g/L in the intervention group.[25] This technique has proven to reduce blood product transfusion, limiting the results.[31]

The randomised controlled trial of Capraro et al. was the only study that demonstrated a significant increase in the transfusion of platelet concentrate during the immediate recovery period (1 h after

TABLE 4 Blood component usage

Study	Study design	Effect measure	Subanalysis	Control	Intervention	OR (95% CI), *p*-value	Risk of bias[a]
All blood component transfusion							
Bilecen et al.	RC	Mean	Units transfused per patient	1.46	1.29	NS	Serious
		%	Patients transfused	33	31	0.74 (0.60–0.92)	
Capraro et al.	RCT	Mean (SD)	Units transfused during total hospitalisation	14.4 (14.0)	17.2 (17.2)	NS	High
Karkouti et al.	RC	Median (IQR)	Units transfused per patient	15 [9–24]	14 [7–25]	NS	Critical
McRae et al.	RC	%	Patients transfused	89	44	0.04	Serious
Rosenthal et al.	PC	%	Patients transfused	40.5	18.2	<0.05	Critical
Red blood cells							
Bilecen et al.	RC	Mean	Units transfused per patient	0.88	0.78	NS	Serious
		%	Patients transfused	29	27	0.69 (0.55–0.86)	
Capraro et al.	RCT	Mean (SD)	Units transfused during total hospitalisation	5.7 (5.6)	6.5 (5.6)	NS	High
Ereth et al.	RC	%	Patients transfused	65.6	49.4	<0.0001	Critical
		Mean (SD)	Units transfused per patient	2.6 (3.62)	1.5 (2.37)	<0.0001	
Karkouti et al.	RC	%	Patients transfused 4–6 units	69	69	NS	Critical
			Patients transfused 7–12 units	20	20		
			Patients transfused >12 units	12	11		
McRae et al.	RC	%	Patients transfused	67	37	NS	Serious
Rosenthal et al.	PC	%	Patients transfused	26.2	10.9	<0.05	Critical
Silva et al.	RC	%	Patients transfused	64	36	<0.001	Critical
Fresh frozen plasma							
Bilecen et al.	RC	Mean	Units transfused per patient	0.47	0.37	NS	Serious
		%	Patients transfused	11	9	0.63 (0.46–0.86)	
Capraro et al.	RCT	%	Patients transfused in recovery period (1 h after surgery)	23.3	10.7	NS	High
		Mean (SD)	Units transfused during total hospitalisation	2.3 (2.3)	2.0 (2.6)	NS	
Ereth et al.	RC	%	Patients transfused	46.8	27.3	<0.0001	Critical
		Mean (SD)	Units transfused per patient	1.7 (2.72)	0.8 (1.89)	<0.0001	
Karkouti et al.	RC	%	Patients transfused 0 units	17	23	<0.05	Critical
			Patients transfused 1–4 units	47	36		
			Patients transfused 5–11 units	27	28		
			Patients transfused >11 units	9	14		
McRae et al.	RC	%	Patients transfused	56	12	NS	Serious
Silva et al.	RC	%	Patients transfused	20	7	<0.001	Critical
Platelets							
Bilecen et al.	RC	Mean	Units transfused per patient	0.12	0.13	NS	Serious
		%	Patients transfused	9	10	NS	
Capraro et al.	RCT	%	Patients transfused in recovery period (1 h after surgery)	10	50	0.001	High
		Mean (SD)	Units transfused during total hospitalisation	6.5 (7.5)	8.7 (10.1)	NS	
Ereth et al.	RC	%	Patients transfused	43.4	26.4	<0.0001	Critical
		Mean (SD)	Units transfused per patient	0.8 (1.31)	0.4 (0.85)	<0.0001	
Karkouti et al.	RC	%	Patients transfused 0 units	29	36	<0.05	Critical
			Patients transfused 1–10 units	54	52		

(Continues)

TABLE 4 (Continued)

Study	Study design	Effect measure	Subanalysis	Control	Intervention	OR (95% CI), *p*-value	Risk of bias[a]
			Patients transfused 11–15 units	8	7		
			Patients transfused >15 units	10	6		
McRae et al.	RC	%	Patients transfused	44	19	NS	Serious
Silva et al.		%	Patients transfused	15	4	<0.001	Critical

Abbreviations: [], interquartile range; (), standard deviation; IQR, interquartile range; PC, prospective cohort; RC, retrospective cohort; RCT, randomised controlled trial; NS, non-significant result.
[a]Risk of bias in non-randomised Studies of Interventions and Cochrane Collaborations Risk of Bias Tool for Randomised Trials.

TABLE 5 Secondary outcomes

Study	Study design	Effect measure	Control	Intervention	*p*-Value	Risk of bias[a]
In-hospital mortality						
Bilicen et al.	RC	%	2.5	2.3	NS	Serious
Capraro et al.	RCT	%	3	0	NS	High
Karkouti et al.	RC	%	8.3	6.0	NS	Critical
Silva et al.	RC	%	3	3	NS	Critical
Rethoracotomy						
Bilicen et al.	RC	%	8.2	9.5	NS	Serious
Capraro et al.	RCT	%	23	21	NS	High
Ereth et al.	RC	%	3.2	3.1	NS	Critical
Karkouti et al.	RC	%	27	26	NS	Critical
Silva et al.	RC	%	18	15	NS	Critical
Chest tube drainage during post-operative period						
Capraro et al.	RCT		Exact number not reported	Exact number not reported	NS	High
Ereth et al.	RC	ml mean (SD)	498 (533)	335 (323)	<0.0001	Critical
McRae et al.	RC		Exact number not reported	Exact number not reported	NS	Serious

Abbreviations: (), standard deviation; NS, non-significant result; RC, retrospective cohort; RCT, randomised controlled trial.
[a]Risk of Bias in Non-randomised Studies of Interventions and Cochrane Collaborations Risk of Bias Tool for Randomised Trials.

surgery). However, the haemostasis management algorithm was solely implemented during the first post-operative hour. Additionally, the number of patients undergoing combined procedures was significantly higher in the intervention group, which likely contributed to increased thrombocyte transfusion.[21,32] Furthermore, with the exception of the studies of Bilecen et al., McRae et al., and Capraro et al. it was unclear which conventional coagulation tests were performed in the control group.[21,22,25]

Another limitation of our review is that most studies were limited by their retrospective sequential design and subsequent risk of bias. The implementation of a transfusion algorithm and conduct of a study raises awareness for patient blood management, which might bias the results in non-randomised studies (Hawthorn effect). This is a bias to be considered in all of the included observational studies. This hypothesis is substantiated by previous studies, which have shown that patient blood management education programmes by themselves lead to a reduction in blood component utilisation.[33] Notably, Silva et al. introduced such an educational campaign among their healthcare personnel (i.e., surgical, anaesthesia, and intensive therapy teams) in the intervention group.[27] This increase in awareness of the implemented transfusion protocol may have contributed to fewer patients requiring transfusion. Furthermore, the study of Silva et al. reported that several patients had undergone CABG without cardiopulmonary bypass. As procedures without cardiopulmonary bypass are associated with reduced transfusion requirements, this could have influenced the results.[34] However, off-pump coronary artery bypass patients were evenly distributed among the groups, reducing the risk of bias.[27] Still, a study solely including on- or off-pump surgery would have been of higher quality.

Additionally, protocol adherence was not assessed in the included studies. It has been shown that adherence to introduced haemostasis algorithms is frequently modest, which might result in a reduced effect of the intervention.[35] Finally, in two of the included studies, only the abstract information was available, leading to a lack of relevant information concerning the details of their transfusion algorithm and additional interventions.[23,26] All of the above-mentioned

limitations, in addition to evaluation of the risk on bias as shown in Table 3, lead to the fact that no conclusion can be drawn on the additive value of transfusion protocols not based on POC tests.

A recent survey did not show any wide-spread implementation of transfusion protocols in cardiac surgery. The survey was performed among Australian cardiac surgeons, cardiac anaesthesiologists, and perfusionists and reported that just over half of the respondents (54%) use a haemostasis management algorithm.[36] Frequently, POC haemostasis tests are used to guide haemostasis transfusion protocols. In the last decades, great emphasis has been placed on the use of these devices to provide rapid assessment of haemostasis and guidance of bleeding management. However, POC tests of coagulation are still not routinely available in many medical centres.[6]

Various meta-analyses on the use of these devices suggested a significant reduction in transfusion requirements. However, also these reviews are limited by the low quality of the available evidence.[9–11] Furthermore, whether the reduced transfusion rate is a result of the implementation of POC testing or due to simultaneous implementation of a structural transfusion and haemostasis management protocol remains unclear in various studies.[12–16]

In conclusion, due to the high heterogeneity and a substantial risk of bias of the included studies, no conclusion can be drawn on the additive value of the implementation of a cardiac surgery-specific non-POC transfusion and haemostasis management algorithm compared to experience-based practice. To define the exact impact of a transfusion protocol on blood product transfusion, bleeding, and adverse events, well-designed prospective clinical trials are required.

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CONFLICT OF INTEREST

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

Reinier P. J. Boxma: Data selection, drafting, data processing, assessment of included studies, and manuscript revision. **Robert P. Garnier**: Data selection, manuscript revision. **Carolien S. E. Bulte**: Assessment of included studies, manuscript revision. **Michael I. Meesters**: Coordination of data selection and drafting, reviewing, and editing of 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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Knowledge, attitudes and risk perception surrounding blood donation and receipt in two high income Caribbean countries

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Abstract

Objectives: To compare knowledge, attitudes and risk perception related to blood donation and transfusion in Trinidad and Tobago and Bahamas.

Background: Trinidad and Tobago and the Bahamas are two Caribbean countries whose national blood transfusion systems are heavily reliant (76.2% and 76%) on family replacement donors. The Pan American Health Organisation/World Health Organisation recommends blood collection from exclusively voluntary nonremunerated donors on the grounds that family replacement donor-based blood systems are unsafe and inadequate compared to those based on voluntary nonremunerated blood donors.

Methods/Materials: A 23-item questionnaire was distributed online by snowball sampling in these two countries to assess knowledge, attitudes, risk perception and behaviour. SPSS version 24 was used for interpretative and descriptive data analysis, chi-square to measure significance and linear regression the strength of associations. $p < 0.05$ was used to define statistical significance.

Results: Four hundred and fifty three (453) responses were obtained from Trinidad and Tobago and 101 from the Bahamas. Knowledge and positive attitudes were high in both countries (75.5% vs. 80.2%, $p < 0.001$ and 96.6% vs. 100%, $p < 0.001$). A substantial proportion of respondents held the perception that the local blood donation system was safe or very safe (26.4 and 61.4%, $p < 0.001$) that was linked to the misconception that the prevalent method of blood donation was voluntary nonremunerated (27.8 and 51.4%, $p < 0.001$). Concerns about receiving blood were underpinned by mistrust of transfusion-related procedures.

Conclusion: A social interface to transfer information between blood transfusion services and the community could encourage voluntary nonremunerated blood donation and reduce concerns about receiving transfusion.

KEYWORDS
blood donation, Caribbean, risk perception

1 | INTRODUCTION

International blood transfusion organisations recommend blood collection from exclusively voluntary nonremunerated blood donors (VNRD) to ensure blood safety and adequacy.[1–4] However, blood transfusion services in the English Caribbean continue to rely heavily on family replacement donors (FRD).[5–7] FRD ranks may be infiltrated by professional donors (PD) who are paid in

secret by patients and relatives which further compromises blood safety.[8]

Trinidad and Tobago (TTO) and the Bahamas (BAH) are two English Caribbean countries in the Pan American Health Organisation/World Health Organisation (PAHO/WHO) Region of the Americas whose blood transfusion services rely heavily on FRDs. The annual blood donation rate is 1.5 percent of the total population in both countries and FRDs constitute 76.0 and 76.2% respectively.[9] Converting from predominant FRD to 100% VNRDs has been achieved in a low income country (LIC) in this PAHO/WHO region by changing regulatory pathways and using intense education to establish a new pool of VNRDs and convert existing FRDs to VNRDs.[10]

1.1 | Country data for TTO and BAH

TTO has a multi-ethnic (roughly 40% Indian, 40% African, 20% mixed ancestry), multi-religious (50% Christian, 22% Hindu, 7% Muslim) population of 1.3 million. Gross Domestic Product per capita is US $ 31300.00 and internet users comprise 87.3% of the population.[11] Adult Human Immunodeficiency Virus (HIV) prevalence rate is 1.2%.[12] The BAH population of 385 640 is more homogeneous by race (>90% African ancestry) and religion (95% Christian). Gross Domestic Product per capita is US $ 32400.00 and internet usage is 85% of the population. HIV prevalence in the adult population is 1.8%.[13] TAH and BAH are therefore high income countries [HIC] with high HIV prevalence and FRD-based blood donation systems.

1.2 | Risk perception, blood donation and transfusion acceptability

Objective risks associated with blood donation may affect donor motivation and retention.[14] Donors may be excluded from the donation cycle at interview based on permanent deferral criteria or after donation because transfusion transmissible pathogens are detected in their blood. These may cause pre-donation fear and anxiety that deter initial donation and return. The blood taken at donation is processed and transfused to a patient to complete the donation-transfusion cycle. This physical link between donation and transfusion leads to psychological linking of risks[15] For example, HIV transmission by transfusion is linked to blood donation as the source of infection and, mistakenly, to a risk of acquiring HIV from blood donation.

1.3 | Knowledge, confidence and risk perception

Knowledge can be categorised as objective (what people actually know) and calibrated (what people think they know). What people think they know rather than what they actually know influences risk perception of transfusion[16] The degree of confidence in this knowledge also contributes to risk perception[17] Persons with high confidence in knowledge perceive less risk[18]

1.4 | Actual risk of infection from FRDs and VNRDs

Risk perception surrounding blood transfusion is high even in developed countries with exclusive VNRD where the actual risk of transmission is virtually zero[19] Fear of contracting HIV remains the main fear for transfusion recipients following widely publicised 'tainted blood scandals' in the 1980s and 1990s. This affects willingness to accept transfusions and ultimately, patient care[20]

Although the prevalence of transfusion transmissible infections (TTIs - Hepatitis B virus; HBV, Hepatitis C Virus; HCV and HIV) is higher in FRD than VNRD,[21] the actual risk of acquiring an infection from transfusion is less well known in FRD-predominant countries. In the Caribbean, such estimation has been hindered by the sporadic nature of replacement donations and an absence of follow-up studies on chronically transfused patients[22] Actual risk of transmission in Subsaharan Africa was estimated using data from the general population[23] A 1: 9969 residual risk of acquiring HIV has been calculated for one FRD-based blood transfusion system in the Region of the Americas[24]

1.5 | Donation rate and donor HIV in TTO and BAH

Annual blood donation rates are lower (15:1000) and prevalence of HIV in the donor pool higher (0.2 and 0.14% respectively) for TTO and BAH than countries in the same PAHO/WHO region with 100% VNRDs (> 36:1000 donation rate, 0% donor HIV in Curacao and Aruba)[25]

1.6 | Previous studies from the English Caribbean

Previous studies from TTO have shown low blood donation rates in all sociodemographic groups that are underpinned by lack of information and inconvenient donor settings.[6,26] Males outnumber females as prospective donors and high deferral rates are associated with poor knowledge of donor eligibility criteria[27] These studies recommended increased information and improved access to increase voluntary nonremunerated blood donation. Another KAP study showed high risk perception regarding transfusion receipt that affected willingness to both donate and accept blood. The authors recommended inclusion of blood transfusion information in campaigns to promote voluntary nonremunerated blood donation.[28] The present study was conducted to reassess blood donation and transfusion knowledge, attitudes, and risk perception in TTO and investigate them in BAH for the first time. The ultimate aim is to scientifically analyse data to identify perceptions affecting blood donor behaviour and recommend strategies to increase VNRDs and acceptability of transfusion.

2 | MATERIALS AND METHODS

A cross-sectional, descriptive, questionnaire-based survey was conducted in TTO and BAH between 26 January and 2 June 2019. The researchers were second year medical students from the Faculty of Medical Sciences (FMS), The University of the West Indies (The UWI), St. Augustine, Trinidad and Tobago. The questionnaire was devised de novo using 'e-Survey Creator' and pretested on 20 adults in each country. Snowball sampling was used to recruit participants. The questionnaire was distributed online via an internet Uniform Resource Locator (URL). Researchers shared the e-Survey link with all their WhatsApp contacts and Facebook followers aged 18–65 and resident in TTO and BAH respectively who were asked to forward the link to all their contacts and followers aged 18–65 resident in the country under study. The URL link ensured anonymity of responses and the settings were adjusted to allow each participant to complete the questionnaire only once.

The questionnaire consisted of 24 close-ended items with parts on demographics (7), attitudes and risk perception (6), knowledge (10) and confidence in knowledge (1). Scoring systems were used for blood transfusion knowledge and disease transmission knowledge questions. One mark was awarded for a correct response and zero marks with no penalty for an incorrect response. The maximum attainable score for blood transfusion knowledge was 16. Scores between 10 and 16 were taken to signify high and <10 low blood transfusion knowledge. Disease transmission knowledge was assessed in a nine-part question about eligibility to donate blood in nine scenarios including having HIV, HBV or HCV, male homosexual sex, sex with prostitutes, tattoos, piercings, multiple sex partners and a history of intravenous drug use. Maximum attainable score in this category was 9. Scores between 5–9 were considered high and 0–4 low disease transmission knowledge. Knowledge of the window period for detecting HIV in blood was assessed in a best response multiple-choice question. At the end of the survey, participants' confidence in their knowledge was assessed in a single question. The questionnaire is attached as Appendix I. Data were collated on Microsoft Excel spreadsheets and analysed using Statistics Package for Social Sciences (SPSS) version 24.0 for descriptive and inferential analysis, Chi-square for significance of associations and logistic regression for strength of associations. A *p*-value <0.05 was used to define statistical significance. Ethics approval for the study was obtained from The UWI Ethics Committee.

2.1 | Sample size

The sample size was calculated at 50 percent, using the equation

$$n = \frac{Z/2^2 * p\,(1\text{-}p)}{d^2}$$

where:

n = The recommended sample size.

Z = 1.96 at a confidence level of 95%.

p = prevalence = 0.5.

d = confidence interval = ±0.05 (5%).

from which a sample size of 384 was determined.

3 | RESULTS

For 524 responses received in TTO, 57 were incomplete and 14 from respondents <18 years old, who therefore did not meet the age criteria for the study. This left 453 (86.4%) eligible responses. Of 116 responses in BAH, 101 (87.4%) were suitable for analysis since 14 were incomplete and one respondent was <18 years old. The final sample analysed was therefore 554 in total.

3.1 | Demographics

Table 1 shows that the majority of respondents were young adults in the age category 18–33 years, female, single, Christian, employed and educated to the tertiary level. Most participants were of Indian

TABLE 1 Demographics of participants for TTO (n = 347) and BAH (n = 101)

Characteristic	TTO (%)	BAH (%)	Total (%)	*p*-value
Age				
18–33	82.6	72.9	80.7	0.018
34–49	13.0	12.9	13.9	0.967
50–65	4.4	14.9	6.3	< 0.001
Gender				
Male	25.2	47.5	29.2	<0.001
Female	74.8	52.5	70.8	<0.001
Race				
African	21.2	90.1	33.8	<0.001
Indian	57.4	1.0	47.1	<0.001
Mixed	20.3	6.9	17.9	<0.001
Religion				
Muslim	18.3	0.0	15.0	<0.001
Hindu	22.5	1.0	18.8	<0.001
Christian	55.4	90.1	61.2	<0.001
Marital status				
Single	78.4	77.2	78.2	0.802
Married	17.7	17.8	21.8	0.969
Education				
≤Secondary	33.8	18.8	31.0	<0.001
Tertiary	66.2	81.2	69.0	<0.001
Employment status				
Employed	61.8	67.3	43.5	0.300
Unemployed	38.2	32.7	56.5	0.300

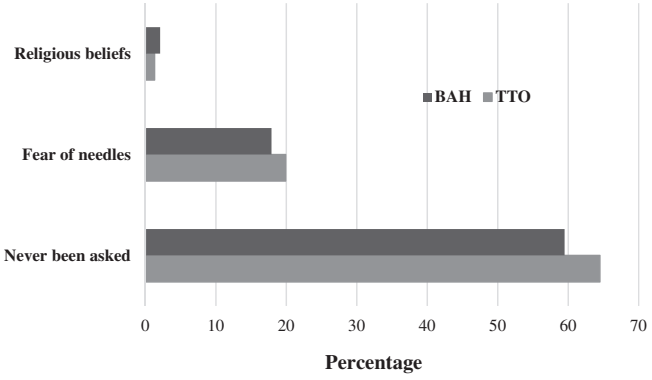

FIGURE 1 Reasons for not donating blood among nondonors in TTO (351) and BAH (n = 54)

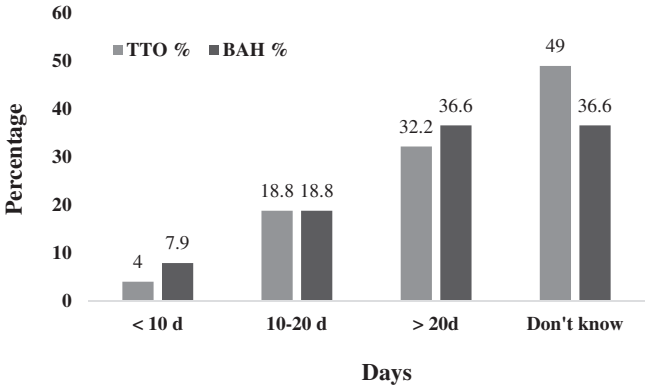

FIGURE 2 Knowledge of HIV window period in TTO (n = 453) and BAH (n = 101)

ancestry in TTO and African ancestry in BAH. This illustrates the high selectivity of snowball sampling towards participants who shared the same characteristics as the researchers. There is no published data on the sociodemographic composition of the national donor panel in either BAH or TTO to draw comparisons.

3.2 | Previous donation/transfusion history

A minority of participants in both countries (16.1% in TTO and 43.6% in BAH, $p < 0.001$) had previously donated blood. Smaller proportions in both countries (7.3% TTO and 5.0% BAH, $p = 0.633$) had previously received blood. Logistic regression analysis demonstrated weak correlations between previous blood donation and age ($r = -0.284$, $p < 0.001$) or gender ($r = 0.189$, $p < 0.001$) in TTO respondents. Among BAH respondents there was a weak correlation between previous donation and age ($r = -0.186$, $p < 0.001$) and a moderate association with gender, males being more likely to have donated in the past ($r = 0.323$, $p < 0.001$). The main reason for not donating among nondonors in the TTO and BAH samples was never having been asked ($p = 0.480$). Fear of needles deterred a substantial proportion of nondonors in both countries ($p = 0.696$) (Figure 1).

