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5. Transfusion Medicine : transfusion practice, thresholds and audits; transfusion efficacy assessment, clinical trials; haemovigilance; non-infectious transfusion adverse events; therapeutic apheresis
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This comprehensive coverage has made the journal essential reading for a wide range of specialists interested in the present state of transfusion research and practice.

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REVIEW ARTICLE

Polycythaemia vera: molecular genetics, diagnostics and therapeutics

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Abstract

Polycythaemia vera is one of several classical myeloproliferative neoplasms that may occur in a juvenile onset or late-onset adult forms. It is linked to specific genetic mutations that cause a deleterious elevation in the patient's red cell mass. The discourse on genetics includes an exposé on the molecular biology of the disease and how a shared *JAK2 V617F* mutation can co-exist among three distinct neoplasms. Concepts of genetics and immunology help define the origin and behaviour of the disease: the tracking of allele burdens of mutations (genetic dosage), the timing or order of acquired mutations, the import of bystander mutations and the onco-inflammatory response; all theories are invoked to explain the progression of disease severity and potential transformational leukaemia. The World Health Organization's diagnostic criteria are accessed to focus on the subtleties of the Hb laboratories and sifting through the challenging listing of differential diagnoses that mimic PV, and our report includes an overview of manual and automated phlebotomy (erythrocytapheresis) procedures, enumerating their clinical indications, significance of *temporary phlebotomy resistance* and optimizing safety/ efficacy, quality and cost. Stratification of low and high-risk disease distinguishes when to commence chemo-cytoreductive therapy in the high-risk patient to prevent thrombotic complications. *Drug resistance* is circumvented by artfully switching drugs or using novel drug designs.

Key words: paediatric/juvenile/adult polycythemia vera, myeloproliferative neoplasms, *JAK2 V617F* mutations, JAK inhibitors, tyrosine kinase inhibitors.

Introduction

Polycythaemia vera (herein referred to as PV) is a myeloid neoplastic multi-clonal stem cell disorder. Cytogenetically, PV is a myeloproliferative neoplasm (herein referred to as MPN), classically categorized as *BCR-ABL1* (Philadelphia chromosome) negative. Essential thrombocythaemia (herein referred to as ET) and myelofibrosis (MF) are categorized as sister diseases, being also *BCR-ABL1* (Philadelphia chromosome) negative.

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[Correction added on 14 April 2022 after first online publication: Reference 42 has been corrected in this version.]

The hallmark of PV is the patient who frequents the clinic cyclically for phlebotomy treatments. This is because of their high red cell mass RCM which can be >25% above the predicted value caused by excess red cell production [1]. Because such patients are at heightened risk for thrombotic complications, it becomes necessary to remove the excess red cells periodically from their circulation.

With a male predominance, polycythaemia vera can start in juveniles (age < 20) or adults (age 60–65), although more commonly in the latter [2,3]. In later life after the diagnosis of PV, some patients transform to a diagnosis of MF and have an increase in grade 2 bone marrow fibrosis. They are at risk of adult onset acute myeloid leukaemia (herein referred to as AML). This malignancy is known to occur in 25–50% of adults with

significant bone marrow fibrosis and has a devastatingly poor prognosis for some patients [4]. Risk factors for leukaemic blast phase crises in PV are myelofibrosis, abnormal karyotype, *TP53* and *TET2* and *DNMT3A* genetic mutations [5–7]. This blast crises occurs in 10% of the patients within 10 years of their diagnosis of PV [6].

Molecular genetics of polycythaemia vera in a mutated Janus kinase 2 gene

The original 2005 discovery of the mutated Janus kinase 2 gene is on human chromosome 9. It effects the abnormal translation of *JAK2* protein kinase associated with MPNs including PV [8,9]. In the schema of the Janus family of non-receptor protein kinases, there are four of them enumerated Janus kinase 1 (*JAK1*), Janus kinase 2 (*JAK2*), Janus kinase 3 (*JAK3*) and tyrosine kinase 2 (*TYK2*) [10].

A principal focus is the derangement of the *JAK2* gene, cytogenetic localization 9p24.1, causing an activated/dysregulated *JAK2* protein kinase and myelosis [11].

When DNA mutations occur in specific peptide coding exons 14 and 12 of the *JAK2* gene, errors are made in protein translation and errant amino acid sequences inserted into the *JAK2* protein kinase. The first discovered mutated protein *JAK2 kinase V617F* is related to a single DNA point mutation *G1849T* of exon 14 from the transversion of guanine to thymine. This leads to the replacement of the amino acid valine by the amino acid phenylalanine at position 617 in protein *JAK2* kinase and occurs in 95–98% of adult PV patients by measurement in granulocyte assays, but only 27% of paediatric patients diagnosed with sporadic PV [2,12,13]. Independently, the occurrence of multiple *JAK2* exon 12 mutations occur in up to 12% of *JAK2 V617F* exon 14 negative adult patients. Exon 14 *V617F*-associated mutations have a reported predilection to erythrocyte, platelet and granulocyte cell excess while exon 12 mutations are inclined towards solitary erythrocytosis [13].