3.3 | Blood transfusion knowledge

High transfusion knowledge scores were attained by 79.8% of the total sample (75.5%, mean 18.6 ± 4.6 in TTO and 80.2%, mean 19.3 ± 4.8 in BAH, $p = 0.429$). High knowledge scores were associated with female gender ($p = 0.011$, OR = 1.822) and previous blood donation or receipt ($p = 0.017$, OR = 2.051) in TTO but not in BAH. The great majority of participants in both TTO (73.3%) and BAH (73.3%) also obtained high disease transmission knowledge scores. However, a substantial proportion of respondents (more in TTO; $p = 0.024$) admitted to not knowing the window period for HIV detection or gave incorrect answers ($p = 0.392$) (Figure 2).

3.4 | Awareness of blood shortage

There was substantial unawareness of national blood shortage (34.2%) with no difference between TTO (34.2%) and BAH (34.7%) participants ($p = 0.900$). Whereas awareness of blood shortage was weakly associated with a positive attitude to blood donation in TTO ($p = 0.001$, OR = 0.264), no association was found for BAH.

3.5 | Attitudes and intention

As many as 97.3% of participants, 96.6% in TTO and 100.0% in BAH ($p = 0.074$) considered blood donation to be of high importance. Overall, 82.8% were willing to donate blood (81.8% TTO and 88.0% BAH, $p = 0.203$) and 49.4% (49.4% TTO and 49.5%, $p = 0.992$) to accept transfusion.

3.6 | Perception of national blood donation system

Adopting the definitions used by PAHO: Paid blood donation = blood donation in return of cash or a market value equivalent; Voluntary nonremunerated blood donation = blood donation for nothing in return other than satisfaction and appreciation or small tokens for promotional purposes; Replacement blood donation = blood donation to replace blood used to transfuse a friend or family member, a substantial proportion of participants (32.1%) believed their country's FRD-based blood donation system to be voluntary nonremunerated. This misperception was more prevalent in BAH ($p < 0.001$) (Figure 3).

3.7 | Risk perception

A substantial proportion of participants correctly identified paid donation as carrying the highest risk of transmitting infections (49.6% total, 47.7% TTO and 58.4% BAH, $p < 0.001$) and voluntary nonremunerated

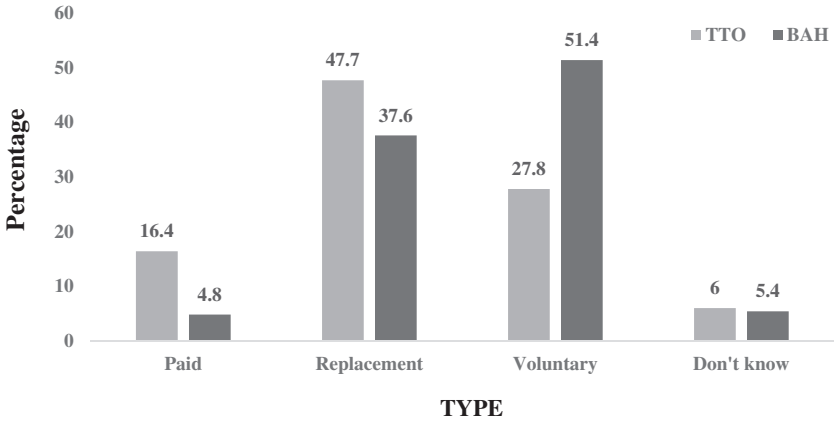

FIGURE 3 Participants' perception of safety of blood donation system in TTO (n = 453) and BAH (n = 101)

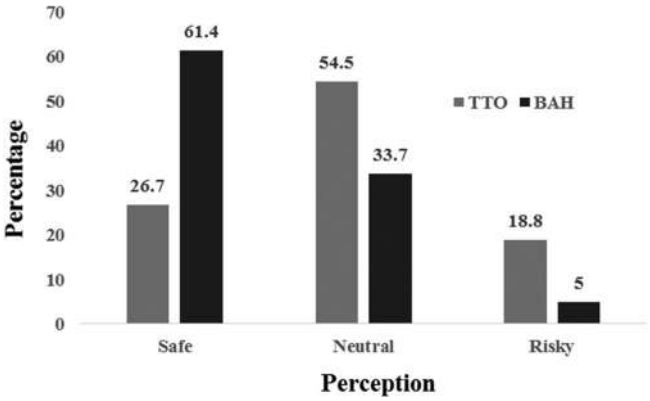

FIGURE 4 Participants' perception of the type of national blood donation system in TTO (n = 453) and BAH (n = 101)

blood donation the lowest (40.1% total, 37.1% TTO, 53.5% BAH, $p < 0.001$). More BAH respondents (61.4% vs. 26.7%, $p < 0.001$) considered their national blood donation system to be safe and conversely, fewer (5.0% vs. 15.3%, $p = 0.002$) thought it to be risky (Figure 4). Logistic regression analysis showed that among TTO participants, young adults (18–33-year-old) were more likely to perceive the blood donation system as risky ($p = 0.002$, OR = 1.563) and that there was a correlation between perceiving the blood donation system as risky and unwillingness to either donate or receive blood ($p = 0.001$, OR = 4.635). No correlation between risk perception of the blood donation system and either age or willingness to donate or receive blood was found among BAH participants. Logistic regression analysis also demonstrated a weak correlation between perception of the prevalent method of blood donation and perception of blood system safety among respondents in both TTO ($r = 0.173$, $p < 0.001$) and BAH ($r = 0.082$, $p = 0.416$) signifying failure to link blood donation method with safety.

3.8 | Comparison between TTO and BAH

Table 2 demonstrates significant differences between the TTO and BAH respondents who had never received transfusion. It shows greater trust in hospital-related procedures among BAH respondents.

3.9 | Confidence in blood donation-transfusion knowledge

At the end of the survey, the majority of TTO participants (52.5%) was confident about only some of their answers whereas the largest proportion in BAH (43.6%) was confident about most of the answers. There was no correlation between knowledge score and confidence in level of knowledge in either TTO or BAH.

4 | DISCUSSION

High knowledge, positive attitudes and willingness to donate or accept blood were demonstrated in a highly selected cohort in two HIC with FRD- based blood donation systems. Pre-donation fear and the lack of an opportunity were the main demotivators to donation. Fear and mistrust of procedures underpinned most concerns about receiving blood. Knowledge of the window period for HIV was low and there was substantial misperception surrounding the definition of voluntary nonremunerated blood donation and its relative safety.

4.1 | Relevance of demographics

Young, single, highly educated, employed females were overrepresented in this survey, reflecting the traits of researchers' social contacts. It illustrates a huge potential target group for donor motivation interventions. Has that been noted and will that be used? Bring that in the discussion section. There is little variation in blood donation knowledge, attitudes and practise across sociodemographic groups in TTO[6] so the results of this survey may be more generalizable in that country. Recruiting FRDs is based on a family's selection of one of its members to give blood rather than the individual response to an anonymous appeal that occurs with voluntary nonremunerated blood donation.[29] Most transfusion recipients in HIC with adequate blood supplies are >60 years old to support complex, expensive procedures such as cardiac surgery and chemotherapy that are less available in LIC. Therefore, previous donors and recipients of blood may be underrepresented in this sample of predominantly young females

Concern	TTO%	BAH%	*p*-value
Fear of hospitals/clinical environments	24.9	9.9	**0.015**
Fear of infection from hospital equipment	17.7	12.9	**0.022**
Fear of complications after transfusion	17.2	18.8	0.708
No confidence in hospital screening of blood	37.5	14.9	**<0.001**
Low confidence in medical expertise	20.3	9.9	0.080

TABLE 2 Concerns among respondents never transfused in TTO (n = 420) and BAH n = 57)

Note: Bold values indicate fear of hospital/clinical environments was significantly higher among TTO respondents

who are less likely to be selected as FRDs because of gender and less likely have been transfused because of age.

4.2 | Knowledge, attitudes and practises

High knowledge, positive attitudes and willingness to donate VNR blood have been demonstrated among all sociodemographic groups in two community KAP studies from TTO.[6,26] A study of existing FRDs in that country also revealed willingness to convert to VNRDs.[30] The message from these studies was that filling specific knowledge gaps, improving communication and enhancing donation arrangements could convert this willingness to actual voluntary nonremunerated donation.[6,26,27] As a follow-up, research, information-sharing, effective communication, interpersonal interaction and convenience were used to generate a pool of predominantly young, female and repeat VNRDs in TTO.[31] No association between awareness of blood shortage and previous donation in BAH is an unexpected finding since knowledge of need is a widely reported motivator to blood donation.[32,33] The absence of a correlation between knowledge and previous donation or transfusion in BAH is also surprising since experience brings knowledge and knowledge encourages participation. However, the phenomenon has been witnessed in other developing countries.[34–36]

4.3 | Determinants of risk perception

In developed countries with VNRD-based systems, high risk perception is associated with female gender, lower education, married status and no history of blood donation or receipt.[16] Similar studies in FRD-based systems are rarer but risk perception was higher among older males in Saudi Arabia[37] and among young females in TTO.[28] The latter finding in TTO has been reinforced in this survey. Although this information is useful for planning motivation of VNRDs in this age group, it may bear little relevance to transfusion receipt which is more often out of necessity than by choice.

4.4 | Income, donation rate and %VNRD

Both TTO and BAH fall short of the median annual whole blood donation rate of 32 per 1000 inhabitants, and %VNRD (> 95%) for HIC globally.[38] However, they suffer from the blood shortages typical of low and middle income countries [LMIC] with FRD-based blood donation systems that are ascribed to the lack of **a** long-term blood donation culture, lower education and less resources.[26] Our findings suggest failure to develop a culture of voluntary nonremunerated blood donation even where education and financial resources are not impediments.

4.5 | Interpretation of voluntary blood donation

Blood donation practises in the English Caribbean are strongly influenced by what happens in the United States of America and the United Kingdom with which it shares strong historical and geographical links. Whereas all blood is collected from VNRDs in the United Kingdom, Federal Drug Administration (FDA) regulations in the United States of America do not prohibit paying donors provided that all blood for transfusion is labelled as 'paid donor' or 'volunteer donor'. Source plasma for further manufacturing use is routinely collected from paid donors.[39] During the period of transitioning from paid to VNRD in the USA a 'family credit system' allowed donors to reclaim their donations for family members without costs.[40]

Interpretations of voluntary blood donation vary among developing countries. In some Asian countries, donors are considered voluntary even if they receive some forms of payment. Direct payment could include items of significant monetary value. Indirect payment may include blood for the donor or relatives if the need arises (similar to the 'family credit system') or preferential admission to hospital. In some cases, even replacement donors are considered voluntary.[41] FRD donations are certainly to an extent voluntary, but are not strictly of the potential donor's own free will as there is always an element of coercion. In Ghana, for example, >70% of FRD consider themselves voluntary donors[42] In order to promote behaviour change, terminology and messaging must be appropriate, accurate, consistent and understood[43] For example, the widely used term 'recruitment' in reference to blood donors could carry connotations of aggressive enlistment and should be replaced by 'motivation' which is based on individual perception and choice. This study revealed confusion about the understanding of the terms 'voluntary' and 'replacement' in assessing safety of existing national blood donation systems. Misunderstanding of the concept of voluntary nonremunerated blood donation and how it differs from replacement donation could blur the target for decision-makers, message-framers, community members and donors aiming for 100% VNRD.[44]

4.6 | Social interface between the blood bank and the community

Overall, data from these studies reveal the effect of a poorly developed societal interface for transfer of information and knowledge between the blood transfusion service and the community that has prevented uptake of voluntary nonremunerated blood donation. Resolving predonation fear can be approached by continuous public education to create appropriate awareness of the safety of donating blood. The perception of lack of opportunity to donate could be removed by improving communication and public relations to increase visibility of the donation centre in the community. Potential donors could be approached through the design and implementation of a properly functioning societal interface to increase public information and school education about blood donation and transfusion including the annual blood donation rate, the types of blood donor, organisation of the blood donation system, the meaning of voluntary nonremunerated blood donation, its safety relative to family replacement donation, blood screening techniques, the HIV window period, the expertise of donor centre and hospital personnel as well as aseptic techniques used to minimise infections in the donation and transfusion processes.[45,46] Appropriate messaging, messenger and medium (pamphlets, posters, billboards, radio, television, newspapers, internet) must be informed by social science research to identify knowledge gaps specific to sociodemographic groups then fill them to shape individual perceptions and encourage the desired social action of voluntary nonremunerated blood donation.[47] In addition to creating mutual trust and confidence, this intervention promotes clear understanding of the responsibilities of the blood bank and of the blood bank and the community with its potential blood donors for a safe blood supply. Greater trust in transfusion procedures in BAH is very likely due to closer interaction between hospital and community in a smaller island setting than TTO. Success from establishing a social interface between a blood transfusion centre and a university community has been demonstrated in TTO. Information from KAP studies in the potential donor age group was used to educate, motivate and retain VNRDs to develop a safe blood donor panel[31] This model is potentially applicable to donation centres nationally to allow persons to make an altruistic choice to donate as VNRDs and provide an impetus for supportive legislation. In parallel, regulatory changes that permit existing FRDs to first convert to anonymous replacement donors (family donors or FD) and then to regular VNRDs are being encouraged. FDs donate blood to boost hospital supplies rather than to benefit a specific individual. They are accessible, reliable, likely to repeat and constitute a valuable resource in transitioning.[48] FDs and VNRDs are of equivalent value but terminology must be adjusted in a stepwise fashion through the change to 100% VNRDs.

5 | LIMITATIONS

The weakness of snowball sampling through a defined group of second year medical students using their WhatsApp and Facebook contacts and friends is a serious obstacle for the interpretation and representativity of the study. Whilst it provided rapid results in a cost-effective manner within the short time period (3 months) available for data collection, its nonrandom nature means that the findings may not be generalizable because the sample was biased towards persons with traits similar to the researchers. The response rate could not be determined since the number of contacts and followers invited to participate is not known. In addition, responses from persons <18 years old demonstrates the uncontrollability of the sample.

There is no guarantee that all respondents were resident in TTO or BAH. Importantly, data on the sociodemographic composition of the national blood donor pool in BAH and TTO were unavailable for comparison. Although validity of the findings may have been increased by exceeding the recommended sample size, this could have resulted in unnecessary use of resources.

6 | SUMMARY AND RECOMMENDATIONS

High knowledge, positive attitudes and willingness to donate voluntarily without remuneration or receive blood were demonstrated in a highly selective cohort from two HIC Caribbean countries. However, knowledge gaps and mistrust prevent uptake of voluntary nonremunerated blood donation and sustain anxieties about transfusion. Development of societal interface to share information could encourage voluntary nonremunerated blood donation and reduce the fear of receiving blood.

AUTHOR CONTRIBUTION

Charles KS – conceptualised the study, supervised the research, analysed data and wrote the paper. **Bain T** and **Beharry T** – group leaders, collected and analysed data, wrote first draughtof paper. **Baksh H**, **Bernard A**, **Bernard C**, **Bhagoutie S** – helped construct questionnaire, conducted interviews, collected data. **Chantry AD** – helped conceptualise study, reviewed final draught.

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APPENDIX I: STUDY QUESTIONNAIRE

Section I: Demographics

1. Age: .....
2. Sex:
   ☐Male ☐Female
3. Ethnicity:
   ☐African ☐Indian ☐Mixed ☐Other
4. Marital Status:
   ☐Single ☐Married ☐Other
5. Highest level of education attained:
   ☐Primary ☐Secondary ☐Tertiary ☐None
6. Are you currently employed?:
   ☐Yes ☐No
7. Your religious is:
   ☐Islam ☐Hinduism ☐Christianity ☐Other

Section II: Risk Perception and Attitudes

8. Have you ever donated blood?
   ☐Yes ☐No
9. Have you ever received blood?
   ☐Yes ☐No
10. If no to either a or b above, please tick "✔" all that apply:

Never received blood:

☐ I have a fear of acquiring an infection because of unsterile equipment.
☐ I am concerned with complications after transfusion.
☐ I am not confident in the hospital's blood screening process.
☐ I have a fear of hospitals or clinical environments.
☐ I am concerned with medical staff expertise.

Never donated blood:

☐ It is against my religious beliefs.
☐ I have a fear of needles.
☐ I have never been asked to donate blood to anyone.
☐ I am afraid to cath an infection from donating

Attitudes

11. Are you willing to:
    ☐Donate blood ☐Receive blood ☐Both ☐Neither
12. Overall, how would you rate the importance of blood donation?
    ☐None ☐Low ☐High.
13. Overall, how would you rate the blood donation system for safety?
    ☐Very risky ☐Risky ☐Neutral ☐Safe ☐Very safe

Section III: Knowledge

14. Have you ever been educated on the importance of blood donation?:
    ☐Yes ☐No
15. Which of the following correctly lists the four (4) main blood groups?
    ☐A, R, HB, O ☐HB, B, OA, H ☐A, B, AB, O ☐I do not know
16. What is the minimum age for blood donation?
    ☐15 ☐18 ☐20 ☐I do not know
17. What is the maximum age for blood donation?
    ☐35 ☐50 ☐65 ☐I do not know
18. What is the required weight in lbs for blood donation?
    ☐>80 ☐>110 ☐>135 ☐I do not know
19. Can persons with the following conditions donate blood?:

(a) Diseases *(please tick the appropriate box)*	Yes	No	I do not know
• Common cold			
• HIV/AIDS			
• Hepatitis B			
• Hepatitis C			
• Diabetes			
• Hypertension			
• Epilepsy			
• Heart disease			
• Cancer			
• Asthma			

(b) Other criteria *(please tick the appropriate box)* A donor who:	Yes	No	I do not know
• is a man who has sex with another man			
• is a prostitute or had sex with a prostitute			
• has multiple sex partners			
• injected drugs not prescribed by a doctor			
• had sex with a carrier of hepatitis B or C			
• donated blood within the previous 6 weeks			

	Yes	No	I do not know
• is pregnant			
• has been injured with a sharp object contaminated with blood of another person within the past year			
• had a tattoo within the past year			
• had an ear or skin piercing within the past year			

20. How long does it take for HIV to show up on a blood screening test after a person has contracted the virus?
☐ <10 days ☐ 10–20 days ☐ >20 days ☐ I do not know
21. Which donation method is most prevalent in your country?
☐ Paid ☐ Voluntary nonremunerated ☐ Replacement ☐ I do not know
22. Which donation method carries:
(a) the highest risk of infection to the recipient?
☐ Paid ☐ Voluntary nonremunerated ☐ Replacement
(b) the lowest risk of infection to the recipient?
☐ Paid ☐ Voluntary nonremunerated ☐ Replacement
23. Are you aware of a current shortage of blood in your country?
☐ Yes ☐ No

Section IV: Feedback

24. After completing this survey, how confident are you in your level of knowledge about blood donation and transfusion?
☐ I know all there is to know about blood donation; all my answers above are correct.
☐ I knew most of the answers; only some were unclear.
☐ I only knew some of the answers.
☐ I did not know the answers to most of these.

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ORIGINAL ARTICLE

TRANSFUSION MEDICINE WILEY

High B-cell activating factor levels in multi-transfused thalassemia patients

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Abstract

Objectives: To assess the associations between B-cell activating factor (BAFF) and alloimmunisation in multi-transfused thalassemia.

Background: Red blood cell (RBC) alloimmunisation is a complication of multi-transfused thalassemia. BAFF is promoting B cells that produce alloantibodies.

Methods/Materials: Multi-transfused thalassemia, 15 years or older, were recruited in the cohort study. Alloantibodies and BAFF levels were analysed.

Results: Of 114 patients, the overall prevalence of RBC alloimmunisation was 29.8%. The most common alloantibodies were anti-E, anti-Mi^a and anti-c. BAFF levels were different among the three groups; the patients with baseline alloantibodies (median ± interquartile range 1251 ± 474 pg/ml), without alloantibodies (1098 ± 453) and healthy controls (719 ± 306), $p < 0.001$. The BAFF level was elevated in the >25 years old patients (vs. the <25, $p = 0.011$) and the buffy-coat-reduced blood recipients (vs. the pre-storage leukocyte-depletion, $p = 0.005$). Absolute lymphocyte count was higher in the patients without baseline alloantibodies (vs. with baseline alloantibodies, $p = 0.049$) and the splenectomised patients (vs. the non-splenectomised patients, $p < 0.001$). Of the 72 patients without baseline antibodies, four who developed new antibodies showed no statistically different BAFF levels compared with those without new antibodies after 40-month follow-up (1296 ± 734 vs. 1062 ± 460, $p = 0.491$). In multivariate analysis, BAFF to absolute lymphocyte ratio was independently associated with RBC alloimmunisation (odds ratio 3.07, 95% confidence interval 1.124–8.369, $p = 0.029$).

Conclusion: B-cell activating factor (BAFF) levels were elevated in multi-transfused thalassemia and the BAFF to absolute lymphocyte ratio was associated with red blood cell (RBC) alloimmunisation.

KEYWORDS
BAFF, multi-transfused thalassemia, red blood cell alloantibody

1 | INTRODUCTION

Thalassemia is the most common inherited red blood cell (RBC) disorder in Thailand. It has a wide spectrum of clinical severities depending on the degree of globin chain imbalance. Regular blood transfusions for thalassemia patients aim to correct anaemia, suppress the ineffective erythropoiesis, and prevent growth and developmental delay. Developments of RBC alloantibodies and autoantibodies are the most

important complications of multiple transfusions which may lead to haemolytic transfusion reactions or may complicate the pre-transfusion testing that causes a delay in patient care.[1]

RBC alloantibody formation is associated with the discrepancy between the RBC antigens of the donor and the recipient. Only ABO- and RhD-matched RBC units are routinely transfused to the patients. Therefore, the recipients are frequently exposed to other RBC antigens. Prevalence of RBC alloimmunisation in thalassemia ranges from 4% to 37%, compared with 1% to 4% in the general population.[2] The reasons why only a subgroup of patients (responders) develop alloantibodies remain to be determined. The risk of alloimmunisation depends on the genetic homogeneity of the donors and recipients in a population, the policy of transfusion management of each institute and host factors.[2] Recent studies showed the roles of $CD4^+$ T cells, B cells and inflammation in RBC alloimmunisation.[3–6] In a mouse model, anti-CD20 immunotherapy can prevent RBC antibody development.[7] The immune mechanisms of RBC alloantibody production have not been clearly identified. Reliable predictors for RBC alloimmunisation will be helpful to prioritise the extended antigen-matched RBC units for the responders.