There is an orderly mechanism of the proliferation of myeloid cells through a JAK2/STAT signalling pathway (a critical signal transducer activator of transcription proteins). The JAK2 gene encodes production of the JAK2 tyrosine kinase, which phosphorylates the STAT proteins (transcription factors), causing these to homodimerize and translocate into the nucleus of the cells. Translocation of these homodimers upregulate or repress the transcription of target genes that control cellular proliferation. The pathway has been further described elsewhere [14].

Mutations located in exons 14 or 12 and a mutated *JAK2* protein kinase cause activated dysregulation of the JAK2/STAT pathway and uncontrolled cellular proliferation. The JAK2 kinase has various domains of structure relevant to this discussion (Fig. 1). These include attention on the JH2 pseudokinase domain and the neighbouring JH1 active catalytic site for phosphorylation of *STAT* transcription factors that promote cellular proliferation.

The *JAK2 V617F* point mutation of exon 14 and the various exon 12 mutations, which are in or near the pseudokinase JH2 domain, cause anomalous domain to domain protein expression. Given the putative *JAK2 V617F* mutation, the replacement of the smaller amino acid valine by the larger phenylalanine at codon 617 is predicted by molecular modelling to destabilize the pseudokinase domain JH2 [15]. Structural destabilization of the pseudokinase domain is thought to favour gain-up (constitutive activation) of the adjacent JH1 catalytic domain [4,16]. The gain in function is associated with increased disease activity as reported by several investigators manifested by pruritus, erythrocytosis, required advanced treatment intensity by cytoreductive therapy, and the propensity to fibrosis [8,17,18].

An interesting observation is that some cases of PV are negative for *JAK2* mutations raising the possibility of other mutations elsewhere in association with the disorder. There are reported true cases of PV with *TET2*-first mutations, cytogenetic localization 4q 24, but negative for the *JAK2* mutations [19].

Developmental theories of the myeloproliferative neoplasms

As separate disorders, an interesting question is the relationships between ET, PV and MF, given many MPN patients having a shared *JAK2 V617F* mutation. One theory is the report of mutant allele burdens that cumulate in the same patient with disease transformation over time, as highlighted below [20]. The quantitative allele burden is defined as the ratio of the mutated allele over the wild type (non-mutated). In one young woman in her early 20s, ET was diagnosed (disease time-line 0–3·5 years, Plt: $800 \times 10^9\ l^{-1}$), transformed to PV (disease time-line 3·5–10 years, Hb: 18·3 g/dl, *JAK2 V617F* allele burden: 24·8%) bHb transformed to MF (disease time-line 10–12 years, WBC: $19 \times 10^9\ l^{-1}$, *JAK2 V617F* allele burden 63·3%). The escalated gene dosage of mutated *JAK2* correlated historically with a staged progression from thrombocytosis to erythrocytosis to granulocytosis.

Enigmatically, some ET cases bypass transformation to PV. Some of these ET patients whether mutated or not evolve to fibrosis and AML directly and the thrombotic risk escalates when age ≥60 with *JAK2* mutation [21]. A plausible explanation why disease sometimes jumps steps is that the specific type and timing or order of somatic mutations in the population define the neoplasm [22]. In