B-cell activating factor (BAFF) is an essential cytokine for normal B-cell maturation and survival. A physiologic BAFF level is relative to the number of B cells and is thought to eliminate the autoreactive B cells. The autoreactive B cells are not able to effectively compete with normal B cells for the available BAFF. In contrast, excessive BAFF promotes autoreactive B cells.[8,9] An overabundance of BAFF rescues peripheral autoreactive B cells from deletional tolerance or anergy, resulting in the development of autoimmunity.[10,11] Moreover, a link between increased serum BAFF level and alloimmunisation was suggested and could be explained by alloreactive B-cell activation. A previous study has shown elevated serum BAFF levels and increased numbers of activated B cells in anti-HLA antibody positive patients before renal transplantation. However, after adjusting for age, phenotype, and immunising events, BAFF level was not independently associated with the presence of pre-transplantation HLA antibodies.[12] Another study suggested that BAFF constituted a risk factor for renal graft dysfunction and donor-specific antibody development after transplantation.[13] Elevated BAFF levels also contribute to B-cell activation in patients with active chronic graft-versus-host disease.[14]

RBC alloimmunisation could also be influenced by patient's age, sex, splenectomy status, the age of onset at time of transfusion, and cumulative number of units transfused.[1,15,16] Recent studies in Thailand found the association between RBC alloimmunisation with splenectomy status and patient's age.[17,18] The aims of this study were to determine the associations of high BAFF levels with RBC alloimmunisation in multi-transfused thalassemia patients and to investigate the relationships between BAFF and candidate risk factors relating to RBC alloimmunisation, such as patient's age, splenectomy status, absolute lymphocyte count, and type of leuko-reduced RBC transfusion.

2 | MATERIALS AND METHODS

2.1 | Study design and patient characteristics

This was a cohort study of thalassemia patients at a tertiary care hospital in Bangkok, Thailand. This study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University, Thailand (Approval number: 689/59).

All thalassemia patients who received blood transfusions at the outpatient clinic between July and December 2016 were recruited and followed for 40 months. All patients were previously diagnosed with thalassemia based on clinical and laboratory examinations. The patients aged below 15 years were excluded from the study because of the low incidence of RBC alloimmunisation from receiving less units of RBC transfusion. Patients with active infection were also excluded from the study. Active infection was defined as body temperature higher than 37.5°C or any sign and symptom of infection on the visit date. The persons who requested ABO and RhD tests during their annual wellness visit at our check-up clinic were recruited as healthy controls.

The patients received leuko-reduced blood within 14 days of donation according to our routine practice. There were two types of leuko-reduced RBC units which included pre-storage leukocyte-depletion or conventional buffy-coat-reduced methods depended on patient financial statuses and reimbursement schemes. The residual white blood cell (WBC) count of pre-storage leukocyte-depletion and buffy-coat-reduced RBC units were below 1.0×10^6 and 5.0×10^9 cells/unit, respectively. The patients were transfused with only ABO and RhD-matched units. The extended antigen-matched RBC units, including Rh (C, c, E, e), Mi^a and corresponding antigens, were transfused to the patients who developed RBC alloantibodies.

The patient's age, sex, history of splenectomy, ABO and RhD blood group, and type of transfused leuko-reduced blood were recorded. RBC antibodies were routinely tested before the transfusion using the conventional tube test or gel cards (Bio-Rad; Cressier, Switzerland). The history of RBC antibodies were determined at the baseline and at the 40-month follow-up visit. The BAFF level was determined at the baseline.

2.2 | Soluble BAFF measurement

The EDTA blood samples were collected at the same time as the pre-transfusion compatibility testing and complete blood count (CBC) for thalassemia patients. For healthy controls, the blood samples were collected at the same time as the ABO and RhD blood tests. All blood samples were centrifuged within 24 h after collection and plasma samples were stored at −80°C until testing. The plasma BAFF level was determined by using ELISA following the manufacturer's instruction (Human BAFF/BLyS/TNFSF13B Quantikine ELISA Kit, R&D Systems, Minneapolis, MN).

2.3 | Statistical analysis

Categorical data were presented as frequencies and percentages. Comparisons of categorical variables among groups used the Chi-square test. Continuous variables were described by medians ± interquartile ranges (IQR) or ranges where appropriate. Mann–Whitney U test and Kruskal–Wallis test were used to compare BAFF levels in the different categories of thalassemia patients. The association between continuous variable was evaluated using Spearman's rank correlation. Binary logistic regression analysis was performed to investigate the candidate risk factors for RBC alloimmunisation. The continuous variables were directly entered in the multivariate analysis. The results were presented as odds ratio (OR) and 95% confidence interval (CI). Two-sided p values <0.05 were considered statistically significant. All statistical analyses were calculated by SPSS version 22.0 (Statistical Package for the Social Sciences, Chicago, IL, United States).

3 | RESULTS

3.1 | Patient characteristics

A total of 114 patients were enrolled, 62 (54.4%) were female and 52 (45.6%) were male. The median age was 19 years (15–81 years). There were 104 (91.2%) patients with Hb E/β-thalassemia, 8 (7.0%) patients with homozygous β-thalassemia, and 2 (1.8%) patients with AE Bart's disease. Fifty-four (47.4%) patients had a splenectomy. All patients received regular transfusion at 2–12 week-intervals. The most common blood group was O RhD+ (47 patients, 41.2%), followed by B RhD+ (33 patients, 28.9%), A RhD+ (27 patients, 23.7%) and AB RhD+ (7 patients, 6.2%).

3.2 | Frequency of RBC antibodies

Thirty (26.3%) patients developed RBC antibodies before enrolment (baseline RBC antibodies), of which seven had three or more antibodies, nine had two antibodies and 14 had a single antibody. The alloimmunisation frequencies in female and male were 32.3% (20/62) and 19.2% (10/52), respectively. Three patients expired and 16 patients went to other hospitals for follow-up. There were 95 patients that completed the follow-up, of which 23 had baseline antibodies and 72 without baseline antibodies. Among the 30 patients with baseline antibodies, 23 had been followed until the end of the study, six were treated at other hospitals and one expired. Seven patients developed new RBC antibodies, of which three already had RBC antibodies at the baseline. Therefore, the overall prevalence of RBC antibodies in this study was 29.8% (34/114). There were 60 alloantibodies and nine unidentified antibodies detected in these 34 patients. The most common antibodies were anti-E (30.0%, 18/60), anti-Mi^a (18.3%, 11/60) and anti-c (11.7%, 7/60) as shown in Table S1. Autoantibodies were detected in three patients (2.6%) who also had RBC alloantibodies.

3.3 | Relationships between BAFF level and RBC alloimmunisation

The BAFF level of thalassemia patients (median 1119 ± IQR 471 pg/ml, $n = 114$) was significantly higher than that of healthy subjects (719 ± 306 pg/ml, $n = 14$, $p < 0.001$). Comparison of BAFF levels between the thalassemia patients with baseline RBC antibody (1251 ± 474 pg/ml, $n = 30$), without baseline RBC antibody (1098 ± 453 pg/ml, $n = 84$) and healthy controls also showed a significant difference ($p < 0.001$). As shown in Figure 1, the statistical differences between thalassemia patients with baseline RBC antibody and those without baseline RBC antibody could not be demonstrated. Moreover, there was no difference in the BAFF level of the patients with three or more baseline antibodies (1409 ± 559 pg/ml, $n = 7$) versus one to two antibodies (1218 ± 453 pg/ml, $n = 23$, $p = 0.607$).

To examine whether BAFF is important for the promotion of RBC antibody production in thalassemia, the 95 patients were followed-up for 40 months for new RBC antibody development. Of the 72 patients without baseline antibody, four developed new antibodies. The BAFF level of the patients who developed new antibodies (1296 ± 734 pg/ml, $n = 4$) was not statistically different compared with those without new antibody (1062 ± 460 pg/ml, $n = 68$), $p = 0.491$.

3.4 | Relationships between BAFF level and patient characteristics

A significant difference in BAFF level between different age groups was demonstrated. The younger age group (15–25 years old) had lower BAFF levels (1027 ± 478 pg/ml, $n = 55$) than the patients over 25 years (1225 ± 477 pg/ml, $n = 59$), $p = 0.011$. However, there was

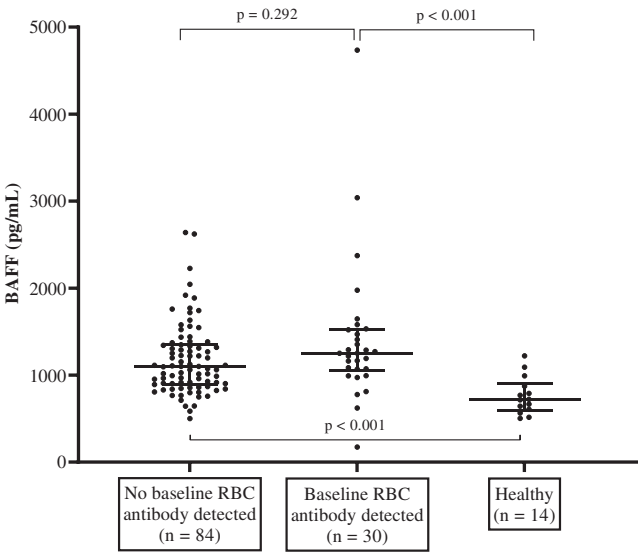

FIGURE 1 B-cell activating factor (BAFF) levels in thalassemia patients with or without baseline red blood cell (RBC) antibodies and healthy controls. Data are shown as medians with interquartile ranges

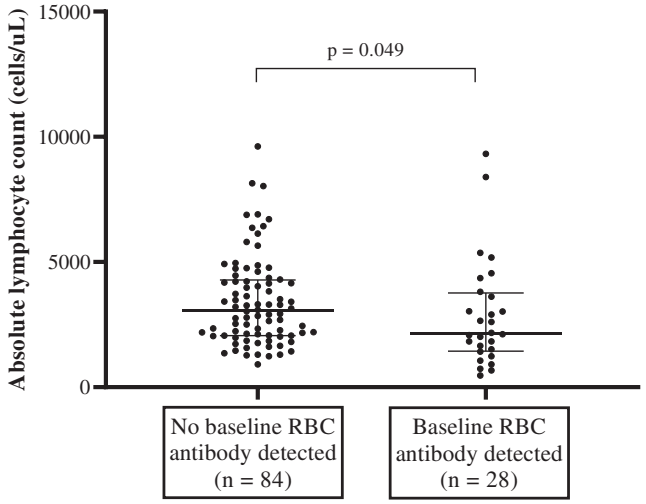

FIGURE 2 Absolute lymphocyte count in thalassemia patients with or without baseline red blood cell (RBC) antibodies. Data are shown as medians with interquartile ranges

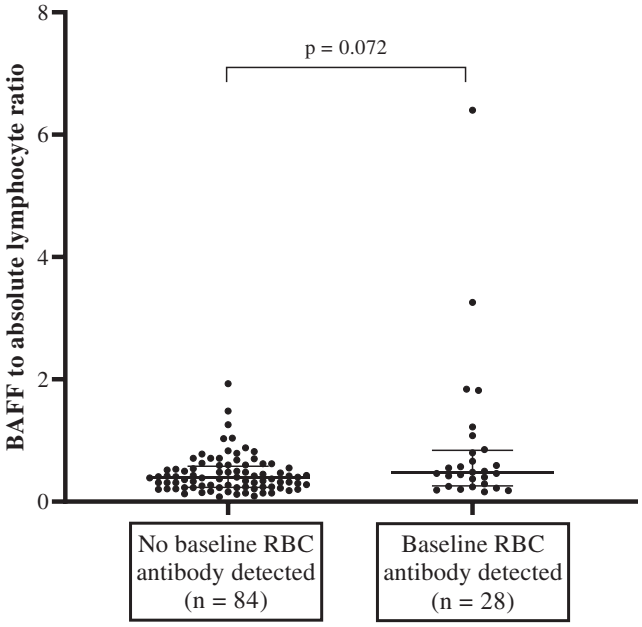

FIGURE 3 B-cell activating factor (BAFF) to absolute lymphocyte count in thalassemia patients with or without baseline red blood cell (RBC) antibodies. Data are shown as medians with interquartile ranges

no difference in the RBC alloimmunisation prevalence between the younger (29.1%, 16/55) and the older groups (30.5%, 18/59). Table S2 showed BAFF levels in different patient groups.

The BAFF level of the patients who received pre-storage leukocyte-depletion transfusions regularly (903 ± 153 pg/ml, $n = 15$) was lower than those who received conventional buffy-coat-reduced blood (1191 ± 472 pg/ml, $n = 99$), $p = 0.005$. The RBC alloimmunisation rate tended to be higher in the conventional buffy-coat-reduced blood transfusion (31.3%, 31/99) than the pre-storage leukocyte-depletion (20.0%, 3/15), although the difference was not statistically significant ($p = 0.547$). In addition, the difference between BAFF level in splenectomized (1110 ± 572 pg/ml) and non-splenectomized patients (1139 ± 429 pg/ml) could not be demonstrated ($p = 0.955$).

Because BAFF binds B cells and promotes B lymphocyte survival, the lower B cell counts may cause the increases in remaining BAFF levels.[19] In this study, the total lymphocytes were used as a surrogate marker for B cell numbers. As shown in Figure 2, the median absolute lymphocyte count in thalassemia patients was 2875 ± 2240 cells/μl ($n = 112$, There were two patients with missing lymphocyte count data.); in which the subgroup with RBC antibody was 2145 ± 2320 cells/μl and 3060 ± 2220 cells/μl in the subgroup without antibody ($p = 0.049$). Only five patients (4.5%) had lymphopenia (absolute lymphocyte count <1000 cells/μl). Absolute lymphocyte count was higher in the splenectomized patients (4230 ± 2340 cells/μl, $n = 53$) than non-splenectomized patients (2120 ± 1200 cells/μl, $n = 59$, $p < 0.001$).

The BAFF to absolute lymphocyte ratio was used to represent the BAFF activity relative to the target cells. As shown in Figure 3, thalassemia patients with baseline RBC alloimmunisation ($n = 28$) have higher BAFF to absolute lymphocyte ratio (0.472 ± 0.580 vs. 0.392 ± 0.350) than those without RBC alloimmunisation ($n = 84$), although the statistical significance was not reached ($p = 0.072$). Absolute lymphocyte count and BAFF to absolute lymphocyte ratio in thalassemia with or without RBC alloimmunisation were shown in Table S3. No correlation was identified between BAFF levels and absolute lymphocyte count ($r_k = -0.048$, $p = 0.615$).

3.5 | Risk factors of RBC alloimmunisation

A multivariate analysis was applied to determine the variables independently associated with baseline RBC alloimmunisation. The age, type of transfused leuko-reduced RBC products, splenectomy status, absolute lymphocyte count, BAFF levels, and BAFF to absolute lymphocyte ratio were analysed. The only variable that showed statistically significant association with RBC alloimmunisation was BAFF to absolute lymphocyte ratio (OR 3.07, 95% CI 1.124–8.369, $p = 0.029$) as shown in Table 1.

4 | DISCUSSION

This is the first study that reports the BAFF level in transfusion-dependent thalassemia patients and its relationships with RBC alloimmunisation. The BAFF level of thalassemia patients was significantly higher than healthy controls although the significant difference between thalassemia patients with RBC alloantibodies and those without alloimmunisation could not be identified. Notably, the thalassemia patients with RBC alloantibodies had lower absolute lymphocyte count than those without alloimmunisation. Interestingly, only BAFF to absolute lymphocyte ratio was independently associated with RBC alloimmunisation.

TABLE 1 Multivariate analysis of factors associated with baseline red blood cell antibody in thalassemia patients

	Odds ratio	95% Confidence interval	*p* value
Age	1.00	0.969–1.034	0.878
Transfused leuko-reduced RBC products	0.45	0.087–2.280	0.341
Splenectomy status	0.34	0.094–1.212	0.205
Absolute lymphocyte count	1.00	1.000–1.001	0.770
BAFF levels	1.00	0.999–1.001	0.999
BAFF to absolute lymphocyte ratio	3.07	1.124–8.369	0.029

Abbreviations: BAFF, B-cell activating factor; RBC, red blood cell.

The overall RBC alloimmunisation rate in this study was 29.8% (34/114) during the 40-month follow-up. The prevalence was comparable to a study in Bangkok by Thedsawad et al.[20] but higher than previous reports in transfusion-dependent thalassemia in Northern and Northeastern Thailand.[17,18] The most common RBC alloantibodies in this study were anti-E, anti-Mi^a and anti-c. Several factors contribute to the rates of RBC alloimmunisation, such as the differences in RBC antigens between the recipients and donors, the preemptive antigen-matched protocol of the institute before antibody development, the total number of transfused units, the immunomodulatory roles of WBC in transfused blood and the recipient's immunity.[1,2]

Among the recipient's immune components, B-lymphocytes play an important role in the immune system through antibody production and participation in antigen presentation to T-lymphocytes. Increased absolute lymphocyte count was found in splenectomized patients compared to non-splenectomized patients. Consistently, the increased absolute lymphocyte count, increased CD19$^+$ B cells and a decrease in percentage of CD3$^+$ T cells were reported in splenectomized B thalassemia Hb E patients.[21] Furthermore, regulatory B cells (CD19$^+$CD38^{hi}CD24^{hi}) are significantly increased in β–thalassemia major patients, both with and without alloimmunisation, compared with healthy controls.[22] The numbers of regulatory T cells, which have an important role in regulation of immune responses, are also increased in β–thalassemia major, both with and without alloimmunisation, compared with healthy controls.[23] The levels of IL-6, IL-10 and transforming growth factor beta (TGF-β) are higher in transfusion-dependent thalassemia (TDT) compared with non-transfusion dependent thalassemia (NTDT).[24] These findings suggest that transfusion in thalassemia could lead to an inflammatory suppressor function of regulatory T cells through these cytokines.

The results of this study showed an elevated BAFF level in the multi-transfused thalassemia patients which indicated a humoral immune activation. A previous study also showed that the thalassemia major patients with gingivitis had a higher serum BAFF level than non-thalassemia patients with gingivitis.[25] The lower BAFF level of younger patients (15–25 years) in this study may be related to the shorter duration of antigenic stimulations by allogeneic blood than the older patients (>25 years old).

Interestingly, only the correlation of alloimmunisation with BAFF to absolute lymphocyte ratio was revealed in the multivariate analysis. This cytokine level relative to its target cells might play a role in RBC antibody production. This result was different from previous studies in Thailand showing that splenectomy and patient's age were associated with alloantibody formation in thalassemia patients.[17,18] Additionally, absolute lymphocyte count was lower in the thalassemia patients with alloimmunisation. Taken together, we hypothesised that the altered BAFF and lymphocyte homeostasis, especially B cells, could contribute to B cell activation that trigger RBC alloantibody production in thalassemia. Future investigation on the number of B lymphocytes related to BAFF level should be explored.

Identifying a biomarker that can predict RBC alloimmunisation is helpful in clinical practice. Preemptive antigen-matched RBC should be considered in the patients who are at higher risk of antibody production. In this study, the BAFF level in the patients with new antibody development tended to be higher than those without new antibody development. However, the statistical significance could not be demonstrated. This might be because the number of the patients that developed new alloantibody was low. Therefore, a larger sample size is required in future investigations. In addition, BAFF level was determined only once at the baseline. Serial testing of BAFF levels in thalassemia patients may be helpful to predict alloimmunisation. Furthermore, other B cell cytokines should be investigated in further studies.

A lower BAFF level was detected in the patients who received pre-storage leukocyte-depletion blood. This finding might be associated with a lower rate of alloimmunisation. BAFF is produced by dendritic cells, follicular dendritic cells, monocytes, and neutrophils.[9,26,27] An animal study showed that donor dendritic cells were required for the alloimmunisation process.[28] A previous study showed that leuko-reduced blood was associated with decreased RBC alloimmunisation.[29] However, a recent multicentre study showed that pre-storage leukocyte-depletion blood did not alter the development of clinically significant RBC alloantibody compared with buffy-coat-reduced RBC units.[30] Leukoreduction of blood components is also effective in preventing immunisation against human leukocyte antigens and human platelet antigens.[31] In addition, pre-storage leukocyte-depletion may mitigate transfusion-related immunomodulation.[32–34] Donor's major histocompatibility complex (MHC) II molecules on residual antigen-presenting cells can present antigens and activate recipient's lymphocytes after transfusion. It could result in either alloimmunisation or immune tolerance.[35] Multi-transfused thalassemia patients receiving pre-storage leukocyte-depletion blood with lower number of BAFF producing cells may result in lower BAFF levels. The roles of BAFF and immunomodulatory effects should be explored in future studies.

The overall prevalence of RBC alloantibodies (29.8%) in our study was higher than those from Northern (17.5%) and Northeastern (19.3%) Thailand.[17,18] The possible reason was the exclusion of paediatric patients from our cohort. On the other hand, the overall prevalence of RBC alloantibodies from a Thai study which included patients older than 17 years was 33.9%.[20] Paediatric patients had lower prevalence of RBC alloantibody because of the lower number of RBC transfusions. A study in India by Pahuja and colleagues[36] which mainly included the patients aged 20 years or lower, reported a low prevalence of alloimmunisation (3.79%). The study used the RBC units that were matched only for ABO and RhD antigens similar to our study.

Regarding the alloantibody specificity, the top three most common alloantibodies were anti-E, anti-Mi^a and anti-c. This finding is similar to the previous reports from Thailand, China and Taiwan.[17,18,37,38] In contrast, Caucasian patients with thalassemia were more likely to have anti-K.[36,39,40] The prevalence of Mi^a antigen in the Southeast Asian and Chinese is up to 15% compared with less than 0.01% in other populations.[41]

There were some limitations in this study. First, this was a single institution study. Second, direct antiglobulin test (DAT) was not included in routine pre-transfusion testing at our institute. This could lead to a low incidence of RBC autoantibody in our study compared with another study in Thai multi-transfused thalassemia patients.[20] Third, our study included only adult patients who already had higher prevalence of alloantibody. Therefore, the number of the patients with new antibody development was limited. Fourth, BAFF levels were measured only once. Based on recent studies, serum BAFF levels were rather stable over time in the majority of SLE and rheumatoid arthritis patients.[42,43] However, there was a high intra-individual variability of BAFF levels over time in patients with anti-Jo1 positive polymyositis and dermatomyositis.[44] The variation of BAFF levels in thalassemia deserves further studies. Fifth, the number of B lymphocytes was not measured in our study. Our study provides a framework for novel exploration of the mechanism of BAFF, B cells, and RBC alloimmunisation.

5 | CONCLUSION

An increased BAFF level was identified in regularly transfused thalassemia patients. Thalassemia patients with RBC alloantibody tended to have higher BAFF to absolute lymphocyte ratio than those without antibody. Our study highlights the possibility of the BAFF and lymphocyte in the promotion of RBC alloimmunisation. The roles of BAFF in modulating and/or predicting RBC antibody development deserve further investigations.

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CONFLICT OF INTEREST

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

Phandee Watanaboonyongcharoen: Designed the experiments, analysed the data, prepared and edited the manuscript. **Benjaporn Akkawat:** Performed the laboratory testing. **Thanida Tohthong:** Collected patient's samples. **Ponlapat Rojnuckarin:** Reviewed and approved 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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Pain control and functional improvement in patients treated by autologous conditioned serum after failure of platelet rich plasma treatments in knee osteoarthritis

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Abstract

Objective: To assess the efficacy of autologous conditioned serum (ACS) for the treatment of patients with knee osteoarthritis after failure of medical treatments and platelet rich plasma (PRP) injections.