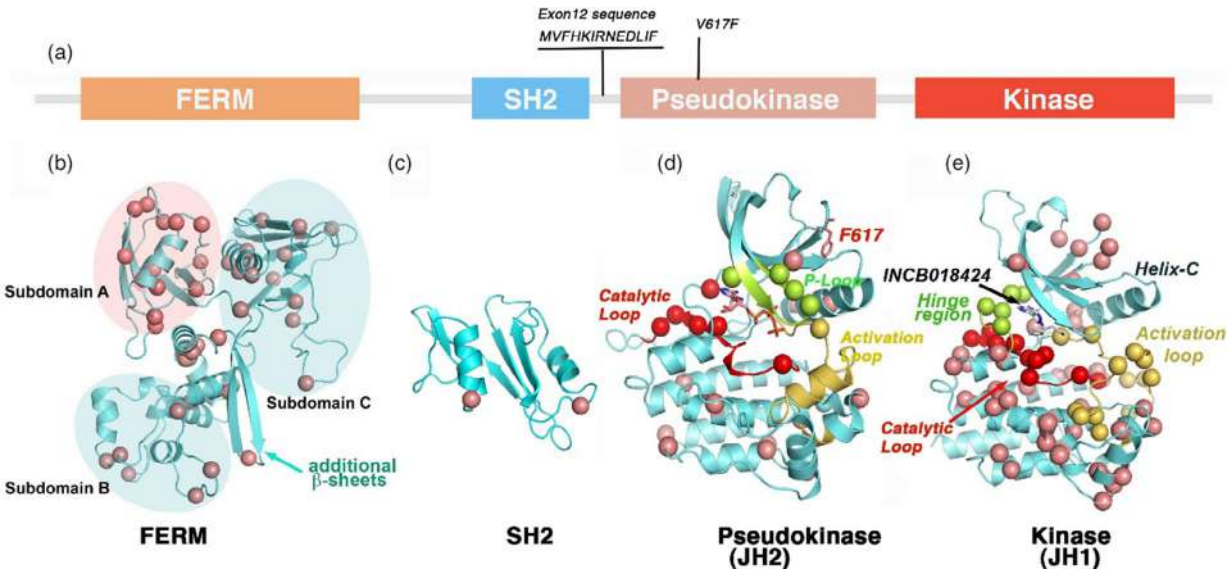

Fig. 1 Dual pseudokinase JH2 and JH1 domains wherein an errant 617F phenylalanine substitution shown in JH2 causes a gain-up of activation of the contiguous ATP/catalytic site. Gaining access to catalysis, the ATP/catalytic pocket is in the catalytic loop shown in JH1. The DNA location, exon 12, is shown by the sequence mapped on a horizontal line while exon 14 incorporates V617F. [Colour figure can be viewed at wileyonlinelibrary.com]

a smaller cohort study of 24 total patients with chronic phase MPNs, those with acquired *JAK2*-first mutations were compared to 12 patients with acquired *TET2* first mutations (22% associated frequency with PV) [17,22,23]. Clonal studies of the mutations were performed in cultured burst forming erythroid units BFU-E. Those with *JAK2*-first had a predilection to a diagnosis of PV, 7 of the 12 patients with the balance of 5 patients ascribed to ET and MF. Those with *TET2*-first mutations were inclined towards a diagnosis of ET and MF regarding 8 of 12 patients, with the residual balance of 4 patients diagnosed to PV. In the *JAK2*-first patients, the degree of homozygosity for the *JAK2 V617F* mutation in *JAK2*-first cultured cells is detected as 57·8% vs. 1·4% in *TET2*-first mutated cultured cells.

Given the overlap of above diagnoses regardless of mutational order, admittedly the timing of these specific mutations does not explicitly define the neoplasm. From the Darwinian perspective, conceivably there is a potential competitive advantage to clonal expansion of *JAK2*-first mutations from hematopoietic stem cells and manifested by greater phenotypic expression of PV [19,22]. This raises the distinct possibility that there are other somatic mutations beyond *JAK2* and *TET2* which drive these neoplasms and explain why ET can transition directly to MF rather than through PV. Candidate genetic alterations reported in association with PV for future study of mutational order are *ASXL1* (12% associated frequency with PV) and *SH2B3* (9% frequency) [23].

Clinical presentation and diagnostic criteria

A plethora of symptoms are associated with PV including numbness of the extremities and tingling (acroparesthesias); burning pain, warmth and redness of the extremities (typifying the erythromelalgia of myeloproliferative disease); unexplained epigastric pain and weight loss; dyspnoea; visual and autonomic-like disturbances of sweating; vague symptoms of dizziness; and the disquieting pruritus associated with hot baths.

When generalized clinical symptoms of PV are evident, there are two characteristic profiles of the blood; (1) an increased red cell mass; and (2) associated blood hyperviscosity. Classic hyper-viscosity syndrome of PV manifests as a triad of mucosal bleeding, visual disturbances and neurological symptoms. Acute obligatory therapy includes phlebotomy or erythrocytapheresis. The elevated viscosity levels at which patients become symptomatic are variable and measured by a viscosimeter in centipoise (cP), with a normal reading being less than 2.0 cP [24].

We have included the World Health Organization, WHO Diagnostic Criteria for Polycythaemia vera, 2016, Table 1 [1]. The original WHO 2008 inclusion criteria are revised, Hb > 18·5 g/dl in adult males lowered to >16·5 dl^{-1}; 16·5 g/dl in adult females lowered to >16·0 g/dl [1,25,26]. The relevance of these modifications to major criteria 1 is that some cases of ET and unclassified MPNs with borderline Hb levels will be classified as having 'masked' or 'prodromic' PV [27]. Prodromic PV

patients are at a substantially increased risk for progression to MF and AML compared to the well-defined PV; and younger patients may be at a higher risk of thrombosis [28]. As >70% of juveniles have negative *JAK2* markers, their diagnosis of PV may remain elusive for some time, not yet satisfying enough major and minor criteria of the disease [2].