Background: Knee osteoarthritis is the most common form of arthritis. Prior to prosthetic surgery these patients might benefit from medical treatments, physiotherapy, and in case of their ineffectiveness, from autologous blood component injections.

Methodology: We have treated 30 patients with Kellgren-Lawrence I-III knee osteoarthritis with ACS after failure of standard medical treatments/physiotherapy and platelet rich plasma (PRP) injections for a full cycle, within the previous year from enrollment. *Results:* ACS administration was performed in all patients with mild side effects and produced prompt (1 month) improvements of VAS and Lequesne scales in 67% of patients and this result persisted at 6 and 12 months. No relationship between the rate of response and Kellgren-Lawrence scale at enrollment was observed whilst responders had a significantly higher amount of interleukin-1 receptor antagonist (IL1-RA) in ACS as compared to nonresponders.

Conclusion: The present study confirms the efficacy of ACS in pain control and functional recovery of patients with knee osteoarthritis resistant to medical and PRP treatment. These results were obtained in a well-defined cohort of resistant patients and seem to be related with IL1-RA content in injected ACS.

KEYWORDS
autologous conditioned serum, knee osteoarthritis, pain control

1 | BACKGROUND

Osteoarthritis (OA) is the most common form of arthritis which causes joint pain and stiffness with consequent functional impairment associated with a significant worsening of quality of life.[1,2] In general, OA is the prevalent form of pain in the older subjects and seems to be related to an increased risk of all-cause mortality.[2,3] OA occurs in subjects with a wide range of age, with a peak of incidence in subjects with more than 50 years, and it may be related to several conditions causing joint inflammation and different level of cartilage damage. The incidence of OA is increasing and this fact is in part related to the aging population and to the increased occurrence of obesity in a large numbers of people.[4] OA mainly affects the knees so that >200 millions of people in the world suffer from joint impairment due to symptomatic knee OA,[5] which progresses invariably as a disabling disease, showing different degrees of severity and requiring treatments

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that encompass nonsurgical approaches such as systemic treatments by oral paracetamol and nonsteroidal anti-inflammatory drugs, visco-supplementation by ialuronic acid (IA) intra-articular infiltration, steroid and nonsteroidal local injections and, in cases of pain persistence, opioid administration. At last, end-stage cases of knee OA may benefit from surgery represented by partial or total knee replacement. In these last years an increasing application of intra-articular blood products, such as platelet rich plasma (PRP) and autologous conditioned serum (ACS), has been reported in subjects requiring pain control for knee OA, after failure of pharmacological treatments or visco-supplemetation. In these patients favourable results have been observed by using PRP or ACS even though a clear superiority of these autologous preparations over other medical treatments is still lacking in large controlled studies or it is based on preliminary data.[6] In general, the principle by which products based on autologous blood components should be effective in pain and inflammation control is the presence of anti-inflammatory cytokines and proregenerative factors, released by blood cells, in plasma or serum administered through intra-articular injections.[7] Moreover, differences in techniques for collection and preparation of these blood-derived products confound the data resulting from studies in small cohort of patients treated using different and customised methodologies. Recently, a specific medical device for the production of ACS in amounts that may be administered in single or multiple injections has been produced and proposed as a commercial CE-marked product.[8–10] The availability of specific device for ACS production by overnight activation of blood cells in well-defined conditions and starting from preset autologous blood amounts may serve to standardise the production process and to optimise the bio-availability of relevant autologous factors to be injected to these patients. Here, we report the results obtained with ACS in 30 patients with knee OA who were resistant to medical treatments, visco-supplementation and PRP injections; ACS was produced through the use of a CE-marked dedicated device and administered by three intra-articular injections, in the site of knee OA at weekly intervals. A wide set of cytokines was also evaluated in patients sera and in each ACS product to ascertain whether a possible association exists between autologous cytokine levels and pain/functional response to ACS treatment.

2 | METHODS

2.1 | Patients and study characteristics

The present study is a prospective-interventional study to assess the safety and efficacy of ACS treatment in 30 patients with knee OA who received previous intra-articular treatments by visco-supplementation, autologous PRP and had no chance to obtain pain-control through the application of other nonsurgical approaches. The study was approved by the Lazio 1 Ethical Committee (authorization Prot. N. 2486-12-05-2017). All patients gave their informed written consent to participate in the study. Characteristics of enrolled patients are described in Table 1. Enrollment criteria were as follows: male or female patients with age ≥18 years, with mono-lateral or bilateral knee osteoarthritis, resistant to previous treatments by IA visco-supplementation and subsequent PRP injections (resistance to PRP was defined as the lack of 20 mm reduction of the VAS scale and at least a class in Lequesne scale 1 month after the end of PRP treatment), ranging in the Kellgren-Lawrence scale from 1 to 3 at enrollment (Figures1 and 2). Exclusion criteria included pregnancy, the presence of major neurological diseases, of any active thrombovascular diseases, of solid or haematological neoplasms, of joint infections. Also patients with a body mass index >30 and with serologically documented HIV, HCV and HBV infections were excluded from the study. Briefly, between 05-03-2018 and 18-10-2018 30 symptomatic consecutive patients (with a visual analogical scale, VAS, ≥30 mm) suffering from resistant mono-lateral (no patients with bilateral knee osteoarthritis had been diagnosed and enrolled in this study in spite of enrollment criteria) knee OA after a full treatment with nonsurgical approaches including visco-supplementation and PRP injections (at least one cycle including a minimum of three PRP injections in the involved knee joint), ranging in age from 35 to 77 years, 17 females and 13 males, 15 with grade 3 Kellgren-Lawrence scale, 13 with grade 2 and 2 with grade 1, had been enrolled in the present study consisting in the collection of autologous serum by a CE marked device and production of at least four aliquots of 2 ml of ACS for subsequent injections in the involved knee joints. Primary objectives of the study were:

a. The safety of ACS treatment in terms of adverse events including infections at the site of injections, systemic infections, pain/edema at the injection site for more than 7 days from treatment, local bleeding episodes and allergic reactions to the injected product; adverse events were monitored from the time of first ACS injection.
b. The efficacy of ACS treatment in terms of VAS and Lequesne scales improvements (response was defined as a reduction of 20 mm of the VAS scale and at least a class in Lequesne) from the beginning of treatment (T0), to 1 month (T1) and 6 months (T2) from treatment.

Secondary objectives of the study were:

a. The efficacy of ACS treatment at 12 months (T3) from the beginning of treatment in terms of VAS and Lequesne scales improvements, as compared to T0 and T2.
b. Patients' satisfaction about the ACS treatment by a multidimensional evaluation.

At the start of treatment and at different time points of follow up patients' response to ACS treatment was evaluated in the following way:

a. By a clinical evaluation of the treated knee joint with recording of pain by VAS and Lequesne scales.
b. By recording possible adverse events during and after treatments.
c. Through the administration of a questionnaire to collect patient's satisfaction about the efficacy and tolerability of treatment.

TABLE 1 Patients' characteristics and outcome after ACS treatment

				VAS scale				Lequesne scale			
	Age (years)	Gender	Kellgren-Lawrence	T0	T1	T2	T3	T0	T1	T2	T3
UPN001	77	F	2	50	20	20	20	4	2	2	3
UPN002	64	M	2	50	10	10	10	2	0	1	1
UPN003	72	F	2	80	10	10	10	4	2	2	1
UPN004	47	F	2	70	20	30	50	3	2	2	2
UPN005	41	M	2	70	40	40	50	3	2	2	2
UPN006	66	F	2	80	10	10	10	4	3	3	1
UPN007	55	M	2	70	30	40	30	4	2	2	1
UPN008	57	M	2	50	20	10	10	2	2	1	1
UPN009	**66**	**F**	**2**	**50**	**40**	**50**	**50**	**2**	**2**	**2**	**2**
UPN010	**72**	**M**	**2**	**50**	**50**	**50**	**50**	**2**	**2**	**2**	**2**
UPN011	**69**	**F**	**2**	**30**	**10**	**30**	**30**	**4**	**1**	**4**	**4**
UPN012	**52**	**M**	**2**	**70**	**50**	**70**	**70**	**2**	**1**	**2**	**2**
UPN013	**52**	**F**	**1**	**90**	**80**	**90**	**90**	**3**	**3**	**3**	**3**
UPN014	**53**	**M**	**2**	**60**	**60**	**60**	**60**	**0**	**0**	**0**	**0**
UPN015	**35**	**M**	**1**	**40**	**10**	**10**	**10**	**1**	**0**	**1**	**1**
UPN016	72	F	3	50	20	30	30	2	2	1	1
UPN017	73	F	3	70	10	40	40	4	2	2	1
UPN018	54	F	3	70	30	20	30	3	2	1	1
UPN019	69	F	3	50	10	0	0	2	0	0	0
UPN020	69	F	3	60	20	20	20	3	2	2	1
UPN021	73	F	3	70	20	20	20	4	1	2	2
UPN022	75	M	3	60	20	20	30	3	2	1	1
UPN023	62	M	3	80	10	10	10	3	1	1	1
UPN024	61	F	3	90	60	50	50	5	3	3	3
UPN025	61	F	3	90	30	20	20	4	3	3	3
UPN026	53	F	3	90	70	70	70	3	2	2	2
UPN027	**70**	**M**	**3**	**70**	**70**	**70**	**70**	**3**	**3**	**3**	**3**
UPN028	**67**	**M**	**3**	**50**	**10**	**40**	**50**	**3**	**3**	**3**	**3**
UPN029	**73**	**M**	**3**	**70**	**50**	**60**	**60**	**2**	**2**	**2**	**2**
UPN030	74	F	3	40	10	20	80	4	4	3	4
Median (range)	66(35–77)	17F/13M	3(1–3)	70(30–90)	20(10–80)	30(0–90)	30(0–90)	3(0–5)	2(0–4)	2(0–4)	2(0–4)

Note: Bold values identify nonresponder patients; response was defined as a reduction of 20 mm of the VAS scale and at least a class in Lequesne scale; Kruskal Wallis nonparametric test confirmed in all patients' series a significant decrease of VAS and Lequesne scale during follow up (T1,T2, primary objective; $p < 0.00001$ and $p = 0.00032$, respectively). Posthoc Dunn for Kruskall Wallis test showed that a significant decrease in VAS and Lequesne scales occurred early at T1 and this result was maintained at later time points (T0 vs. T1 $p = 0.000001$, T0 vs. T2 $p = 0.000015$, T0 vs. T3 $p = 0.00013$, whilst $p > 0.05$ for T1 vs. T2, T1 vs. T3 and T2 vs. T3).

Abbreviations: T0, at enrollment, T1, 1-month follow up; T2, 6-month follow up; T3, 12-month follow up; UPN, unique patient number.

d. By recording patient's need of analgesics and anti-inflammatory drugs.

2.2 | Autologous blood collection, ACS preparation, storage and administration

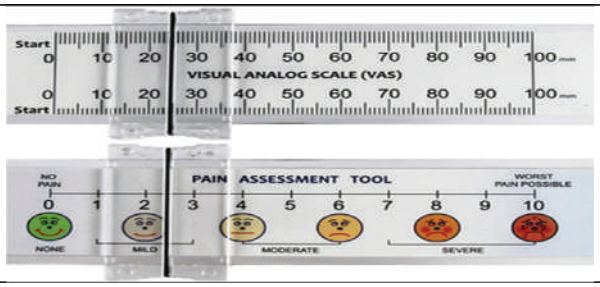
Sixty millilitres of autologous venous blood had been collected in each enrolled patient by a 60 ml dedicated syringe containing glass beads (Orthokine II, Orthogen Lab Services GmbH, Dusseldorf) in the absence of anticoagulation. Concomitant samples of 8 ml of venous blood had been collected in Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) to perform serological tests for HIV, HBV and HCV, to confirm the absence of current or previous viral infections, and blood counts with leukocyte differentials. Syringes containing whole blood and beads were directly incubated at 37°C in a 95% air–5% CO_2 incubator for 24 h and then centrifuged at 3000 g for 10 min to separate and collect ACS from the rest of whole blood.

Kellgren-Lawrence Scale

Grade	Radiologic findings
0	No radiologic findings
I	Doubtful narrowing of joint space and possible ostephytic lipping
II	Definite osteophytes and possible narrowing of joint space
III	Moderate multiple ostepohytes-definite narrowing of joint space-small pseudocystic areas with sclerotic walls and possible deformity of bone contour
IV	Large ostephytes-marked narrowing of joint space-severe sclerosis and definite deformity of bone contour

FIGURE 1 Kellgren-Lawrence scale to stage initial severity of patients' knee osteoarthritis

VAS scale



Lequesne scale

Parameter	Finding	Points
Pain of discomfort during nocturnal bedrest	None Only on movement or in certain positions Without movement	0 1 2
Duration of morning stiffness or pain after getting up	None < 15 min >= 15 min	0 1 2
Remaining standing for 30 min increases pain	No yes	0 1
Pain on walking	None Only after walking some distance Early after starting	0 1 2
Pain or discomfort after getting up from sitting without use of arms	No yes	0 1

FIGURE 2 VAS and Lequesne scale used to assess the degree of patients' response to ACS [Color figure can be viewed at wileyonlinelibrary.com]

ACS were aliquoted in five sterile syringes (Texium® Syringe, Becton Dickinson) with a nominal capacity of 5 ml, containing a volume of 2 ml of ACS each. An additional aliquot of ACS of 5 ml was subjected to sterility tests for aerobics, anaerobics and fungi. The ACS aliquots of 2 ml were then stored at −20°C until their use for intra-articular administration. Prior to use, ACS aliquots were thawed at room temperature. Each patient was treated by a single 2 ml aliquot injection in the involved knee joint per week, for a total period of treatment of four consecutive weeks. Each aliquot of 2 ml of ACS was injected by the Texium Syringes connected with a filter of 0.22 um (Millex GP, Merck Millipore, Burlington, MA, USA) and a proper needle after that a sterile field had been obtained on the site of injection following standard disinfection procedures.

2.3 | Cytokine concentration assay in patients' serum and ACS

Orthokine system allows the production of ACS enriched in anti-inflammatory cytokines and in particular in the Interleukin-1 Receptor Antagonist (IL-1RA), a glycoprotein produced by monocytes which has an inhibitory action against interleukin-1, a proinflammatory cytokine involved in inflammatory processes of arthritic diseases. Therefore aliquots of patient serum before treatment and all ACS productions were collected and kept frozen at −80°C until testing the cytokine panel. The following set of soluble factors were quantified by Luminex ProcartaPlex immunoassays (ThermoFisher Scientific, Waltham, MA, USA): interleukin-10 (IL-10), IL1-RA, tumour necrosis factor alfa (TNF-a), interleukin-6 (IL-6) and interleukin-1 beta (IL1-b).

2.4 | Statistical analysis

Result analysis has been carried out by SPSS 15.0 software for Windows (Statistical Package for Social Science, SPSS Inc., Chicago, IL, USA). Shapiro–Wilk normality test was initially used to confirm that our data were not normally distributed, so that non parametric tests were subsequently used for data comparison . The whole results observed for Lequesne and VAS scales were compared at T0, T1, T2 (primary objectives) and T3 (secondary objective) by Kruskal Wallis

non parametric test. This test was also applied after patients' stratification for Kellgren-Lawrence scale, grouping patients in 1–2 and 3 series, to assess if clinical improvement was related to initial disease severity. Then, Wilcoxon test for paired data was applied to compare VAS and Lequesne data on responder patients by comparing both T0 and T3 and T2 and T3 to verify the persistence of response at 12-month follow up. Fisher exact test was used to evaluate the occurrence of adverse events related to ACS treatment in all patients. Mann–Whitney and Wilcoxon tests were used to compare cytokine concentrations in ACS of responder and nonresponder patients and between basal serum and ACS of all patients, respectively. A $p < 0.05$ was considered as significant in all statistical analysis.

3 | RESULTS

3.1 | Adverse events

All patients completed the treatment receiving four subsequent ACS injections in four consecutive weeks (a single injection per week). Adverse events were monitored from the time of first ACS injections and prior and after subsequent ACS injections. In nine patients out of 30 (30%; $p = 0.001$ at Fischer exact test) we observed mild adverse reactions after the first ACS, consisting in pain and knee edema in seven cases and knee edema only in two cases. In six cases these conditions required treatment by ice application at the site of pain/edema (two cases), analgesics alone (one case) or analgesics and ice application (three cases). After second ACS injection seven patients (these patients were part of those who experienced adverse events after the first ACS injection; 23% $p = 0.010$) experienced mild adverse reactions which were edema in two cases, pain only in one case and pain and edema in four cases. In five cases these clinical conditions required treatments which consisted in ice application in four cases and analgesics in one case. After the third and fourth ACS injections we observed five and one adverse reactions (these patients were part of those who experienced adverse events after the first and second ACS injections), respectively, ($p > 0.05$) which did not require any treatment except ice application in two and one cases, respectively.

3.2 | Rate of response

The rate of response at T1 and T2 of whole patients' series were 67% for both time points with 20 patients in the responder group and ten patients in nonresponders (Table 1). This result was also confirmed at T3, after a follow up of 12 months. The median value of VAS scale decreased from 70 to 30 and of Lequesne from 3 to 2 for all patients at any follow up time point. The median value of VAS scale decreased from 70 to 20 and of Lequense from 3 to 1 in responder patients at any time point, including at last follow up of T3 (1 year; Figure 3). Kruskal Wallis non parametric test confirmed in all patients' series a significant decrease of VAS and Lequesne scale during follow up (T1,T2, primary objective; $p < 0.000$ and $p < 0.000$, respectively). Post-hoc Dunn for Kruskall Wallis test showed that a significant decrease in VAS and Lequesne scales occurred early at T1 and this result was maintained at any later follow up time point (T0 vs. T1 $p < 0.000$, T0 vs. T2 $p < 0.000$, T0 vs. T3 $p < 0.000$, whilst $p > 0.05$ for T1 vs. T2, T1 vs. T3 and T2 vs. T3). In responder patients Wilcoxon test for paired data demonstrated that also at 1 year follow up (T3, secondary objective) VAS and Lequesne scales decreased significantly ($p < 0.000$ for both scale). In responders, comparison of VAS and Lequesne scales between T2 and T3 showed no significant differences, indicating a persistence of the therapeutic effect at the latest follow up ($p > 0.05$). When patients were stratified for Kellgren-Lawrence scale at enrollment, we observed a significant decrease in Kellgren-Lawrence 1–2 and 3 groups for both VAS and Lequesne scale ($p = 0.004$ and $p < 0.000$ for VAS scale in Kellgren-Lawrence 1–2 and 3, respectively; $p = 0.044$ and $p = 0.004$ for Lequesne scale in Kellgren-Lawrence 1–2 and 3, respectively; Figure 4). These results indicated no significant

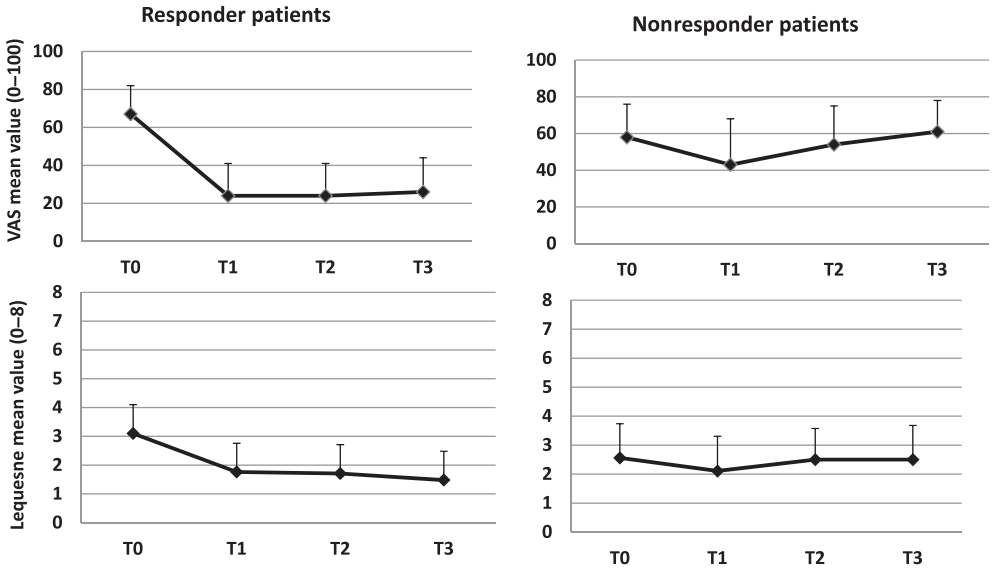


FIGURE 3 VAS and Lequesne scale values in responder and nonresponder patients' at different time points. Results are presented as the mean value (+*SD*). T0, enrollment; T1, 1 month, T2, 6 months, T3, 12 months from treatment. ($p = 0.00014$ between T0 and T3 for both VAS and Lequesne scales at Wilcoxon test in responder patients; $p > 0.05$ between T0 and T3 for both VAS and Lequesne scales at Wilcoxon test in nonresponder patients)

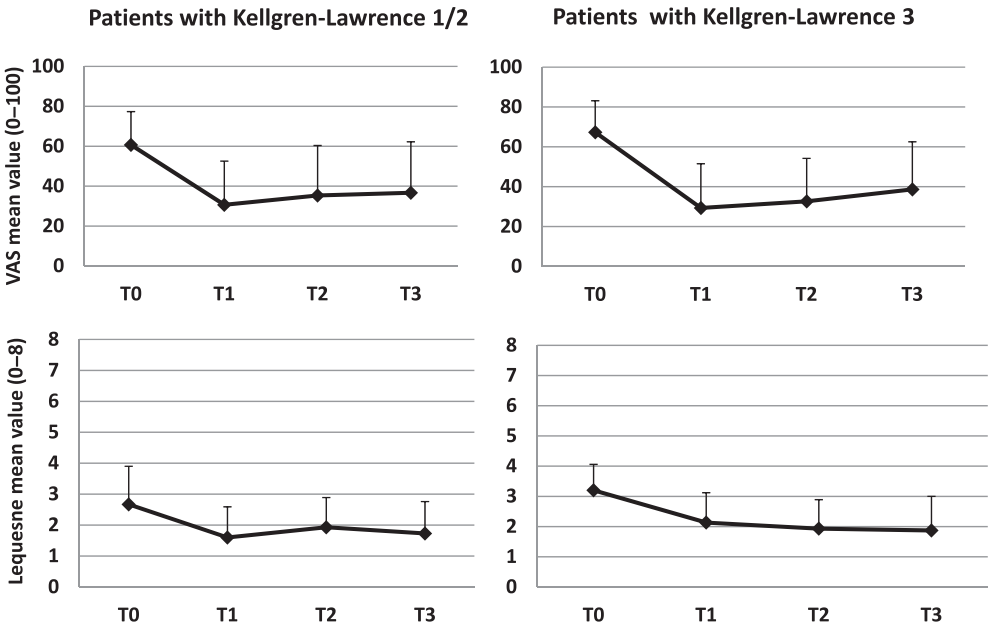


FIGURE 4 VAS and Lequesne scale values in Kellgren-Lawrence 1–2 and 3 at different time points for all patients' series (responders and nonresponders). Results are presented as the mean value (+*SD*). T0, enrollment; T1, 1 month, T2, 6 months, T3, 12 months from treatment. ($p = 0.00446$ and $p = 0.00023$ for VAS scale in Kellgren-Lawrence 1–2 and 3, respectively, at Kruskall Wallis test; $p = 0.04491$ and $p = 0.00498$ for Lequesne scale in Kellgren-Lawrence 1–2 and 3, respectively, at Kruskall Wallis test)

relationship between the severity status of knee OA and response to ACS treatment at any time point. All responders patients discontinued medical treatments and all avoided surgery and maintained response at a median follow up of 24 months. No significant relationship was found between response and patients' gender, age, weight, duration of disease prior ACS injection, degree of edema at enrollment or the traumatic or nontraumatic nature of the disease (indeed, the rate of response in nontraumatic OA was 60% vs. 80% in traumatic, a trend that, in any case, did not reach statistical significance; $p = 0.246$ at Fischer exact test). Finally, we administered a questionnaire to all treated subjects to assess the level of patients' satisfaction with respect to ACS treatment after 6 months of follow up. A six-dimensional evaluation with a score ranging from 1 (total dissatisfaction/clear worsening) to 5 (full satisfaction/great improvement) for the following questions gave the following results: (a) pain control, median score 4; (b) autonomy in routine daily activities, median score 4; (c) treatment efficacy, median score 4; (d) treatment tolerability, median score 5; (e) treatment expectations, median score 4; (f) treatment reliability, median score 4.

3.3 | Cytokine concentration in ACS and patients' serum at baseline

Several cytokines were assayed in patients' basal serum and in aliquots of their ACS by Luminex High Performance assay. The concentration of the following biological factors were quantified: IL-10, IL1-RA, TNF-a, IL-6 and IL1-b. Relevant differences in concentrations were observed between basal serum and the corresponding ACS for all cytokine tested at Wilcoxon paired test (Figure 5(A)). Notably, IL1-RA showed a higher concentration in ACS of responder patients, as compared to nonresponders, with an average concentration of 930 and 200 pg/ml, respectively. However, no significant differences

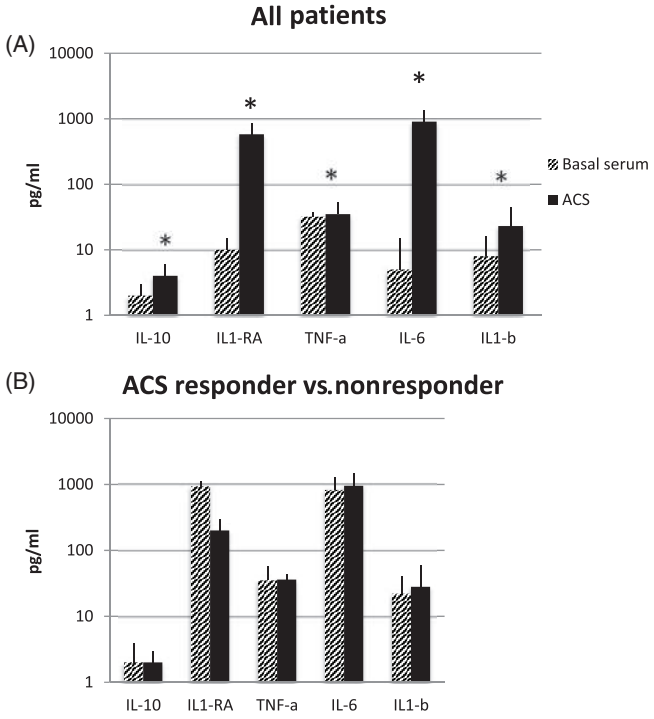


FIGURE 5 (A) Cytokine level in basal serum and ACS by Luminex ProcartaPlex immunoassays. Results are presented as the mean values +/-*SD*. *Statistically significant at Wilcoxon paired test ($p = 0.0009$ for IL-10, $p < 0.00001$ for IL1-RA, $p = 0.00138$ for TNF-a, $p = 0.00096$ for IL-6 and $p = 0.00018$ for IL-1b). (B) Cytokine level in ACS of responders (20 patients) and nonresponders (10 patients). Results are presented as the mean values +/-*SD*. $p > 0.05$ between any cytokine concentration of responders and nonresponders at Mann–Whitney U test

were observed between cytokine concentrations of ACS in responders and nonresponders, including IL1-RA at Mann–Whitney U test (Figure 5(B)).

4 | DISCUSSION

Knee OA is a frequent and disabling disease of joints that occurs in subjects with a wide age range, being related to several pre-existing conditions, including the increasing occurrence of obesity in several populations of developed countries.[1–5] This disease is progressive and when medical treatments are ineffective it may require surgical intervention consisting in the placement of knee prosthesis. In these last years, injection of ialuronic acid or autologous blood products has contributed to delay surgery in those patients who did not respond to standard medical treatments for pain control.[6,8–11] In particular, the injection of PRP in knee joints of patients with OA resistant to standard medical treatments produces long-lasting pain control in more than half of treated patients, as reported by several studies.[6] On the other hand, patients who show resistance to PRP injection for pain control seems to have no chance of cure other than prosthetic surgery. The principle by which products based on autologous blood components should be effective in pain and inflammation control is the presence of anti-inflammatory cytokines and proregenerative factors, released by autologous blood cells, which are administered through intra-articular injections.[7] Failure of these blood-based treatments may reside in an ineffective release of these factors in autologous blood components due to an insufficient individual capacity of patients to transfer relevant factors from the intracellular compartments to plasma, associated or not with particular local joint conditions. Local treatments by blood-components for knee OA may be also attempted using innovative technologies aimed to potentiate factor release through ex vivo stimulation of patients' blood cells.[12] The application of this approach produces a patient serum which is reported to be enriched in anti-inflammatory factors, especially in IL1-RA. Local injection of this form of ACS seems to have potent anti-inflammatory activity, leading to pain control in several forms of OA.[8–10] Here we have investigated the effect of the Orthokine ACS production technology to treat 30 patients who failed pain control by standard medical treatments and a full cycle of PRP injection and who had as the sole chance of cure the placement of knee prosthesis. This study showed several important results:

a. All patients completed the planned scheme of therapy and around 70% of treated patients had prompt response to ACS treatment with few side effects.
b. The response was long-lasting since all responder patients maintained pain control and a recovered functional activity at 6 months and 1 year follow up.
c. Response was not related to Kellgren-Lawrence scale at enrollment so that both one–two and three grade patients had a similar proportion and duration of response to ACS.

All responder patients (20 patients out 30 treated) have postponed any surgery over the time with discontinuation of all medical treatments in all of them. This study is the first report of ACS treatment in a very well-defined group of patients with knee OA, refractory to non-surgical treatments, including recent PRP injections. A recent publication reported the use of ACS, compared to PRP, in an earlier stage of treatment of patients with knee OA, but it did not include patients who failed a blood component-based treatment, as in the present study.[13] Our cohort of patients represents the worse series that can be treated by a nonsurgical approach and this fact gives a preliminary demonstration of some relevant circumstances:

a. ACS can rescue patients who failed medical treatments and PRP injections.
b. ACS may control pain in critical patients with knee OA and is able to postpone knee prosthesis placement in these patients.

Furthermore, this study indicates that a higher amount of IL-RA may be found in ACS of responders, as compared to nonresponders. The average IL-RA level of responders was 930 pg/ml (200 pg/ml in nonresponders). However, this difference was not statistically significant due to the small size of comparison and to the very high individual variability of all cytokine concentrations in ACS. On the other hand, it may assumed, as preliminary observation, that an increased IL1-RA level in ACS might predict response. Indeed, also nonresponders increased IL1-RA level in their ACS as compared to baseline and experienced an initial mild response. Likely, suboptimal IL1-RA increase combined with a higher, albeit not statistically significant, concentration of IL-6 (951 pg/ml vs. 823 pg/ml, on average) in nonresponders ACS affected the persistence of the anti-inflammatory effect during time. In reference to this, an increased IL-6 concentration in synovial fluids has been related to worse symptoms and outcome in knee OA.[14,15] It is not so trivial to hypothesize that the balance between anti−/proinflammatory cytokines in synovial fluids makes the difference in long-lasting pain control and to support this view we found an IL1-RA/IL-6 average ratio of 1.13 and 0.21 in responders and nonresponders, respectively. In other words, a proper cytokine profile of ACS may turn off inflammation the first weeks after injection then allowing a local response of synovia which regulates production of inflammatory molecules during time. The characteristics of the cytokine profile of nonresponders support the hypothesis that these patients are unable to produce a prevalent amount of anti-inflammatory molecules at any time of their disease, with no chance to control pain with the planned four or more ACS injections.

5 | CONCLUSIONS

Overall, the present study indicates and confirms that ACS is a safe and effective treatment for knee OA;[9,16,17] particularly, it is able to rescue a good proportion of patients from orthopaedic surgery, once medical and PRP treatments have failed. On the other hand, to widen these results to the general population of patients with knee OA, as well as to replace PRP treatment with ACS after failure of standard medical interventions, larger controlled studies are required to confirm our encouraging data and to determine whether ACS may be a standard of treatment when current medical treatments fail.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTION

RL, AD and LP planned and designed the study; RL and AD enrolled patients' by clinical and laboratory evaluation, collected and produced autologous conditioned serum and evaluated patients' response during follow up; LP and MAI performed statistical analyses and LP wrote the manuscript; GC and FR made orthopaedic examinations and administered autologous conditioned serum by intra-articular injections; PI and DF performed cytokine assays and quality controls. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

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Impact of the use of hydroxyethyl starch in granulocyte apheresis using Spectra Optia

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Abstract

Objectives: To determine the impact of the use of hydroxyethyl starch (HES) in granulocyte apheresis using Spectra Optia.

Background: Granulocyte transfusion (GT) is a therapeutic option for neutropenic patients with severe bacterial or fungal infections. Recent studies in emergency medicine have shown the potential risk of using HES, which is routinely used in granulocyte apheresis to increase yield by sedimenting red blood cells. We hypothesized that the use of a newer device (Spectra Optia) would spare the need for HES.

Methods: We retrospectively compared granulocyte apheresis with HES (HES group, $n = 89$) and without HES (non-HES group, $n = 36$) using Spectra Optia.

Results: The granulocyte yield was significantly higher in the HES group (7.3×10^{10} vs. 2.0×10, $p < 0.01$) and was attributed to the difference in collection efficiency (36% vs. 7.7%, $p < 0.01$). The absolute neutrophil count on the following morning of GT was significantly higher in the HES group than in the non-HES group (2460/μl vs. 505/μl, $p < 0.01$). There were no significant differences in the occurrence of adverse events between the HES and non-HES groups. The renal function was unchanged in both groups after apheresis.

Conclusions: We demonstrated that the advantage of using HES remained unchanged in granulocyte apheresis using Spectra Optia.

KEYWORDS
apheresis, collection efficiency, granulocyte transfusion, hydroxyethyl starch

1 | INTRODUCTION

Bacterial and fungal infections during the neutropenic period remain one of the most important causes of mortality in patients with aplastic anaemia or in those who undergo intensive chemotherapy or stem cell transplantation.[1,2] Recent studies have clearly shown that the risk of severe infections, particularly fungal infections, is strongly associated with the degree of neutropenia (i.e., the duration and depth of neutropenia), which is indicated by the D-index, defined as the area surrounded by the neutrophil curve and the horizontal line at a neutrophil count of 500/μl.[3–5]

Thus, granulocyte transfusion (GT) has been recognised as a rational therapeutic option for neutropenic patients. In GT, granulocyte concentrates are collected from healthy donors after mobilisation with granulocyte colony-stimulating factor (G-CSF) with or without corticosteroids.[6,7] Numerous case series and reports have shown that GT could provide a substantial increase in the absolute neutrophil count (ANC) of the patient, resulting in clinical efficacy.[8–10] However, the most recent

and largest randomised controlled study ('RING study') failed to confirm clinical efficacy.[11] One of the conceivable reasons for this failure might be that more than one-quarter of patients received a mean dose less than the intended dose of granulocytes (4.0×10^{10} granulocyte per transfusion, that is, $\sim 0.6 \times 10^{9}$ cells/kg for a 70 kg subject).

The collection of granulocytes from healthy donors consists of two major phases: mobilisation and apheresis. In our previous study, we showed that the use of high-molecular-weight hydroxyethyl starch (HES) in granulocyte apheresis was associated with a significant increase in the yields of granulocytes, as well as with a reduction of contamination by red blood cells (RBCs) and platelets (PLTs) in the granulocyte concentrates.[12] The use of HES in this setting has been applied to promote the separation of granulocytes from RBCs by sedimenting the RBCs in the separation chamber of a cell separator device. On the other hand, HES has long been used in the field of emergency medicine as a volume expander in patients with sepsis or other critically ill patients.[13] Recent studies have shown the development of severe adverse effects of HES, including kidney injury or dysfunction, bleeding, and increased mortality.[14–17] Even though the actual volume of HES infused to the healthy granulocyte donor is remarkably small, it would be preferable if non-HES granulocyte apheresis were feasible.

Moreover, several studies have shown that a novel cell separator (Spectra Optia) could provide greater granulocyte yields than an earlier system (COBE Spectra), which was used in our previous study.[18,19] We hypothesized that the use of a newer device would spare the need for using HES. Thus, we modified the protocol of granulocyte collections to allow apheresis without the use of HES in GT for less critical patients who have infections, but not of a life-threatening severity, with the discretion of donors. Here, we conducted a retrospective analysis comparing the collections of granulocytes with or without HES when using a new cell separator, Spectra Optia.

2 | MATERIALS AND METHODS

2.1 | Donors

All consecutive granulocyte collections that were performed from November 2013 to September 2018 were included in this study. Details of the eligibility criteria for GT donors have been described in a previous study.[12] Donors underwent a thorough history check, physical examination, blood tests, chest radiography, electrocardiography, and abdominal echography prior to the first granulocyte mobilisation. Eligibility was judged by two haematologists who were not engaged in the treatment of the recipients. All donors signed an informed consent form prior to the procedures. This study was approved by the Institutional Review Board of the Hyogo College of Medicine.

2.2 | Granulocyte mobilisation

All GT donors underwent granulocyte mobilisation with G-CSF alone, by receiving 600 μg of filgrastim subcutaneously 12–18 h before granulocyte collection. Granulocyte collections were allowed to be performed three times per episode from a single donor. In repeated collections, the time intervals from the previous collections were equal to or greater than 3 days. In several cases, peripheral blood stem cell (PBSC) donors also served as granulocyte donors (PBSC-GT donors). In those cases, PBSC-GT donors received an evening dose of G-CSF (200 μg/m^2 of filgrastim or 5 μg/kg of lenograstim) on the day of the last session of PBSC collection and underwent granulocyte collection the following morning.[12] Donors who underwent three sessions of PBSC collection were not allowed to serve as GT donors to limit the exposure to G-CSF for up to 5 days, an approach routinely used in PBSC donors, which has been proved to be safe in previous studies.[20,21]

2.3 | Granulocyte apheresis

Granulocytes were harvested via peripheral venous access using a blood cell separator (Spectra Optia, Terumo BCT, Tokyo, Japan) with a granulocyte collection program (v11.3 PMN collection protocol). In the apheresis using HES (HES group), high-molecular-weight HES (Hespan, Braun Medical, Irvine, CA, USA) was used as an RBC-sedimenting agent. As an anticoagulant, 30 ml of trisodium citrate was added to 500 ml of HES in the HES group, and ACD was used in apheresis without HES (non-HES group). The target process volume of whole blood was set at 7 L; however, the collections were completed when 500 ml of HES was consumed in the HES group. The PMN collection program of Optia has an automated interface monitoring system placed in the chamber, which performs real-time interface monitoring, interpretation, and adjustment for flexible procedural control.[18] However, collection preference needs to be optimised in the non-HES group because the clear interface is not formed in the absence of HES. Thus, the collection preference was set automatically in the HES group and at 35 in the non-HES group after optimization. For other parameters, the default settings were used.

2.4 | Definitions and statistical analysis

Pre-apheresis ANC (pre-ANC) was defined as ANC examined just prior to the granulocyte collection. All data were provided as median and range. The collection efficiency 2 (CE2) was calculated as follows:

$$\mathrm{CE2}\,(\%) = (\text{granulocyte yield}\,(\text{cells})/(\text{pre}-\text{ANC}\,(/\mu l) \times \text{whole blood volume processed}\,(\text{ml}) \times 10^{3})) \times 10^{2}.$$

Comparisons between independent groups (i.e., HES group and non-HES group) were performed by using Mann–Whitney *U* test, and those between dependent groups (i.e., before and after apheresis) were performed by using Wilcoxon signed-rank test.

3 | RESULTS

3.1 | Donor characteristics

In the study period, 125 granulocyte collections were performed from 97 donors. The donor characteristics are shown in Table 1 with collection-based numbers. Eighty-nine granulocyte apheresis procedures were performed using HES (HES group), whereas 36 apheresis procedures were performed without HES (non-HES group). No statistical differences were found between groups in baseline parameters, including body weight, baseline WBC, baseline ANC, and baseline PLT count.

3.2 | Granulocyte collections

One collection in the non-HES group was terminated early due to a problem with venous access when 2 L of blood was processed, and it was thus excluded from further analyses. Although the processed blood volumes during apheresis in the HES group ($n = 89$) were shown to be significantly smaller than those in the non-HES group ($n = 35$) due to the restriction in the volume of HES, the observed difference was small (7.0 L, range 4.2–7.5 L vs. 7.5 L, range 6.5–8.1 L, $p < 0.01$). Consequently, the volumes of the granulocyte concentrate in the HES group were significantly smaller than those in the non-HES group (505 ml, range 264–811 ml vs. 571 ml, range 464–592 ml, $p < 0.01$).

3.3 | Granulocyte yields and collection efficiency

Granulocyte yields and CE2 are shown in Figure 1A,B, respectively. Granulocyte yields in the non-HES group were shown to be significantly reduced than those in the HES group (2.0×10^{10} range $0.39–7.9 \times 10^{10}$ vs. 7.3×10^{10} range $1.8–24 \times 10^{10}$ $p < 0.01$), which was attributable to the significant difference in CE2 (7.7%, range 2.2%–32% vs. 36%, range 16%–84%, $p < 0.01$).

TABLE 1 Granulocyte donor characteristics

	HES group ($n = 89$)	Non-HES group ($n = 36$)
Median age, years (range)	43 (19–65)	36.5 (19–59)
Sex (male/female)	34/55	23/13
Body weight (kg)	59.5 (40.7–91.2)	60.8 (40.7–111)
Baseline WBC ($\times 10^9$/L)	5.8 ± 1.8	6.5 ± 1.5
Baseline ANC ($\times 10^9$/L)	3.6 ± 0.2	4.0 ± 0.1
Baseline PLT count ($\times 10^9$/L)	237 ± 50.8	211 ± 61.4
PBST-GT donors (no/yes)	83/6	33/3
Number of the previous collections		
0	57	25
1	23	6
2	4	5
3 or more	5	0

Abbreviations: ANC, absolute neutrophil count; GT, granulocyte transfusion; HES, hydroxyethyl starch; PLT, platelet.

3.4 | Contamination of RBCs and PLTs in granulocyte concentrates and effects on donors

The volume of RBCs in granulocyte concentrates was shown to be similar in the HES and non-HES group, as shown in Figure 2A (43 ml, range 10–132 ml vs. 33 ml, range 18–135 ml, $p = 0.16$). In contrast, the number of contaminated PLTs was observed to be significantly higher in the non-HES group than that in the HES group, as shown in Figure 2B (31×10^{10} range $12–63 \times 10^{10}$ vs. 11×10^{10} range $2.1–22 \times 10^{10}$ $p < 0.01$). The haemoglobin and PLT levels after apheresis were significantly lower than those before apheresis in both HES and non-HES groups, as shown in Figure 3A,B. While a decrease in haemoglobin levels was more evident in the HES group, the decrease in PLT levels was more evident in the non-HES group.

3.5 | Adverse events in granulocyte donors

The adverse events occurring during granulocyte apheresis are shown in Table 2. There were no significant differences in the occurrence of adverse events between the HES and non-HES groups. Moreover, to examine the possible effect of HES on renal function, the creatinine levels between the baseline and follow-up visit (~1 month after granulocyte collections) were compared in patients in whom both data were available ($n = 24$ in the HES group and $n = 33$ in the non-HES group). As shown in Figure 4, the renal function was unchanged in both the HES and non-HES groups. Pruritis was reported in one patient in the HES group at the follow-up visit.

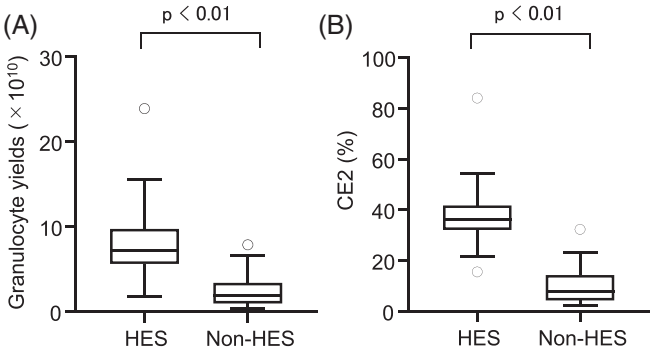

FIGURE 1 Effect of the use of hydroxyethyl starch (HES) in granulocyte apheresis. Box-and-whisker plots are shown with Tukey's method. Granulocyte yields (A) and CE2 (B). The use of HES was associated with significantly improved granulocyte yields

3.6 | Increase in the number of neutrophils in recipients of granulocyte concentrates

The ANC on the morning of GT (pre-GT) and the following morning (post-GT) in recipients of granulocyte concentrates were compared between the HES and non-HES groups (Figure 5). The pre-GT ANC was observed to not be statistically different between the HES and non-HES groups (30/μl, range 0–1330/μl vs. 20/μl, range 0–1000/μl). In contrast, the post-GT ANC was demonstrated to be significantly higher in the HES compared with the non-HES group (2460/μl, range 270–17 860/μl vs. 505/μl, range 60–2490/μl, $p < 0.01$).