Regarding diagnostic criteria to measure total volume of erythrocytic 'red cell mass', there are comparative advantages and disadvantages of radiolabeling techniques that have been described [29]. For ^{51}Cr-labelled erythrocytes, the RCM determinations are accurate to make a diagnosis of PV when relying upon experienced technicians. The problem surfaces that the ^{51}Cr testing is complicated to perform estimated 2·6 times more expensive than simpler radio-iodine-labelled human serum albumin ^{125}I technique and not always readily accessible to clinicians. Given these considerations, RCM determinations in adults may be unnecessary in cases of persistently elevated Hb and Hct levels above the diagnostic threshold.

Differential diagnoses

Juveniles

Precision diagnosis and optimal treatment of PV demands careful attention to the clinical differential of disorders. True polycythaemia in neonates, children and juveniles needs to be differentiated from transitory elevated Hb in the neonatal period, twin-twin transfusion syndrome, maternal-foetal bleeds, trisomy syndromes, adrenal hyperplasia, erythropoietin secreting tumours, polycythaemia of the newborn seen with diabetic mothers and congenital causes of erythrocytosis [2].

The juvenile form of PV is distinguishable from juvenile ET with respect to platelet counts. The characteristic thrombocytosis for juveniles seen in ET presents at the mean, $1{\cdot}109 \times 10^9\ l^{-1}$ compared to the significantly lower counts seen in juvenile PV in the range $207–394 \times 10^9\ l^{-1}$ [2]. With respect to platelet counts in adults, higher median counts of $811 \times 10^9\ l^{-1}$ are reported in ET compared to $686 \times 10^9\ l^{-1}$ seen in PV [30]. Across the broad spectrum of age for PV patients, these data suggest a general upward trend of the platelet levels between youth through adulthood. Platelet counts are logical biological markers to follow serially as possible indicators of disease progression.

Adults

Extending the differential diagnoses to adult PV, the list includes chronic hypoxaemia related to COPD and heavy cigarette smoking; morbid obesity associated with chronic obstructive sleep apnoea; chronic habitation at high altitude; doping; various erythropoietin secreting tumours; states of blood volume contraction due to dehydration; autosomal dominant benign familial polycythaemia; recurrent bleeding states treated episodically by iron infusions and triggering exuberant RBC regeneration; middle age obese male hypertensive patients who smoke and are treated with diuretics, identified as Gaisbock syndrome or pseudo-polycythaemia; and patients with testosterone replacement therapy [31].

In adult patients, it is important to differentiate PV from ET otherwise clinicians may inadvertently apply phlebotomy to the latter group and cause unnecessary iron deficiencies. With respect to ET, it may distinguish itself from PV by manifest platelet counts in excess of $450 \times 10^9\ l^{-1}$, bone marrow biopsy megakaryocyte proliferation and loose clusters, and positive molecular diagnostics of *JAK2, calreticulin CALR* and MPL proto-oncogene (thrombopoietin receptor mutations) [21]. Normal or elevated serum erythropoietin levels should discriminate out PV as the putative diagnosis. ^{51}Chromium isotopic red cell mass determinations can differentiate ET from PV in adult patients, the latter having a higher risk for thrombosis and myelofibrosis complications [32].

Table 1 World Health Organization, WHO Diagnostic Criteria for Polycythaemia vera, 2016* [1].

Major 1 criterion	Hb > 16·5 g/dl male; >16·0 g/dl female
Major 1 criterion	or Hct > 49% adult male; >48% adult female; paediatrics**
Major 1 criterion	or red cell mass, RCM > 25% above the predicted value
Major 2 criterion	Positive genetic markers *JAK2 V617F* point mutation in exon 14 or various *JAK2* mutations in exon 12
Major 3 criterion	Hypercellular bone with proliferation of erythroid cells, megakaryocytes of different sizes and granulocytosis
Minor 1 criterion	A subnormal erythropoietin level

*a. Two major criteria and one minor criterion are sufficient to diagnose PV.

**b. While not part of the WHO diagnostic criteria, which are specific to adults, in paediatric PV, reported are Hct criterion equivalent to 53·2–55% [12,58].

Disease severity

Adult complications

Genetically, the evolution to aggressivity and complications of PV has been implicated to a loss of heterozygosity (LOH) on the short arm of chromosome 9 (9pLOH) [33]. This is likely because of a process of mitotic recombination in somatic cells and suggests a localized dominant mutation therein. The heterozygous form of the mutation is more prevalent and phenotypically less expressive, compared to the homozygous state. The explanation for this dichotomy of disease severity is rooted in the tenets of Mendelian genetics. The heterozygous form appears to compete with the wild type. A pairing of the mutated alleles in homozygosity amplifies gene expression phenotypically.

The onco-inflammatory response and bone marrow fibrosis

The ensuing development of MF in some adult PV patients is hypothesized to be linked to a chronic inflammatory milieu attendant to the co-existing neoplastic clones of cells, carrying mutations of *JAK2*. As the load of inflammatory driven reticulin fibrosis in the bone marrow escalates to grade 2, this pathology correlates to an increased risk of lymphoproliferative disease, AML [34]. An inflammatory cascade is triggered by various cytokines/chemokines released into the stromal microenvironments of the bone marrow and causal to fibrosis. When the neoplastic associated chronic inflammatory process is aborted after stem cell transplantation, there is noted a significant regression of bone marrow fibrosis associated with normalization of haematopoiesis [34].