4 | DISCUSSION

Although we expected that the use of Spectra Optia might partly compensate for the necessity of HES in granulocyte apheresis, the present study demonstrated the advantage of using HES in terms of granulocyte yields. Indeed, CE2, which is the most direct marker for the evaluation of the effect of HES, was demonstrated to be less than one-third in the non-HES group (7.7% vs. 36%). Consequently, a significant difference was observed in granulocyte yields. Previous

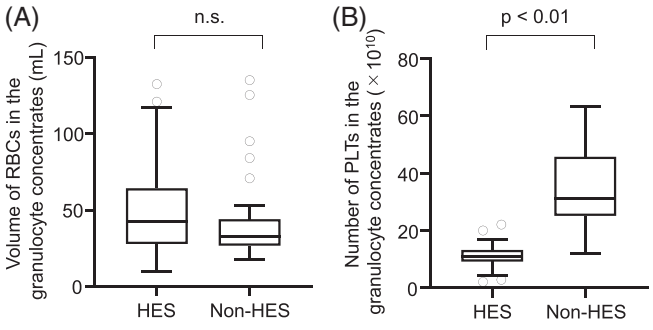

FIGURE 2 Effect of the use of hydroxyethyl starch (HES) on the contamination of granulocyte concentrates by other blood components. Volume of RBCs (A) and number of platelets (PLTs) (B). The use of HES was associated with significantly reduced contamination by PLTs

TABLE 2 Adverse events during granulocyte apheresis

	HES group ($n = 89$)	Non-HES group ($n = 36$)
Vasovagal reflux	2 (2.2%)	1 (2.7%)
Venous access problems	1 (1.1%)	1 (2.7%)
Citrate reactions	0 (0%)	1 (2.7%)
Malaise	1 (1.1%)	0 (0%)

Abbreviation: HES, hydroxyethyl starch.

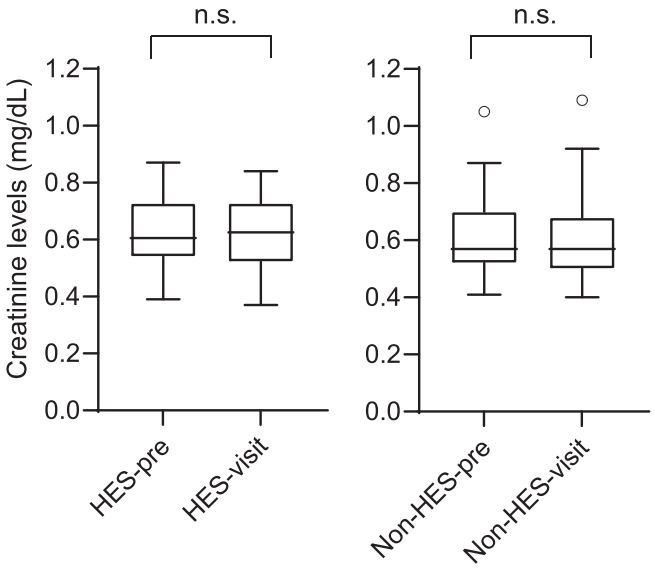

FIGURE 4 Serum creatinine levels at the baseline and follow-up visits, ~1 month after granulocyte collection

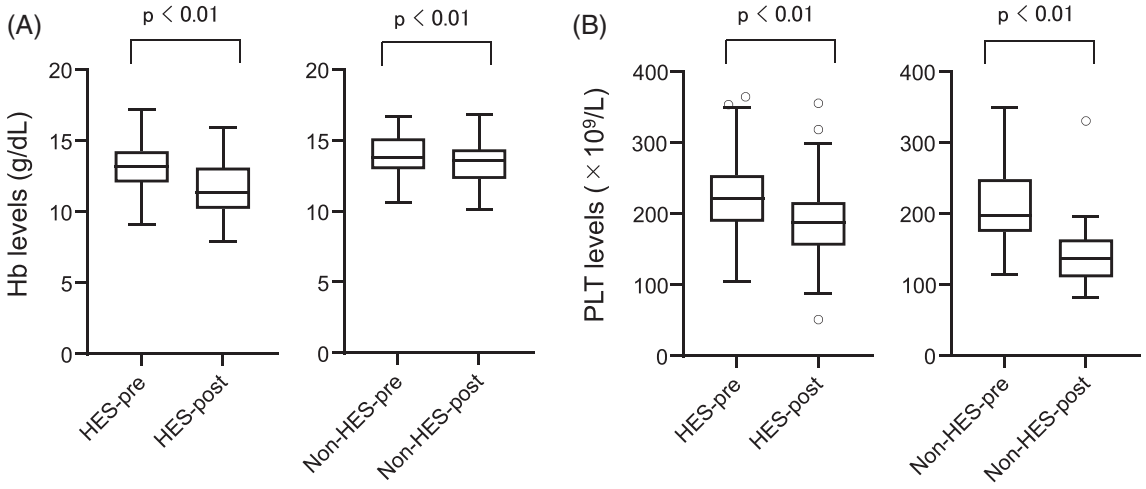

FIGURE 3 Haemoglobin (A) and platelet (PLT) (B) levels before and after granulocyte apheresis

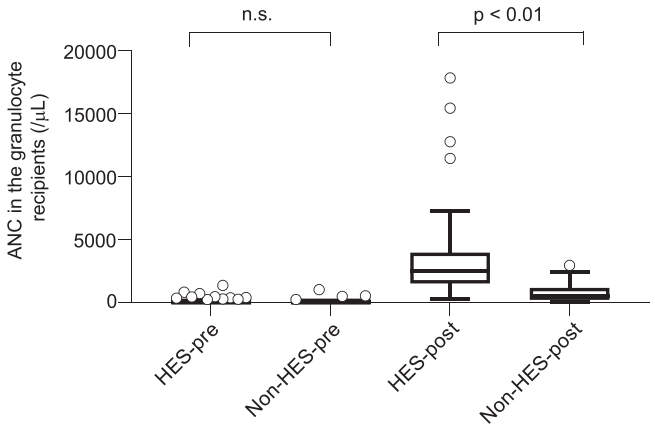

FIGURE 5 Effect of the use of hydroxyethyl starch (HES) on absolute neutrophil count (ANC) in recipients of granulocyte concentrates. The use of HES was associated with higher ANC in recipients on the following morning of granulocyte transfusion (GT)

studies have shown the clinical efficacy of GT to be highly dependent on transfusing an appropriately high dose of granulocytes. Whereas 95% of the granulocyte collections in the HES group exceeded the targeted dose of the RING study (i.e., 4×10^{10} granulocytes), only 14% was demonstrated to exceed it in the non-HES group. Indeed, we showed that the difference in granulocyte yields translated into the different increase in ANC in patients receiving GT. Although the current study did not intend to evaluate the clinical efficacy in recipients of granulocytes, it would be reasonable to assume that the usage of HES in granulocyte apheresis might be associated with better clinical efficacy.

Regarding the adverse events in granulocyte donors, in general, there were no significant differences between HES and non-HES groups. In both HES and non-HES groups, the haemoglobin and PLT levels after apheresis were significantly lower than those before apheresis. While a decrease in the haemoglobin levels was more evident in the HES group, reflecting the effects of HES as a volume expander, a decrease in the PLT levels was more evident in the non-HES group, reflecting the larger amount of PLT contamination in the granulocyte concentrates in non-HES. It is worth noting that we demonstrated that there were no significant changes in the creatinine levels between the baseline and follow-up visits (~1 month after granulocyte collection) in both the groups. While renal damage is one of the most important adverse effects with the use of large amounts of HES, the current finding confirmed that the use of a small amount of HES in granulocyte donors does not affect renal function. Pruritis, which is a common adverse event with the use of HES,[22] was reported in only one patient in the study; however, it might be because of under-reporting of mild events.

Several other studies have explored other RBC-sedimenting agents apart from high-molecular-weight HES, which has been the standard and was used in the current study. Recently, Nannya et al. reported the efficacy of 130 kDa HES, which is categorised as low- to medium-molecular-weight HES, in granulocyte collections from seven donors.[23] They performed granulocyte apheresis for two consecutive days using Spectra Optia; the CE2 on days 1 and 2 of apheresis was reported to be 14.5% ± 2.8% and 13.7% ± 2.8%, respectively. Although 130 kDa HES has superior accessibility compared with high-molecular-weight HES in several countries, including Japan, the collection efficiency in that study was shown to be moderate and similar to that of the non-HES group in the present study. Nonetheless, the granulocyte yields in their study ($5.27 \pm 3.10 \times 10^{10}$ and $2.91 \pm 2.92 \times 10^{10}$ on days 1 and 2 of apheresis, respectively) were better than that of the non-HES group in our study, which is attributable to the difference in the mobilisation protocol (i.e., the use of dexamethasone in their study). Additionally, although 130 kDa HES is considered to have fewer adverse events than those of high-molecular-weight HES, the use of 130 kDa HES has been shown to be associated with a higher mortality rate in critically ill patients than with Ringer's acetate or saline.[14,15] Thus, the risk–benefit profile of using 130 kDa-HES in granulocyte apheresis seems to be somewhat arguable.

Alternatively, Dullinger et al. used modified fluid gelatin (MFG) as an RBC-sedimenting agent in granulocyte apheresis, also performed for two consecutive days, and retrospectively compared it with the use of high-molecular-weight HES.[24,25] The median WBC collection efficiencies were reported to be lower with MFG than with HES on both day 1 (24% vs. 43%) and day 2 (15% vs. 37%).

In conclusion, our study demonstrated the advantage of using high-molecular-weight HES in terms of granulocyte yields in granulocyte apheresis using Spectra Optia. Although the present study showed no differences in the occurrence of adverse events between the HES and non-HES groups, the safety profile of HES in the setting of granulocyte apheresis should be further examined. In addition, alternative approaches, including the use of other RBC-sedimentation agents, other than HES, should be explored in future.

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CONFLICT OF INTEREST

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

Satoshi Yoshihara and **Junko Ikemoto:** Designed the study; **Keiko Fukunaga**, **Kyoko Yoshihara**, **Katsuji Kaida**, **Kazuhiro Ikegame**, **Hiroya Tamaki**, **Masaya Okada**, **Yuko Osugi**, **Kenichi Yamahara**, and **Satoshi Higasa:** Contributed to the data collection; **Satoshi Yoshihara**, **Junko Ikemoto**, **Hitomi Onomoto**, **Hiroki Sugiyama**, **Noriko Okuda**, and **Yoshihiro Fujimori:** Analysed the data; and **Satoshi Yoshihara**, **Junko Ikemoto**, and **Yoshihiro Fujimori:** Wrote the paper.

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ORIGINAL ARTICLE

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Molecular validation of pathogen-reduction technologies using rolling-circle amplification coupled with real-time PCR for torquetenovirus DNA quantification

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Abstract

Background: Pathogen reduction technologies (PRT) based on nucleic-acid damaging chemicals and/or irradiation are increasingly being used to increase safety of blood components against emerging pathogens, such as convalescent plasma in the ongoing COVID-19 pandemic. Current methods for PRT validation are limited by the resources available to the blood component manufacturer, and quality control rely over pathogen spiking and hence invariably require sacrifice of the tested blood units: quantitative real-time PCR is the current pathogen detection method but, due to the high likelihood of detecting nonviable fragments, requires downstream pathogen culture. We propose here a new molecular validation of PRT based on the highly prevalent human symbiont torquetenovirus (TTV) and rolling circle amplification (RCA).

Materials and methods: Serial apheresis plasma donations were tested for TTV before and after inactivation with Intercept® PRT using real-time quantitative PCR (conventional validation), RCA followed by real-time PCR (our validation), and reverse PCR (for cross-validation).

Results: While only 20% of inactivated units showed significant decrease in TTV viral load using real-time qPCR, all donations tested with RCA followed by real-time PCR showed TTV reductions. As further validation, 2 units were additionally tested with reverse PCR, which confirmed absence of entire circular genomes.

Discussion: We have described and validated a conservative and easy-to-setup protocol for molecular validation of PRT based on RCA and real-time PCR for TTV.

KEYWORDS

pathogen inactivation, pathogen reduction technologies, rolling circle amplification, torquetenovirus, validation

1 | INTRODUCTION

Pathogen reduction technologies (PRT) are being increasingly used to ensure safety of critical blood components such as plasma and platelets: progressive deployment has been often accelerated by emerging epidemics, such as Zika virus,[1,2] Dengue virus,[3,4] Chikungunya virus,[5] Yellow fever virus,[6] Middle East respiratory syndrome coronavirus[7] and severe acute respiratory syndrome (SARS)-1 coronavirus.[8] Different PRT brands exist on the market, having interstrand cross-links and fragmentation of nucleic acids in both blood cells and different classes

of pathogens as the common mechanism of action (MOA): when photochemicals are used, DNA fragmentation is triggered by ultraviolet irradiation (photoactivation) of the active ingredient[9]: formation of adducts between the photochemical and nucleic acid block replication, transcription and translation. Since both platelets and red blood cells do not harbour a nucleus, those useful blood cell types are not severely damaged (although there are evidences of lower post-transfusion platelet[10] and red blood cell[11] increments, and damage to platelet-derived microparticles (PMPs)[12]), and the blood unit can be safely transfused.

PRT has been mandated in several countries (e.g., Italy) to produce convalescent plasma under clinical trials for the ongoing COVID-19 pandemic.[13,14] This has led to many hospitals introducing PRT under emergency scenarios, with no time or facilities for in-house validation. In fact, PRT validation currently relies on pathogen spiking at definite concentrations,[15,16] followed by quantitative measure of pathogen loads before and after PRT, typically by quantitative real-time PCR.[17] The same adducts block the amplification of nucleic acids using PCR, in a manner that correlates with the number of adducts formed. Since the MOA of PRT includes breaking double-stranded nucleic acid regions, if the spiked pathogen is a single-stranded virus, the qPCR primers should target a hairpin region and be large enough to statistically ensure that at least one breakage occur within that region, which is often not the case with commercial qPCR kits. Additionally, spiking with some human pathogens should be done under BSL-3 rooms, which are not routinely available at blood banks.

We report here robust and fast validation of the Intercept® (Cerus Corp) PRT using rolling-circle amplification (RCA) followed by qPCR targeting the highly prevalent (>70%) human virome component torquetenovirus (TTV), a symbiont and highly prevalent circular single-stranded DNA virus.[18] This method does not require BSL-3 rooms and relies over PCR kits that are already commercially available in part.

2 | MATERIALS AND METHODS

2.1 | In silico study of secondary structure of TTV genome

The reference nucleotide sequences of human TTV species (in accordance with International Committee on Taxonomy of Viruses, ICTV) were retrieved from the National Center for Biotechnology Information nucleotide database (access number: AB008394, AY666122, AB041957, AF345523, AF435014, AF261761, AB054647, DQ187006, AB064607, AF34552424, AB045524, AB045526, AB045526, AB028668, AB017613, AX025830, AX025718, AB025946, AB060594, AF348409, AX174942, AB049607, AB060597, AB064595, AB064598, AB038621).

The secondary DNA structures of the genomes were predicted using the RNAfold algorithm (Vienna RNA package version 2.4.11). The analyses were conducted using the *-p -d2–noLP–circ–noconv* options and the DNA energy parameters were used for the prediction of the secondary structures.[19] The secondary structures obtained were then visualised using the VARNA software (version 3.93).

2.2 | Apheresis plasma donation

In preparation of activities for clinical trial NCT04393727 ("transfusion of convalescent plasma for the early treatment of patients with COVID-19 [TSUNAMI]"), the blood bank set up a PRT system based on Intercept® (Cerus Corporation). Briefly, twenty-two 600-ml single plasmapheresis units collected from healthy blood donor were exposed to amotosalen hydrochloride and ultraviolet light in the Int-100 illuminator according to manufacturer instructions. Pre- and post-inactivation plasma aliquots were stored at −20°C for successive studies. Post-inactivation samples were collected at the end of the PRT process, that is, after the compound adsorption device (CAD) step.

2.3 | TTV real-time PCR

Viral DNA was extracted from 200 μl of pre- and post-inactivation plasma samples by using QIAamp DNA Mini kit (QIAGEN, Chatsworth) associated with a QIAsymphony SP/AS instrument (QIAGEN, Chatsworth), according to the manufacturer's instructions. Extracted DNA was amplified by real-time PCR using TTV R-GENE® kit (BioMerieux, Marcy-l'Etolle) on a 7500 Fast Real Time System instrument (Applied Biosystems). The assay detects and quantifies TTV DNA in samples by using the 5′nuclease TaqMan technology, it is commercialised in the format of the ready-to-use amplification mixture, and it amplifies a 128-nucleotide long fragment within the untranslated region of the TTV genome, highly conserved among all the species in which TTV is actually classified by ICTV.

2.4 | Rolling circle amplification

Rolling circle amplification (RCA) is a rapid, very sensitive and isothermal single-stranded DNA amplification technique used for high-performance amplification of circular DNA viral genomes without the need of specific primers. RCA reaction was standardised carrying out in a 20 μl format by using an optimised mix with few nanograms of sample DNA (~10 ng), 25 μM of exonuclease-resistant random primer, 4 mM of dNTPs, and 10 U of φ29 DNA polymerase. Amplification was performed at 30°C for 17 h, followed by inactivation of φ29 DNA polymerase at 65°C for 10 min. The resulting linear double-stranded DNA product was spectrophotometrically quantified by NanoDrop Lite instrument (Thermo Fisher Scientific) and 10-fold diluted (range 100–1000 ng) to be used as the template in the TTV real-time PCR and/or universal TTV inverse-PCR for TTV quantification and full-length genome detection, respectively.

2.5 | TTV inverse PCR

Inverse PCR "cross validation" serves to demonstrate that intact TTV genome is present/absent in pre- and post-PRT treated samples.

Universal TTV inverse-PCR was performed in 50 μl consisting of 1× PrimeSTAR® GXL Buffer (Takara, Ohtsu), 200 μM dNTP mixture, 0.3 μM forward primer, 0.3 μM reverse primer, 1.25 U of PrimeSTAR® GXL DNA polymerase (Takara), and 500 to 1000 ng of template. Primers used are as follows: 206INV-TTVFor, 5′-CAAGGGGCAATTCGGGCTC-3′; 205INV-TTVRev, 5′-ACTNCGGTGTGTAAACTCACCT-3′. Positions of 5′-end nucleotide of primers in the reference TTV1 species genome (Accession number: NC_002076) were used as primer prefix names with either forward (For) or reverse (Rev) orientation. The PCR protocol was 35 cycles at 98°C for 10 s, 60°C for 15 s, 68°C for 4 min. The PCR products were separated on a 1% agarose gel in 1 x TAE buffer and visualised with Invitrogen SYBR Safe DNA Gel Stain (Thermo Fisher Scientific).

3 | RESULTS

Figure 1 reports the secondary structure of the genome of a representative TTV species, showing hairpin regions and PCR target in the commercial real-time PCR kit. There is huge secondary structure variability among different TTV species, but it can be generally considered that only one to few hairpin motifs are located within the PCR target region, making fragmentation of that region highly unlikely. Accordingly, TTV quantification by real-time PCR poorly differed between pre- and post-PRT samples (Table 1). TTV DNA remained essentially stable in most plasma samples; two samples, which were TTV positive at the first test, showed viral loads levels under the detection limit (< 1.0 Log DNA copies/ml) at the post-PRT sampling point. Only 6 of 22 samples (27%) showed a variation of viral load (e.g., > 0.5 Log; mean ± *SD*: 0.8 ± 0.4 Log) and in the sample that differed most, the change was 1.5 Log between the pre- and post-PRT sampling.

TABLE 1 TTV loads in pre- and post-PRT samples tested by real-time PCR

	TTV load (Log copies/ml)	
Sample	**Pre-PRT**	**Post-PRT**
1600	2.4	1.8
1693	3.7	2.9
2518	2.1	1.8
2534	2.7	2.6
2535	1.8	1.2
2538	2.1	1.7
2239	1.4	1.1
2564	2.7	1.2
2586	3.3	2.9
2682	1.8	1.6
2694	1.5	< 1.0
2725	1.9	1.7
2811	1.7	1.6
2816	1.9	1.8
2910	2.2	2.0
2913	2.1	1.6
2936	2.1	2.1
2939	1.5	1.2
2976	1.8	1.5
3012	1.6	< 1.0
3288	2.5	2.2
3299	3.2	2.6
Total	*2.2 ± 0.6*	*1.8 ± 0.6*

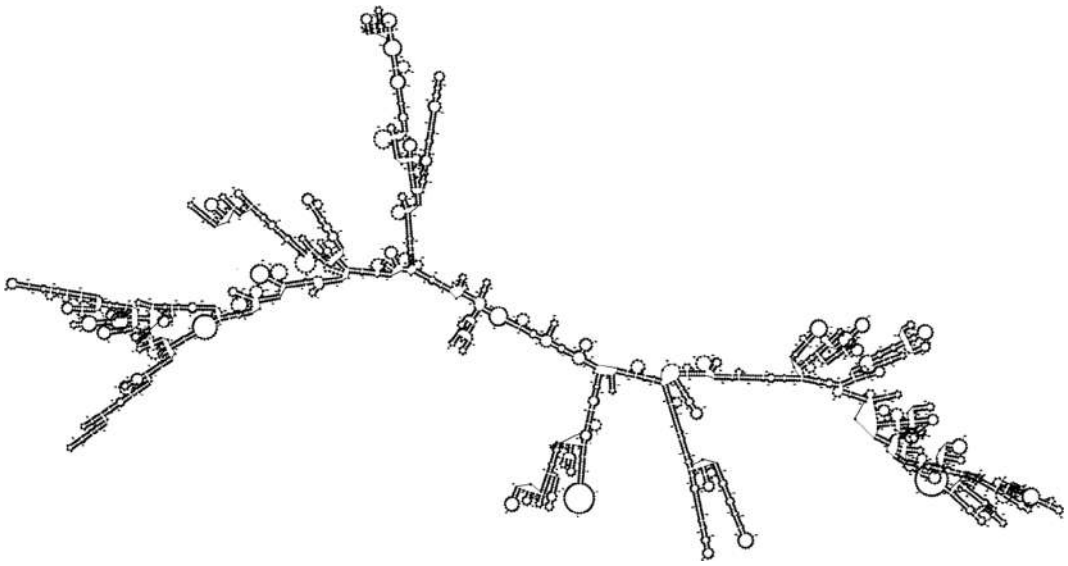

FIGURE 1 Schematic representation of the secondary structure of full-length genome of representative TTV species 1 (accession number: AB008394.1), with hairpins within the real-time PCR target region highlighted in grey

TABLE 2 TTV loads in pre- and post-PRT samples tested by RCA and real-time PCR

	TTV load (Log copies/ml)	
Sample	Pre-PRT	Post-PRT
1600	8.8 ± 0.8	< 1.0
1693	5.5 ± 1.0	2.5 ± 0.9
2586	11.3 ± 1.4	2.4 ± 0.7
3288	10.5 ± 0.9	< 1.0
Total	*9.0 ± 1.0*	*1.7 ± 0.8*

Note: All samples were tested in duplicates.

TABLE 3 Detection of full-length TTV genome in pre- and post-PRT samples tested by inverse PCR

	Detection of full-length TTV genome	
Sample	Pre-PRT	Post-PRT
1693	Positive	Negative
3288	Positive	Negative

Four of 22 samples were selected based on their TTV drop between pre- and post-PRT having a decrease in viral load >0.5 Log (sample 1600 and 1693) and < 0.5 Log (sample 2586 and 3288), respectively. When these samples were first amplified by RCA, the following TTV quantification by real-time PCR largely differed between pre- and post-PRT plasma samples (Table 2). Of the four post-PRT samples, two tested negatives for TTV DNA with a load reduction of more than 8.0 and 10.0 Log, respectively, relative to the corresponding pre-PRT samples. The other two post-PRT samples remained virus positive, but TTV dropped of about 9.0 Log in the sample 2586 and of 3.0 Log in the sample 1693. As shown in Table 3, an inverse PCR method used for amplifying TTV DNA revealed TTV as full-length genome (about 3.8 kb) in pre- but not in post-PRT sample 1693.

4 | DISCUSSION

Any licensed PRT system has already undergone validation as part of the regulatory process, and, on the face of it, further validation by blood banks is not strictly required. However, periodic internal quality controls and external proficiency testings remain in charge of the transfusion services. Current methods to validate or control PRT rely over pathogen spiking in blood units, which implies a need for expensive BSL3 facilities and trained personnel, both of which are not routinely available at peripheral community hospitals and especially within transfusion services. The tested donations must be eliminated and cannot be transfused, which is relevant in the setting of precious blood components.

Another major limit of the current method is reliance over real-time PCR. Real-time PCR is generally prone to amplification of fragments rather than entire genomes, resulting in only small differences between pre- and post-inactivation samples, which could suggest inefficacy of the PRT procedure. PRT using intercalants such as amotosalen only act on double-stranded genome regions by creating interstrand cross-links: for single-stranded viruses, these only occur in hairpin motifs, whose location can stay outside of the region targeted by primers. So, the chances of detecting differences in pre- versus post-PRT samples dramatically drop. Additionally, for viruses having variants in different hosts/donors, these hairpin motifs can occur within or outside the primer region across different species/genotypes, introducing additional variability and making results poorly standardised.

TTV is an anellovirus with extremely high prevalence in blood donors across different continents,[18,20] with prevalence increasing with age and peaking at 70%. The fact that every patient become TTV-positive after immunosuppression argues for universal prevalence, with low viral loads undetected by current methods. Although this lower-than-100% prevalence in healthy subjects makes it a non-universal marker, TTV remains the most prevalent and abundant virus in human peripheral blood,[18,20–23] and testing a minority of PRT-treated units provides reliable data for both quality controls and external proficiency testings. This can be achieved by pre-screening donor/units for TTV positivity. Alternatively, an enrichment step moving from higher volumes can increase the prevalence of TTV and avoid larger screenings.

This study has the limitation of not having run spiking with transfusion-transmitted pathogens to show direct correlation between their inactivation and inactivation of TTV. However, given that we used the same inactivation protocol which has been shown to be effective against clinically significant pathogens in multiple Intercept® registration and post-marketing studies, a correlation can be easily inferred. Additionally, TTV represents a challenging proxy, having been shown in the previous studies as highly resistant to different inactivation strategies.[24]

Anellovirus, being circular viruses, have the unique feature of having a continuous genome whose amplification is impaired by even a single breakage. Hence, a RCA step preliminary to a conventional real-time PCR is enough to detect differences between pre- and post-inactivation samples. This confidence avoids the need for passaging post-inactivation samples over replication-competent cell lines in order to discriminate whether the detected genomes are vital or not. Since the MOA of amotosalen does not involve capsid damage, but only nucleic acid damage, there is no reason to doubt about the vitality of the entire genomes detected by RCA.

Alternatives to TTV as a universal nonnuclear target already exist: several authors have reported endogenous mitochondrial DNA (mtDNA), which is detectable in PLT and plasma units, as a target for the quantification of photochemically induced modifications.[24–28] Human mtDNA is a circular chromosome that includes 16 569 base pairs and encodes 13 proteins: mtDNA is a universal marker and is obviously found in human blood cell products but at much lower loads in human plasma, impairing sensitivity of the method in such blood component. The main limitation of mtDNA when compared to TTV is the length of the genome, which in the case of mtDNA impairs the reliability of the RCA.

Limitations of the current study include the small number of samples that were assessed, and the inability to study a dose–response relationship by varying amotosalen and UV dose. Additionally, although theoretically similar, the results cannot be expanded to different PRT platforms such as Mirasol® or Theraflex®.

5 | CONCLUSION

We have shown that in-house validation of Intecept® PRT can be run conservatively without sacrificing previous blood units (such as convalescent plasma units), using a commercially available PCR kits and several modified PCR that can be easily setup at every microbiology laboratory, without need for BSL3 facilities and skilled personnel for pathogen manipulation, spiking and cell culture.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTION

Daniele Focosi designed the study and wrote the draft manuscript: Lisa Macera and Pietro Giorgio Spezia run virology tests; Fabrizio Maggi, Maria Lanza and Francesca Ceccarelli critically revised the manuscript.

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ORIGINAL ARTICLE

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Impact of microbial contamination of haematopoietic stem cells on post-transplant outcomes: A retrospective study from tertiary care centre in India

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Abstract

Background: Haematopoietic stem cells (HSC) may act as a source of infection for the recipient due to manipulation at multiple levels from collection to infusion. Due to the high risk of contamination cultures are usually taken during multiple steps. The clinical significance of microbial contamination of HSC on the post-transplant course and the role of prophylactic antibiotics is relatively unknown.

Aims and methods: The aim of our study is to investigate the incidence of microbial contamination of haematopoietic stem cell and to assess its impact on the post-transplant febrile neutropenia, engraftment kinetics, hospitalisation and day 100 mortality. Details of all patients admitted in the bone marrow transplantation unit of a tertiary care centre in India between January 2014 and December 2018 were collected from case records.

Results: Of the 1306 stem cell harvests from 503 patients sent for culture, 17 harvests (1.3%) were found to have a culture positive report. Sixteen patients had undergone autologous transplant. Multiple myeloma was most common indication of HSC transplant followed by Non-Hodgkin Lymphoma (NHL). Twelve of 17 HSC cultures were positive at the time of infusion and five were positive at the time of harvest. The five HSC that were culture positive at the time of harvest were culture negative at the time of infusion. Gram-positive organisms were isolated in six cultures and gram-negative in rest. All patients developed febrile neutropenia post-transplantation between day 1 and day 7. The median time of onset of fever was day +5 (1–7), the median duration of fever was 4 days (2–7), the median duration of antibiotic use was 11 days (9–16). Median day for neutrophil engraftment was 11 days (9–16), the median day for platelet engraftment was 14 days (10–25) and median duration of hospitalisation was 15 days (12–78). All patients were alive at day 100 of transplant.

Conclusion: This study shows that there appears to be minimal impact of culture positive HSC on transplant related outcomes in terms of engraftment kinetics, duration of hospitalisation and day 100 mortality. Discarding of contaminated HSC may not be

required, though on development of febrile neutropenia appropriate antibiotics should be administered based on sensitivity pattern of HSC culture. Larger prospective studies are needed to determine the clinical relevance of such contaminations. Emphasis should be laid on better infection control practices to minimise contamination rates.

KEYWORDS
haematopoietic stem cells, HSC, microbial contamination, transplant

1 | INTRODUCTION

Haematopoietic stem cell transplantation (HSCT) is commonly used treatment modality in various haematological malignancies. Haematopoietic stem cells (HSC) are sourced either from the patient or alternative sources namely, matched related donor, matched unrelated donor, haplo-identical donor, or cord blood. Currently peripheral blood HSC obtained after apheresis are most commonly employed due to early engraftment, ease of harvesting and cost-effectiveness.[1–3]

Stem cells can act as a source of infection for the recipient if these are contaminated due to manipulation at multiple levels from collection to infusion. Microbial contamination can be introduced at any stage namely, collection of stem cells by apheresis or other sources, processing, tumour cell purging, cryopreservation, storage, thawing of cryopreserved stem cells, and during infusion into recipient. Sources of microbes may be from contamination from skin flora, non-sterile handling and storage, or infected central line catheters. Due to high risk of contamination cultures are usually taken during multiple steps, immediately after collection and before stem cell infusion.[4,5]

The microbial contamination rate of stem cells varies in different studies.[6–24] The clinical significance of microbial contamination of HSC on post-transplant course is relatively unknown. Impact of microbial contamination on neutrophils and platelet engraftment, duration of febrile neutropenia, and patient morbidity and mortality are unclear. The available literature is scarce to provide clear insight on management of patient receiving contaminated HSC. In the past, few authors have discarded the microbial culture positive HSC. Role of prophylactic antibiotics in such scenario is unknown. Current data suggest no significant adverse impact of transfusing microbial contaminated stem cells on post-transplant outcomes. In the present study, we aimed to investigate the incidence of microbial contamination of haematopoietic stem cell and to assess its impact on the post-transplant febrile neutropenia, engraftment kinetics, duration of hospitalisation and day 100 mortality.

2 | METHODOLOGY

The records of all patients admitted in the bone marrow transplant unit of a tertiary care centre in India between January 2014 and December 2018 were analyzed. The study was conducted after taking approval from the Institute's ethics committee. During the period of review, a total of 1306 stem cell harvests were sent for aerobic bacterial cultures. Blood was drawn aseptically and incubated in the blood culture bottles (Bactec or conventional) after cleaning the cap. Then 10 ml of harvest product was sent from each harvest twice, first one after completion of stem cell harvest and second one just before transfusion of HSC. The blood culture bottles were sub-cultured on blood and Mac-Conkey's agar when they flagged positive or showed visible turbidity. The inoculum was plated across an area measuring 3 x 2 cm near the edge of the plate and spread using a sterile loop. Culture plates were aerobically incubated at 37°C overnight. The isolates were further identified using MALDI TOF-MS or standard biochemical tests. Antibiotic susceptibility testing was done for all clinically significant isolates using the Kirby Bauer disc diffusion method.[25] Those patients with culture positive HSC after harvest or at the time of transfusion were included in the study.

Clinical profile of patients, including diagnosis, comorbidities, indication of the transplant, duration from diagnosis to transplant, disease status at time of transplant, conditioning regimen, source of stem cells (autologous/allogenic), stem cell dose, details of cryopreservation were recorded retrospectively. Microbiological details of the isolated bacteria and their sensitivity was recorded. MDR bacteria were defined as bacteria that are non-susceptible to at least one antimicrobial agent in three or more antimicrobial classes. Details of episodes of febrile neutropenia including day of onset, duration, number of febrile episodes, focus of infection, use of prophylactic antibiotics, first line and further lines of antibiotics, response to antibiotics, and other infection work-up were recorded. Engraftment kinetics, packed red blood cells and single donor platelets transfusion requirement, and any other complication post-transplant were recorded. Response to antibiotic was defined as defervescence of fever within 48 h of initiation of treatment. Prophylactic ciprofloxacin, itraconazole and acyclovir were used in all patients undergoing HSCT as part of institutional policy. Prophylactic antibiotic was upgraded in patients according to sensitivity pattern of HSC culture.

3 | RESULTS

3.1 | Baseline characteristics

Of the 1306 stem cell harvests from 503 patients sent for culture, 17 patients (1.3%) were found to have a culture positive

report. Baseline characteristics of the HSC culture positive patient are shown in Table 1. Sixteen of the 17 patients had undergone autologous transplant and only one patient had allogenic HLA matched sibling transplant. Multiple myeloma was the most common indication of transplantation followed by Non-Hodgkin Lymphoma (NHL). HSC harvests were cryopreserved in eight patients and duration of cryopreservation ranged from 7 to 34 days.

3.2 | Microbiological profile

Five of the 17 cultures were positive at time of apheresis and 12 were positive at time of infusion. Five HSC that were culture positive at the time of apheresis, were sterile when cultures were sent before HSC infusion. Three of these were cryopreserved and the rest were kept at 4°**C** before transfusion. Gram-negative isolates were isolated in four out of five cultures. Out of 12 culture positive HSC at the time of infusion, only 3 had been cryopreserved. Eight of these were gram-negative isolates. Overall, gram-positive organisms were isolated in five and gram negative in rest. None of the harvest had polymicrobial growth.

Source of infection could be localised in only five patients. Two patients had lower respiratory tract infection and one each had urinary tract infection and periodontitis as source of infection. Only one patient had blood culture positivity post-transplant and culture yielded same organism (*Streptococcus viridans*) as HSC culture. Repeat

TABLE 2 Details of febrile neutropenia and engraftment kinetics

Parameter	Median (range)
Day of fever onset	Day +5 (day 1–7)
Duration of fever	4 days (2–7 days)
Duration of antibiotics use	11 days (9–16 days)
Neutrophil engraftment	11 days (9–16 days)
Platelet engraftment	14 days (10–25 days)
Duration of hospitalisation	15 days (12–78 days)
Day 100 mortality	NIL

TABLE 1 Clinical, microbiological profile and outcomes of patients with culture positive HSC

S. No	Age (years)/sex	Diagnosis	Month/Year of positivity	Conditioning regimen	Culture	Febrile neutropenia			NE	PE	Duration of stay in hospital (days)
						Fever onset	Fever duration	Antibiotic duration			
Culture positivity at time of HSC infusion											
1	35/M[a]	HL	September 2018	BEAM	*S. aureus*	4	7	6	9	13	15
2	47/M	MM	August 2018	Melphalan	*E. coli*	5	4	9	11	14	16
3	13/M[a]	AML	July 2018	CY-BU	*Acinetobacter sp*	1	4	14	12	25	15
4	41/F	MM	June 2018	Melphalan	*E. coli*	6	3	6	9	10	15
5	41/M[b]	B-ALL	May 2018	BUCY	*E. faecium*	3	3	20	16		78
6	38/M	MM	March 2018	Melphalan	*Acinetobacter sp*	5	7	9	13	15	18
7	40/F[a]	HL	January 2018	BEAM	*K. pneumonia*	5	2	7	10	11	15
8	48/M	MM	July 2016	Melphalan	*CONS*	6	3	6	12	12	14
9	42/M	MM	September 2017	Melphalan	*Acinetobacter sp*	5	5	10	10	13	12
10	50/F	MM	November 2016	Melphalan	*Acinetobacter sp*	5	4	10	12	15	16
11	30/M[a]	NHL	September 2016	CBV-R	*P. aeroginosa*	4	4	10	12	14	14
12	19/M[a]	PNET	May 2016	BU-MEL	*S. viridans*	5	4	7	9	11	16
Culture positivity at time of HSC apheresis											
13	54/M	MM	December 2018	Melphalan	*K. pneumonia*	7	2	5	11	13	14
14	65/F[a]	PTCL	September 2017	BEAM	*E. faecium*	2	5	15	10	24	37
15	2/F[a]	NB	June 2017	BU MEL	*E. coli*	7	5	14	12	13	17
16	51/F[a]	NHL	December 2015	BEAM	*Acinetobacter sp*	4	5	10	10	14	17
17	43/M	POEMS	May 2015	Melphalan	*E. coli*	4	2	11	11	17	15

Abbreviation: AML, acute myeloid leukaemia; B-ALL, B acute lymphoblastic leukaemia; BEAM, BCNU Etoposide Ara-c Melphalan; BU MEL, busulfan melphalan; CONS, Coagulase negative staphylococcus; CY-BU, cyclophosphamide busulfan; HL, Hodgkin's lymphoma; MM, multiple myeloma; NB, neuroblastoma; NE, Neutrophil engraftment; NHL, non-Hodgkin's lymphoma; PE, platelet engraftment; PNET, primitive neuro-ectodermal tumour; PTCL, peripheral T cell lymphoma.

[a]Cryopreserved harvest.

[b]Allogenic stem cell transplant (platelets not engrafted).

TABLE 3 Review of peripheral blood harvest contamination in published studies

Author, Year	Number of cultures sent	Number of positive cultures (%)	Common organisms	Any excess morbidity or mortality	Impact on outcome	Remarks
Kamble et al, 2005[17]	737 harvests	33 (4.5%) UC: 15% BM: 4.5% PB: 3.9%	CONS-17 *P. acne*-9 MSSA-4 MRSA-1	No	None	Four patients received prophylactic antibiotics. Clinical sequelae were rare after transfusion of contaminated HSC. Routine surveillance of HSC may be avoided.
Kelly et al, 2006[18]	1502	15 (1%)	CONS-8 GPC-3 GPB-2 GNB-2	One (7.7%) patient developed septicemia with CONS	None	13 patients transfused contaminated HSC and two grafts discarded. Only five received pre-emptive antibiotics. Contamination more in autologous HSCT.
Klein et al, 2006[19]	2935	35 (1.2%) UC: 2.0% BM: 1.3% PB: 0.7%	CONS-19 *Bacillus sp*-4	Two (5.7%) patients developed septicemia. One died due to septicemia.	Yes	Contamination was more common in autologous HSCT. Prophylactic antibiotics were given to all patients. Post-transplant blood culture was positive in two patients with same organisms and one died of septicemia.
Padley et al, 2007[20]	7233	119 (1.6%) BM: 3.1% PB: 1.6%	CONS-73 *S. aureus*-7	One (1.4%) patient developed septicemia with same species as graft.	None	No risk associated with infusion of contaminated HSC.
Patah et al, 2007[21]	3078	37 (1.2%)	CONS-32	No patient developed septicemia related to organism grown in harvest	None	22% culture positive HSC graft became culture negative after cryopreservation. Prophylactic antibiotics given in all patients. No infectious complication.
Donmez et al, 2003[22]	491	Post-processing-28 (5.7%), Post-thawing-18 (3.66%)	CONS most common (77%–85%)	Four (20%) patients developed septicemia with same species as graft.	None	Large volume leukapharesis and repeated sampling of HSC were associated with culture positivity. Cryopreservation decreased viability of microbes.
Almeida et al, 2014[23]	837	36 (4.3%)	CONS-20 *S aureus*-6	No	None	22 grafts infused, 14 discarded. 50% culture positive HSC graft became culture negative after cryopreservation.
Skrzypczak et al, 2014[24]	330	9 (2.7%) BM: 5.13% PB: 2.75%	CONS-3 *Bacillus sp*-4 *Pseudomonas*-2	Not available	Not available	Contamination more in BM HSC harvest.
Dal et al, 2016[8]	1552	18 (1.15%)	CONS-13	Two (22.2%) patient developed septicemia with same species as graft.	None	9 patients received contaminated culture and eight developed febrile neutropenia. Contaminated products with antibiotic prophylaxis may be safe in terms of the first day of fever, duration of fever, time of engraftment and duration of hospitalisation.
Present study	1307	17 (1.3%)	GNB-12 GPC-4	One (5.8%) patient developed septicemia with same species as graft.	None	All patients received contaminated HSC. Antibiotic prophylaxis given to all patients. No impact on engraftment kinetics.

Abbreviations: UC, umbilical cord; BM, bone marrow; PB, peripheral blood; CONS, coagulase negative staphylococcus; MSSA, methicillin sensitive *staphylococcus aureus*; MRSA, methicillin resistant *staphylococcus aureus*; GPC, gram-positive cocci; GPB, gram-positive bacilli; GNB, gram-negative bacilli; HSC, haematopoietic stem cell; HSCT, haematopoietic stem cell transplantation.

blood cultures of all patients after development of febrile neutropenia were sterile.

3.3 | Clinical profile

All patients developed febrile neutropenia post transplantation between day +1 and day +7. Duration of febrile episodes ranged from 2 to 7 days. Antibiotics were started in all patients according to hospital antibiotic policy. Empiric treatment for febrile neutropenia in 13 of 17 patients also covered for the organisms grown in the HSC culture. Eight of these patients responded appropriately to the initial treatment while nine patients required the addition of other antibiotics. In four patients, the empiric treatment did not cover the organism grown on pre-transplant HSC culture. These patients did not respond to the empiric treatment but responded to the second line treatment.

Details of febrile neutropenia and engraftment kinetics are mentioned in Table 2. All patients were alive at day 100 of transplant. Two patients had prolonged stay, one had urinary tract infection post neutrophil engraftment and a second patient had acute GVHD, CMV colitis, VOD, and hemorrhagic cystitis post allogenic transplant.

4 | DISCUSSION AND REVIEW OF LITERATURE

The incidence of contamination of HSC has been reported from 0.23%[6] to 26.4%[7] among various studies. Higher rates were reported in older studies, recent studies have reported relatively lower rates of contamination of HSC due to improvement in aseptic measures. Rates of HSC contamination, microbiological profiles and important conclusions from important past studies are summarised in Table 3. Various factors have been attributed to the contaminations of HSC, including, source of graft (umbilical cord/bone marrow/peripheral blood), and contaminants from normal skin flora during collection of HSCs, use of central venous catheters, laboratory processing of the HSC, poor cryopreservation, and repeated sampling for culture.[4–6]

The highest rates were observed with umbilical cord blood harvests followed by bone marrow harvest and least with the peripheral blood apheresis HSC. This may be attributed to multistep processing required in cases of cord blood and bone marrow harvests.[17–20,24] Studies have also observed higher rates of contamination with autologous transplant as compared with allogenic transplants.[19]

The role of cryopreservation in contamination of HSC is debatable. Microbes may not survive the cryopreservation process especially gram-positive organisms and there is substantial reduction in the bacterial load post cryopreservation.[21–23] In our study three harvests which were culture positive after apheresis became culture negative post cryopreservation. It is possible that the organisms did not survive cryopreservation. However, five harvests which were culture negative before cryopreservation, became culture positive after cryopreservation which may be due to contamination during handling of HSC after apheresis.

Majority of the studies show contamination by gram-positive skin colonisers (Table 2).[8,17–24] However, gram-negative organisms were commonly isolated in our series. Of the 12 gram-negative organisms, 6 were multi-drug resistant organisms.[26–28] Data from other transplant centres in India shows predominant gram-negative infections in post-transplant setting. Similarly, high rate of gram-negative sepsis was observed during treatment of acute myeloid leukaemia (AML) patients from our centre.[29] This suggests a possible source of nosocomial contamination and raises possible concern of infection through stem cells by resistant organisms.

Studies have shown no increase in immediate complications post infusion of contaminated HSC.[17,20] Our study showed that contamination was not associated with adverse outcomes. Similar findings were observed in a study by Dal et al.[8] Though in our study we did not analyse patient who received uncontaminated HSC, outcomes are similar to study by Kumar et al. from our centre.[30]

Blood culture positivity rate in febrile episodes in acute leukaemia patients from our institute varied from 19% to 24%. Majority of organism were gram negative (56%–63%) with predominance of *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Escherichia coli* species. These results are comparable to microbiological profile of current study.[29,31]

A policy of discarding contaminated HSC has been adopted in some studies.[9,18] However, with the available evidence, discarding contaminated cultures may not be recommended. It is suggested that such patients should be closely followed up for development of febrile neutropenia. Routine prophylactic antibiotics based on institutional policy should be continued.