Quantifiable blood levels of the inflammatory phase biomarker high sensitivity C-reactive protein, hs-CRP, have been reported to stratify the risk of thrombosis, haematological evolution and death in PV patients and those that transform to MF [34]. It is noteworthy that the *JAK2* exon 14 and exon 12 mutations have an approximate equal probability of associated thrombotic events, fibrotic and leukaemic transformations, and overall survival [35,36]. The occurrence of mutations *ASXL1* and bystanders *SRSF2* and *IDH2* are noted to negatively impact transformation-free survival of PV [23].

Paediatric complications

In the paediatric age group, an increased incidence of testing positive for *JAK2 V617F* has a greater association with thrombotic, haemorrhagic and other malignant conditions. This was reported in two smaller cohort studies. In the first study of 11 patients followed serially in the ensuing decade of life after the diagnosis of paediatric PV, 27·3% of the individuals were *JAK2* mutation positive but with zero reported thrombotic complications and deaths [2,12]. By comparison in a separate study of 36 paediatric PV patients, being *JAK2* positive in 75% of the cohort, 37·5% of the patients collectively included antecedent malignancies; Budd–Chiari Syndrome (causal to hepatic vein stenosis/thrombosis, positive *JAK2 V617F)*; stroke; gangrene; severe haemorrhage; and death [11,37,38].

Guidelines for phlebotomy, reduction of red cell mass in polycythaemia vera

Manual phlebotomy, first-line therapy

Manual phlebotomy merely requires intravenous access by a 16-gauge catheter and associated IV line, followed by drainage of whole blood in the range of 300–450 ml for adults as guided by body weight [39]. An even lower volume range of phlebotomy has been suggested but probably unnecessary because the risk of hypovolaemia is countered by the increased plasma volume and red cell mass of the disease [39].

The typical length of the manual phlebotomy procedure is 10–15 min, though patients are often asked to stay in the infusion clinic for observation to ensure their hemodynamic stability for some time afterwards. It is appropriate to request the patient to consume a cumulative total of one litre of liquids taken orally, divided equally before and after phlebotomy. Initially, the induction treatment frequency is typically every one to two weeks, though may be escalated to twice weekly, and maintenance extended to monthly or even less frequently for some patients to target a Hct of less than 45% [39,40]. For patients presenting with recalcitrant hyper-viscosity syndrome, a lowered Hct in the 40–42% range would be an appropriate intervention [39].

As each manual phlebotomy session decreases the Hct by around 3%, multiple sessions are required to reach a target Hct of less than 45%. It is imperative to titrate the Hct consistently because a chronically high Hct is associated with an increased risk of thrombotic complications [41]. Twenty-five per cent of patients find the repetitive phlebotomies uncomfortable and inconvenient, and as a result, there is the potential for non-compliance, which is observed in up to 8% of PV patients [42]. Though single phlebotomies are estimated to be nearly 71% less expensive compared to single procedure erythrocytapheresis (ECP-not to be confused with extracorporeal photopheresis), proponents of ECP suggest that an overall reduced treatment frequency compensates to equalize costs of the

two modalities [43]. Nonetheless, the majority of red cell depletion procedures for PV continue to employ manual phlebotomy, whereas ECP is relegated to special circumstances.

Large volume isovolaemic erythrocytapheresis, first-line therapy

Erythrocytapheresis is an extracorporeal centrifugation driven technology, primarily intended to reduce red cell mass in patients with PV to normal levels and secondarily to separate red blood cells from whole blood in blood banks. In practice, ECP can be considered an extension of whole blood component preparation by the routine 'bottom and top' technology. Today, preparation by various apheresis technologies is utilized in practice to ensure the highest standards of safety/efficacy and reliability while employing modern automated equipment.

The practicability of ECP is enumerated in Table 2, and there are supportive evidence-based guidelines [5]. Two clinically relevant scenarios qualify for a trial of ECP. (1.) A trial of ECP is indicated for patients who consistently require increased frequency of manual phlebotomy to avoid or delay chemotherapy. Nonetheless, ECP would be excluded, non-indicated in those patients having a known phenomenon of *temporary phlebotomy resistance* (defined rather conservatively as >=3 manual phlebotomies per year transiently) [39]. (2.) In order to prevent intraoperative/perioperative vascular complications of emergent surgery, ECP takes precedence to achieve an Hct of less than 45% in a single treatment session [39].

After receiving ECP treatment, patients experience a rapid reduction in RBCs and WBCs, though platelet counts tend to remain relatively unchanged [42]. After decreasing red cell mass, the resulting iron deficiency does not generally warrant intervention unless severe with coincidental symptoms, because doing so would unfortunately trigger the iron-dependent proliferation of erythropoietic precursor cells and thus reinvigorating red cell production [39,44].

Risk stratification of disease guiding cytoreductive pharmacotherapy

Low-risk disease

These patients are typically less than age 60, have no history of arterial venous thrombosis, but may have some uncontrolled microvascular features of disease such as numbness, tingling, erythromelalgia, pruritus; cardiovascular conditions including hypertension; or haematological aberrations, that is leukocytosis [21]. Consistent with controlled studies, low dose aspirin, ASA 81mgs once or twice a day should be sufficient to reduce thrombotic risk [45]. The decision to treat once or twice daily with low dose ASA is predicated on how well the microvascular symptoms are controlled and the plus/minus presence of comorbidities, that is hypertension and leukocytosis. A double dose of mini-dose ASA to sufficiently suppress thromboxane A2 synthesis is indicated to interdict elevated platelet aggregation in MPN disorders [46].

High-risk disease

Such patients are ≥60 years of age and *JAK2* mutation positivity or a history of arterial venous thrombosis [21]. The initiation of pharmacological cytoreduction intervention is a keynote of these high-risk patients for which hydroxyurea is a prime candidate. Comparable to the low-risk stratification, mini-dose ASA is indicated in single or double dose forms daily because of the drug's antithrombotic properties [21]. Anti-coagulation would be indicated for concomitant deep venous thrombosis of the extremities, pulmonary embolism and thrombotic complications of the liver (Budd–Chiari syndrome). A lytic drug would have value for treating untoward acute arterial strokes guided by medical protocols of administration.

For the high-risk patients, below are characterization of the major benefits and some drawbacks of the recommended first or second-line drugs. We feature the principal role of hydroxyurea, a favourite choice for first-line cytoreductive therapy and busulfan, peginterferon alpha-2a and ruxolitinib as second-line agents.

Hydroxyurea: first-line

Hydroxyurea (HU) also known as hydroxycarbamide (HC) is a cytotoxic antimetabolite with the potential to cause severe myelosuppression. As an older but favoured first-line drug, it is used as an effective cytoreductive agent in the treatment of PV. Based upon tissue culture studies in humans and rats, its principal mode of action is thought to be as a ribonucleotide reductase inhibitor and causing immediate pronounced cessation of DNA synthesis [47].

In the Spanish Registry of Polycythaemia Vera opened in 2011, 890 patients with a history of receiving HC (aka HU) cytoreductive therapy were tracked using the European LeukaemiaNet (ELN) criteria [3]. This included categorization of clinico-haematological response, resistance and intolerance to the drug. Tracking criteria included measuring Hct, Plt and WBC in response to HC therapy while compared to defined laboratory threshold values; need for phlebotomy; symptomatic or excess splenomegaly; uncontrolled myeloproliferation; cytopaenias; and extra-haematological toxicities. In this sizeable study of PV, 78% of patients received a complete or partial clinical

Table 2 Therapeutic erythrocytapheresis: practicable utility in selective polycythaemia vera patients.

First-line therapeutic option, strong grade I recommendation by the ASFA to reduce the red cell mass effectively [5].
Capacity to achieve pronounced red cell extraction with a single session to achieve Hct < 45%
Rapid normalization of the Hct in order to treat or prevent life threatening pro-thrombotic or haemorrhagic complications; and in preparation for emergency surgery [39].
Potential to reduce the number of phlebotomy procedures administered chronically. This is based upon reports of an increased duration between efficient large volume single isovolaemic ECP maintenance treatments, median of 6–7·5 months compared to a 1·0–1·5 month manual phlebotomy regimen [41,59]
Relevant to patients with hemodynamic intolerance to manual phlebotomy; and contraindications or reluctance to undergo chemo-cytoreductive therapies, some of which may predispose to secondary leukaemias [41,43,59]

Technical notes: a. Using automated ECP, the red cells are depleted after apheresis and re-infusion of the plasma, buffy coat cells, and additional crystalloids, ie. isotonic saline and albumin [60]; b. The volume of red blood cells V_R to be removed by ECP can be reasonably estimated by the following formula [44,59]. c. ECP-erythrocytapheresis; d. ASFA-American Society for Apheresis. $\mathrm{VR} = \frac{\mathrm{startingHCT} - \mathrm{desiredHCT}}{79} * \mathrm{bloodvolumein}\frac{\mathrm{mL}}{\mathrm{kg}} * \mathrm{bodywtinkg}$

response and 22% no response. By at least one of the ELN criteria, 15·4% of the total patient series were deemed to be resistant or intolerant to the drug or both. Given the non-responders or those with resistance and/or intolerance, consideration of second-line drug therapy such as busulphan, peginterferon alpha-2a and ruxolitinib is in order.