To conclude, our study shows that there appears to be minimal impact of culture positive HSC on transplant related outcomes in terms of engraftment kinetics, duration of hospitalisation and day 100 mortality. Discarding of contaminated HSC may not be required, though on development of febrile neutropenia appropriate antibiotics should be administered based on sensitivity pattern of HSC culture. There is a need for larger prospective studies to understand the clinical relevance of such contaminations and need for antibiotic prophylaxis in such cases. Emphasis should be laid on better infection control practices to minimise contamination rates.

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SHORT COMMUNICATION

TRANSFUSION MEDICINE WILEY

D--phenotype due to RHD-RHCE hybrid transcript in a case of severe haemolytic disease of newborn with anti-Rh 17(Hr_o) antibodies

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Abstract

Background: D antigen is one among the most immunogenic antigens and is the most common cause of Haemolytic Disease of Fetus and Newborn (HDFN). The D-phenotype is a rare Rh variant in which none of the RhCE antigens are expressed on the red cell surface. Individuals having D-phenotype are capable of producing a rare alloantibody named as anti-Rh17(Hr_o) in response to pregnancy or transfusion and has the potential to react with C/c and E/e antigens causing severe haemolytic transfusion reaction (HTR) and haemolytic disease of fetus and newborn (HDFN).

Case report: We have encountered a case of severe HDFN with an accidental discovery of D- phenotype of the mother with anti-Rh-17 antibodies. D- phenotype has been confirmed with molecular typing along with genotyping of all family members.

Conclusion: Rare phenotypes like D- individuals especially if allo-immunised are of great concern at times of transfusion requirements. Hence, proper identification of these individuals are important to contribute them to the rare donor pool and to adopt adequate patient blood management strategies.

KEYWORDS
allo-antibody, D--phenotype, genotype, RhCE

1 | INTRODUCTION

The Rh blood group system consists of more than 60 antigens but D, C, c, E and e are considered as the main antigens of this system. These five Rh antigens are known to evoke alloantibodies following mismatched blood transfusions and are thereby clinically significant. They are encoded by two highly homologous *RH* genes, that is, *RHD* (D antigen) and *RHCE* (C, c, E, and e antigens) located on chromosome 1p36.11.[1] The D antigen is most immunogenic in nature and is the major cause of haemolytic disease of the fetus and newborn (HDFN). Polymorphic RHCE gene encodes for four antigens, of which C and c antigens vary by four amino acids, whereas E and e antigens have one amino acid variation.[2] The D--phenotype is a rare Rh variant in which none of the RhCcEe antigens are expressed on the red cell surface. In the absence of RhCE protein, D antigen expression enhances leading to the exalted D phenomenon. Rare Rh deficient phenotypes like Rh_{null}, D--, Dc-, and so forth are known to cause alloimmunization due to lack of one or more Rh antigens.[3] Individuals with D--phenotype are capable of producing a rare alloantibody named as anti-Rh17(Hr_o) in response to sensitization during pregnancy or transfusion and has the potential to react with C/c and E/e antigens, causing severe haemolytic transfusion reaction (HTR) and haemolytic disease of the fetus and newborn (HDFN).

1.1 | Case presentation

A 33 years old second gravida (G2P1L1A0) was brought to the obstetric casualty at 38 weeks of gestation following a premature rupture of membranes. She was making regular antenatal visits in a peripheral centre and her antenatal period was uneventful. The yellowish stained liquor at admission was misinterpreted to be meconium-stained and

an immediate caesarean section was performed owing to fetal distress. The birth weight of the baby was 2.74 kg and was born with features suggestive of HDFN. Samples of the baby and mother were sent to the blood centre for HDFN work up.

Routine blood investigations of the baby on cord sample revealed hyperbilirubinemia (S. Bilirubin—17.4 mg/dl) and anaemia (Haemoglobin—9 mg/dl). Peripheral smear showed fragmented red cells, nucleated cells and reticulocytosis (Reticulocyte count—25%). The sepsis screen was negative. The baby initially received quadruple surface phototherapy, which was followed by intravenous immunoglobulin (IVIG) infusion and transfusion of 40 ml of packed red cells. Transfusion was done on postnatal Day 3 and cross-match was done with the baby's serum. Gradually, hyperbilirubinemia and anaemia improved with falling serum bilirubin and rising haemoglobin values.

1.2 | Previous obstetric and medical history

The parents of the proposita had consanguineous marriage. The proposita already has got a healthy 6 years old female child in a non-consanguineous marriage with no previous history of abortions. The antenatal and postnatal period was uneventful and there was no history of previous blood transfusions during the antenatal period or after childbirth. The lady suffered carcinoma of the breast 3 years back for which she has undergone modified radical mastectomy and was on chemotherapy for one more year. There was no history of blood transfusions during surgery and chemotherapy.

1.3 | Immunohaematology work up

Immunohaematological workup of the proposita and her family (husband, first child, newborn, father, mother and sister) was performed (Table 1).

Direct antiglobulin test (DAT) and indirect antiglobulin test (IAT) of the baby were positive. The mother's DAT was negative but IAT was positive. Her Rh extended phenotyping was performed using Rh-extended phenotyping antisera from Ortho clinical diagnostics (Bioclone—Anti-C, c, E, e antisera) in conventional test tube technique and confirmed the finding in Rh-phenotyping cards by Biorad (Diaclon Rh subgroups +K Lot No: 50110.1902). Both these Rh phenotyping agents typed the sample to be D + C − c − E − e− (Figure 1). Suspecting this to be a case of D--, adsorption-elution test using cold-adsorption and heat elution method was performed, which confirmed the absence of C, c, E, e antigens. The maternal serum showed pan-reactivity with three red cell panel (Tulip Diagnostics—Reacell Lot No.722001) and 11 red cell panel (Tulip Diagnostics—Reacell Lot No.741919). Pan reactivity in 3 and 11 cell panel suggested the presence of anti-Rh-17 (Hr_o) antibodies in the patient's serum. We suspect the patient was sensitised during her first pregnancy and developed anti-Rh-17 (Hr_o) antibodies.

For molecular workup, the samples were sent to the ICMR-National Institute of Immunohaematology. DNA was prepared by the phenol-chloroform method. Proposita was screened for the presence of alleles specific for C, c, E and e antigens by polymerase chain reaction with sequence-specific priming (PCR-SSP).[4] PCR-SSP confirmed the absence of coding regions defining C, c, E and e antigens. To understand the molecular basis resulting in the absence of C, c, E and e antigens from the RBC surface, an invaluable technique quantitative multiplex PCR of short fluorescent fragments (QMPSF) was performed. Ten primers specific for *RHD* and *RHCE* exons were co-amplified along with internal controls *HFE* and *F9* in two different tubes. The product was run on a 3730xl DNA sequencer (Genetic Analyzer 3730xl, Applied Biosystems, United States) and data were interpreted using Genemapper software (Thermo Fisher Scientific, United States). Copy number analysis was performed as previously described.[5] *RHD*-QMPSF showed the presence of all 10 *RHD* exons. However, *RHCE*-QMPSF amplified only exon1 and 10, and thus revealing the formation of *RHCE-D*(3-9)-*CE* hybrid as the mechanism responsible for D--phenotype in the patient. Copy number analysis of family members showed both of her parents carrying a single non-functional copy of the RHCE gene. Both the children of the woman were found to be the carriers of D--haplotype.

2 | DISCUSSION

Finding a suitable donor for an individual with rare Rh deficient phenotypes such as Rh_{null}, D--, Dc- is difficult due to the scarcity of blood group-specific donors. Identification of these rare blood types is usually accidental mostly in cases with an unresolved cross-match incompatibility or during pregnancy with HDFN. An individual with D-- lack all four RhCcEe antigens with normal expression of D antigen and has

TABLE 1 Blood grouping, Rh extended phenotype and genotype

	Blood group	Rh extended phenotype	Rh genotype
Mother (Proposita)	A Rh(D) positive	D + C − c − E − e−	*D--/D--*
Newborn	A Rh(D) positive	D + C + c − E − e+	R_1/*D--*
First child	A Rh(D) positive	D + C + c − E − e+	R_1/*D--*
Husband	O Rh(D) positive	D + C + c − E − e+	R_1R_1
Parents and sibling of the index case			
Father	A Rh(D) positive	D + C + c − E − e+	R_1/*D--*
Mother	A Rh(D) positive	D + C + c − E − e+	R_1/*D--*
Sister	A Rh(D) positive	D + C + c − E − e+	R_1R_1

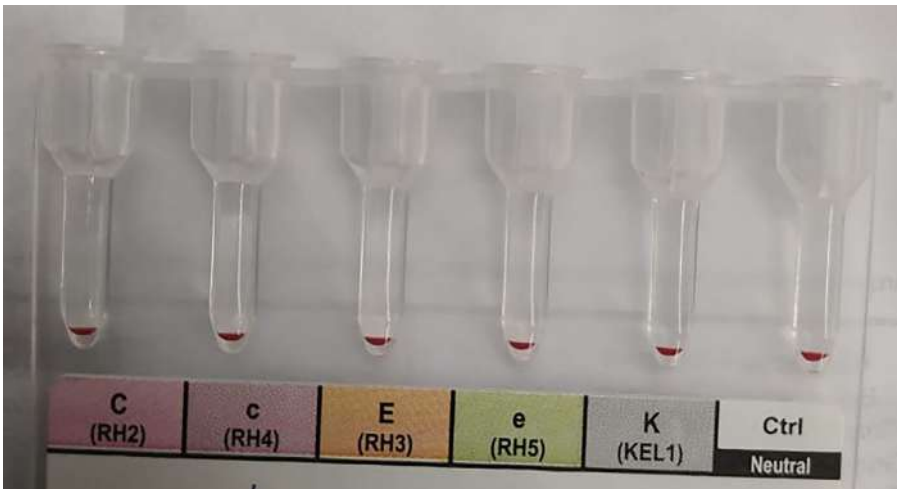

FIGURE 1 Rh extended phenotyping of maternal blood sample [Color figure can be viewed at wileyonlinelibrary.com]

been reported among many populations, including Asians, Caucasians and African Americans.

A woman during the second pregnancy delivered a child with severe HDN. The detailed serological analysis revealed mother is of rare Rh phenotype D-- and none of the RhCcEe antigens were present on the RBC membrane. However, the Rh phenotype of the husband and both the children were R_1R_1. Antibody identification using 11 cell panel detected anti-C and anti-e antibodies in maternal serum, which were probably produced during the first pregnancy. Hirose et al. in their study stated that first pregnancy in a D--/D-- a woman can also get affected as an anamnestic immune response and intrauterine transfusion may be warranted to avoid fetal jeopardy.[6]

Vooght et al. have mentioned the case of an alloimmunised D-- patient who has undergone multiple surgical procedures with the help of pre-deposit autologous blood collection and intraoperative cell salvage .[7] Cases producing Anti-Rh-17 are mostly reported from Japan as there is a relatively high frequency of the D--/D--phenotype (1 in 100 000) in their population.[6]

Various molecular mechanisms such as complete deletion of *RHCE* gene, replacement of the *RHCE* coding regions by *RHD* with *RHD* {*RHCE-D*(3-8)-*CE*, *RHCE-D*(3-9)-*CE*, *RHCE-D*(4-9)-*CE*, etc.}, SNPs like *RHCE**Ce(c.87insT), *RHCE**cE(c.221G>A), and so forth are associated with D--phenotype.[8] Lack of C/c and E/e antigens can be due to the reduced transcriptional activity or as a result of an integrated gene deletion of the RHCE gene. Another possibility is the formation of an *RHD-RHCE* hybrid with the lack of C/c and E/e antigens expression.[9] Replacement of the *RHCE* coding regions by RHD is termed as a "deletion" haplotype, with *RHD* in cis position.[10] In our case, a molecular rearrangement has resulted in the formation of *RHCE-D* (3-9)-*CE* hybrid by replacing *RHCE* exons 3-9 with corresponding RHD exons. Kulkarni et al. have also found *RHCE-D*(3–9)-*D* hybrid as the predominant mechanism responsible for D--phenotype in Indians.[11]

Once identified, the D-- individuals should be enrolled into a rare donor registry to make them available to donate for other individuals of the same phenotype whenever needed. In the case of elective surgeries or pregnancy, either these individuals can be planned up for an autologous blood collection or blood can be made available through a rare donor registry.

3 | CONCLUSION

Rare phenotypes like D-- individuals especially if alloimmunised are of great concern at times of transfusion requirements. Proper identification of these individuals are important not only to contribute to the rare donor pool but also for adequate patient blood management. Effective collaboration of the national and international rare donor registries have to be established to adequately mobilise rare blood units across the globe if required.

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CONFLICT OF INTEREST

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

All authors discussed the results and contributed to the completion of the final manuscript.

DECLARATION OF PATIENT CONSENT

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that

their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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LETTER TO THE EDITOR

The impact of the SARS-CoV-2 pandemic on the ongoing prospective, international, multicentre observational study assessing the preoperative anaemia prevalence in surgical patients (ALICE-trial)

Preoperative anaemia has been defined as an independent risk factor for morbidity and mortality in surgical patients.[1,2] The causes of anaemia are multifactorial, with iron deficiency being the most prominent. Further potential underlying reasons for an inadequate erythropoiesis are lack of vitamin B12 or folate, and bone marrow disease.[3] Anaemia of inflammatory disease is another frequent cause of anaemia and occurs in the context of auto-immune disease, acute and chronic infections and/or cancer.[4] Furthermore, anaemia can be caused by renal dysfunction resulting in a relative deficiency of erythropoietin.

The effectiveness of intravenous iron supplementation to correct iron deficiency anaemia (IDA) has been demonstrated recently.[5,6] However, the PREVENTT trial clearly demonstrated that, if preoperative IV iron supplementation in anaemic patients increases haemoglobin, it does not reduce the frequency of transfusions or the mortality after surgery. Nevertheless, the pragmatic design of the PREVENTT trial precluded extrapolation to specific patients where some factors may have masked the effect of iron supplementation.[7] Indeed, little is known about the proportion of surgical patients who are deficient in vitamin B12 and/or folate and those who suffer from renal anaemia or anaemia of inflammation. The aim of the *Preoperative Anaemia prevaLence In surgiCal patiEnts* (*ALICE*) study is to provide detailed data about the prevalence of preoperative deficiencies in iron, vitamin B12 and/or folate and the presence of underlying renal or chronic diseases in patients undergoing major surgery. The results will facilitate the development of treatment strategies to combat causes of anaemia and to reduce the risk of perioperative complications (NCT03978260).

The ALICE-study is an investigator-initiated world-wide collaboration. Within a self-selected study week between 2019 and 2021, participating hospitals recruit patients undergoing major surgery. As many planned surgical procedures were postponed or cancelled globally in order to compensate for the increasing number of SARS-CoV-2 in-hospital patients, the preparation and execution of the ALICE-study had to be paused in many participating centres. The following survey was conducted to assess the status of the ALICE-study and to elucidate if participating hospitals require additional support.

In total, 120 participating hospitals were contacted in August/September 2020 and 79 among them completed the survey (Figure 1A). Of those centres who responded, 23% ($n = 18$) had already completed and 21% ($n = 17$) planned a study week before the pandemic. Further 41% ($n = 31$) were in the process of finalising ethical approval and paperwork (Figure 1B). During the peak of the pandemic, most planned major procedures were postponed or cancelled in 65% ($n = 50$) of the responding hospitals, whereas some surgeries were reduced in 31% ($n = 25$) of the participating hospitals. Only 3% ($n = 2$) of the centres reported no changes in the daily surgical procedures (Figure 1C). Overall, the COVID-19 pandemic delayed milestones such as site activation or enrolment.

After the peak of the first SARS-CoV-2-wave in their country 11% ($n = 10$) of the participating centres resumed planned procedures and conducted the study week. In addition, 11% ($n = 9$) of participating centres rescheduled the study week. Because of the continuous pandemic, 16% ($n = 13$) did not reschedule a study week (Figure 1D). Approximately three months after the first wave of SARS-CoV-2, 44% ($n = 33$) of the participating hospitals have returned to the status quo regarding their surgical routine. On average half of the hospitals are performing 70–90% ($n = 19$) or 50–70% ($n = 17$) of surgeries compared to before the pandemic and only 8% ($n = 6$) are conducting less than 50% interventions.

Over half of the participating centres (54%, $n = 42$) believe that the SARS-CoV-2 pandemic will not influence the study results. Nearly one third ($n = 22$) assume that it might influence the outcome, but only slightly. Less than 20% ($n = 13$) expect that it will affect the results. Since the SARS-CoV-2 outbreak, the percentage of emergency surgical procedures increased in many hospitals. Depending on the time of patient recruitment, the estimation of the overall prevalence of anaemia might be affected since emergency cases are more frequently anaemic than elective cases.[8] Furthermore, surgical outcome including length of stay and allogenic blood transfusion rate might be altered. The results of this survey indicate that the SARS-CoV-2 pandemic interrupted standard clinical practice. As many preoperative anaemia clinics are closed, diagnosis and treatment of IDA might be impeded,[9] particularly in institutions performing mostly elective surgery such as orthopaedic clinics, where the shift towards urgent/emergent surgery might have been more significant. Elective surgery is also more likely to benefit from pre-operative anaemia detection and treatment programs, whose activity has been disrupted

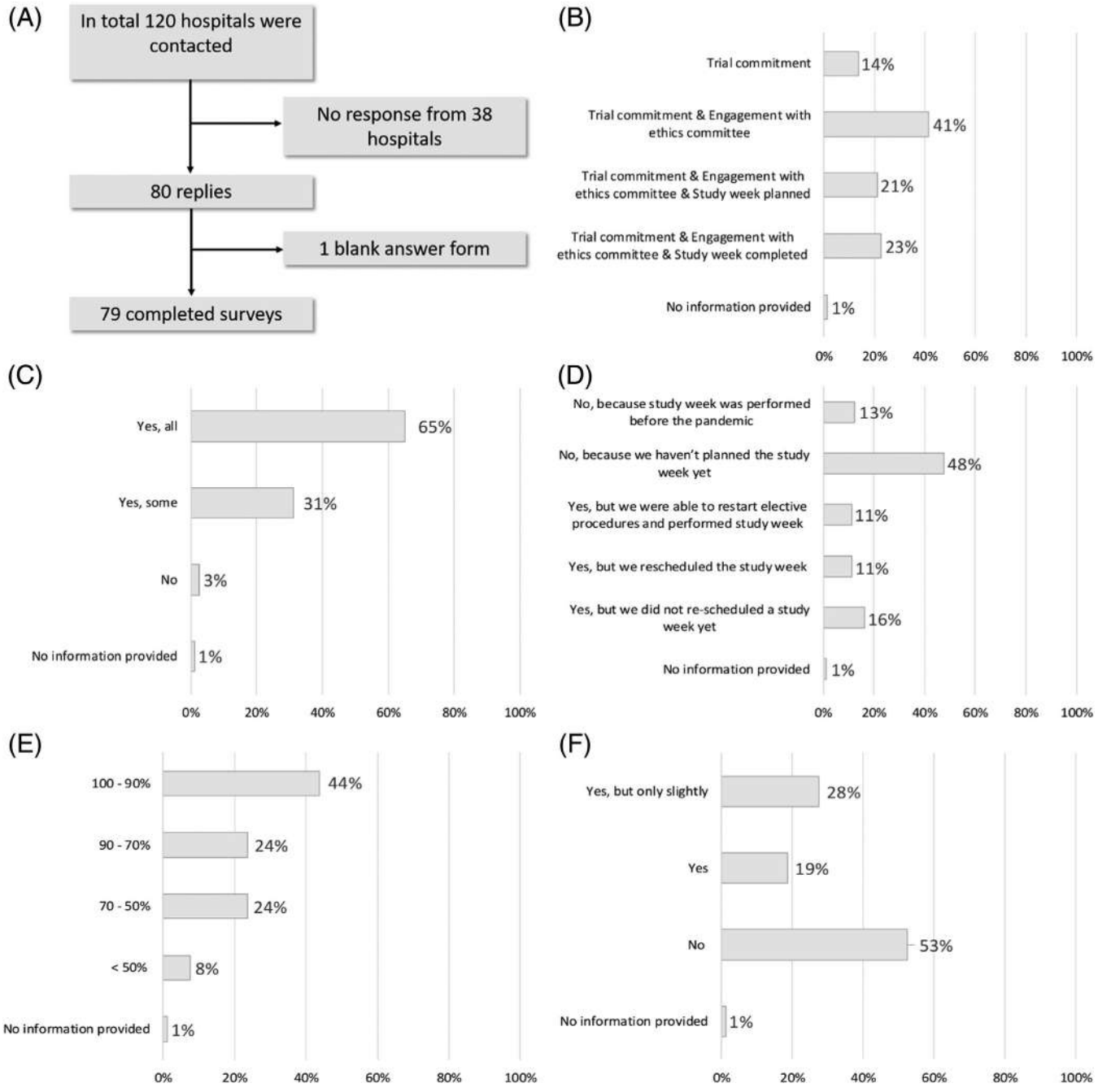

FIGURE 1 The effect of the SARS-CoV-2 pandemic on the ALICE-study. (A) Flow chart, (B) Which milestone(s) has/have been achieved? (C) During the peak of SARS-CoV-2 pandemic, were elective major procedures postponed/cancelled at your hospital? (D) During the SARS-CoV-2 pandemic, was the planned study week postponed? (E) After the first wave of SARS-CoV-2 pandemic and going back to 'routine', do you perform the same amount of surgeries as before the pandemic? (F) Do you believe the SARS-CoV-2 pandemic will influence the study results?

by the pandemic. In addition, oncological patients have been heavily affected by postponed surgeries leading to further tumour growth. These patients often require intravenous iron supplementation and may need to be analysed separately.

Clinical research has been greatly affected by the medical emergency and scientific activities being either suspended or shifted towards SARS-CoV-2-oriented projects. Since only institutions who had already expressed an interest in the Alice-study were contacted for this survey, but recruitment was still ongoing when the pandemic rose, we do not know how many centres might have been discouraged from embarking on a new research project altogether.

The ALICE study provides a unique possibility to evaluate the causes of preoperative anaemia, facilitating treatment strategies and thereby improving patient safety and surgical outcome, even in challenging times of the pandemic.

CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

LJ, DMB, EB, SL, PM, MP, RR, MR, DRS, CS, KZ, SC and PM wrote the manuscript with input from the ALICE-Study-Group. LJ, DMB, EB, SL, PM, MP, RR, MR, DRS, CS, KZ, SC and PM designed survey. LJ, SC, KZ and PM analysed data and wrote first draft 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.