Busulphan: second line

Heretofore, the primary experience to treat PV with busulfan has been by first-line therapy orally. Busulphan was recently tested as a second-line cytoreductive agent to treat HU intolerant or resistant PV and ET patients. Durable haematological responses were achievable in 75% of the combined patient pool, being able to discontinue busulphan. Partial reductions in molecular *JAK2 V617F* allele burdens were observed in 33% of the tested patients [48].

Busulfan is a bifunctional alkylating agent, possessing intense myeloablative activity with associated mutagenic, clastogenic and cytotoxic effects. The pharmaceutical activity of the drug is propagated by dual methyl sulphonates on a short alkyl chain that are hydrolysed to carbonyl ions. These alkylate the DNA non-specifically in the cell cycle resulting in a configuration of DNA rearrangements, fragmentation, cross-linked guanine bases, inhibition of DNA double helix uncoiling, miscoding and inhibition of replication and transcription [49,50]. Inherent to this cocktail of antagonistic actions to DNA, busulphan possesses dose-dependent bone marrow suppression, which requires vigilance. Despite being a DNA alkylator, busulphan has not been well correlated to leukaemic transformation, and if there is risk, it appears to be relatively low, a plus for use of this drug as a therapeutic [21,51].

Peginterferon alpha-2a: second line

Recombinant peginterferon alpha-2a's (peg INF alpha-2a) mechanism of action includes binding to type 1 human interferon receptor, receptor dimerization, activation of the JAK/STAT pathway and having downstream pleiotropic effects on multiple cell types. Peg INF alpha-2a has the capacity to produce pronounced neutropaenia, lymphocytopaenia, RBC anaemia and thrombocytopaenia [52]. It is recognized as a second-line cytoreductive agent in the treatment of PV patients that are refractory or intolerant of HU [21].

The drug has capacity to induce partial molecular remissions of JAK2 mutation burdens (but of debatable clinical import) and observable durable haematological remissions. Therapy alleviates burdensome splenomegaly and pruritus but managing the drug is delicate, dealing with a miscellany of toxic side-effects that relegate it as a second-line agent [21].

Ruxolitinib: second line

The first licensed ATP dual competitive *JAK1/JAK2* inhibitor is ruxolitinib (RUX)), which became FDA approved in 2014. The drug is part of the class of compounds known as tyrosine kinase inhibitors (TKIs). Importantly, there are two common misconceptions about the mechanism of action with respect to the binding of this drug to the JAK1/JAK2 tyrosine kinases. Erroneously, it is thought the kinase needs to be mutated to bind the drug and that the drug complexes to the actual mutated *JAK2 V617F* site. As points of edification, RUX complexes with *JAK* kinases, but is bound to the *JAK2* ATP/catalytic pocket, not the site of mutation [53]. In the treatment of PV, its principal pharmaceutical activity occurs as an inhibitor of the catalytic site of the protein which

overdrives the production of myeloid cell lines. RUX clearly does not markedly improve allele burden of mutant *JAK2*, nor can it reverse bone marrow histopathology [14].

The Janus kinase 1 gene is crucial to the expression of genes that mediate the inflammatory response, and Janus kinase 2 is essential to cytokine and growth factor signalling [18,54]. The partial pharmacological inhibition of JAK1 and JAK2 protein catalytic sites by RUX is valuable as maintenance therapy to mitigate disease severity yet has profound consequences given the crucial roles of the parent genes. Patients are at heightened risk for opportunistic infections, cytopaenias, bleeding and neurological sequelae [55]. If these complications ensue, the clinician must be well attuned to immediately identify them and consideration to lower the dosage of RUX or discontinue it.

Evolution of genetic resistance to tyrosine kinase inhibitors, the molecular biology

The mechanism by which patients become refractory to RUX is of great interest because it has introduced novel kinase inhibitors thought to have activity in the treatment of PV transformation to myelofibrosis.

The following research was supported by one of our contributors, Azam [56]. In an in vitro model of drug-resistant screening using a murine cell line BaF3, it expressed randomly mutagenized *JAK2-V617F* proteins followed by selection with *JAK2* inhibitors RUX or fedratinib [56]. Subsequent analysis identified 211 drug-resistant mutations against RUX clustered in kinase and regulatory domains of *JAK2* (Fig. 2). These mutational hot spots conferred resistance not only to RUX but also showed cross resistance to other *JAK2* inhibitors AZD1480, CYT-387 and lestaurtinib [56]. However, there is no observation of resistant clones against fedratinib. Perhaps more interestingly, all RUX resistant mutations were fully sensitive to fedratinib suggesting a unique mechanism of kinase inhibition which can efficiently suppress genetic resistance.

Further, enzymatic and structural studies revealed that fedratinib binds two different sites in the kinase domain in proximity: (1) ATP-binding and (2) peptide-substrate binding sites. So far, anti-kinase therapy exploits the ATP-binding site for drug targeting which is prone to develop resistance over the course of treatment. Mechanistically, mutations in the ATP-binding sites in the kinases including *JAK2* prevent drug binding without affecting the ATP-binding and catalytic activity, thus conferring drug resistance. In contrast, mutations in the substrate binding pocket kill the kinase activity. To confer drug resistance, the kinase must be catalytically active; therefore, drug-resistant mutations in the substrate binding pocket failed to emerge as these inactivated the kinase [56]. This study provides a proof of concept to target the substrate binding pocket to design resistance free kinase inhibitors. These novel types of TKI inhibitors are being tested in the treatment of myelofibrosis and, in particular, may have application to post-PV transformation [57].

Summary

With respect to the origination of PV, we highlight the principal discovery of the *JAK2 V617F* mutation in a phosphorylating JAK2 kinase protein. The mutation is consequent to a dominant point mutation in the parent Janus kinase 2 gene and phenotypically is most expressive in its homozygous form. These genetic errors translate to

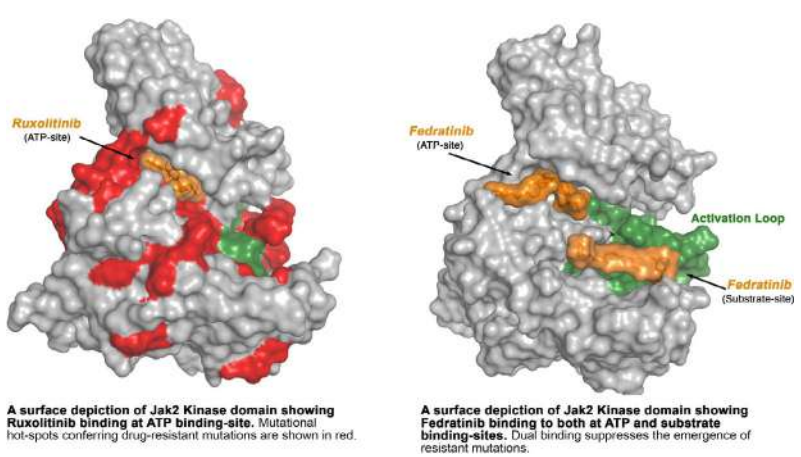

Fig. 2 Two JAK2 kinase drug inhibitors: Molecular mechanisms of kinase resistance to ruxolitinib but gained sensitivity to fedratinib. [Colour figure can be viewed at wileyonlinelibrary.com]

mutations in the JAK2 kinase protein causing uncontrolled gain in activity and excess red cell production.

Disease transformations have been observed in patients followed in excess of 10 years, between ET, PV and MF, the transitions occurring sequentially. Yet the MPN diseases are not always so orderly and paradoxically, some patients matriculate directly from ET to MF directly. The possible explanations for this diversity of disease behaviour include such factors as progressively increasing allele burden of genetic mutations and timing or order of specific mutations.

It is rather important that clinicians be cognisant of the complex differential diagnoses of PV. The WHO provides inclusive criteria of a lower Hb threshold of 16·0–16·5 g/dl, not to miss the diagnosis of PV in a 'prodromic' group of patients known to be at higher risk for leukaemic transformation.

Polycythaemia vera can be stratified into low- and high-risk patients, the latter being age defined ≥60 *JAK2* positive or those with a history of thrombosis. While phlebotomy and low dose ASA are front-line treatments for low-risk patients, chemo-cytoreductive therapy and ASA are introduced in the high-risk class to prevent thrombosis.

While more than five decades old, hydroxyurea by virtue of its' cytoreductive activity has proven to be an essential drug in the treatment of PV. In refractory patients, busulphan and peg INF alpha-2a are good second-line alternatives as cytoreductive agents. RUX, a JAK2 kinase inhibitor, has a relatively smaller role in the maintenance treatment of PV. Novel TKI drugs are being explored (1) to suppress drug resistance and (2) assess their potential therapeutic application in post-PV, when it transforms to MF.

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Conflict of interest

None to report.

References

1 Arber DA, Orazi A, Hasserjian R, *et al.*: The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood 2016;127:2391–405
2 Hofmann I: Myeloproliferative disorders in children. J Hematop 2015;8:143–57
3 Alvarez-Larran A, Kerguelen A, Hernandez-Boluda JC, *et al.*: Frequency and prognostic value of resistance/intolerance to hydroxycarbamide in 890 patients with polycythaemia vera. Br J Haematol 2016;172:786–93
4 Lee S, Ma W, Zhang X, *et al.*: Mechanisms of constitutive activation of Janus Kinase 2–V617F revealed at the atomic level through molecular dynamics simulations. Cancer 2009;115:1692–700
5 Schwartz J, Padmanabhan A, Aqui N, *et al.*: Guidelines on the use of therapeutic apheresis in clinical practice – evidence-based approach from the writing committee of the American Society for apheresis: the eighth special issue. J Clin Apher 2019;34:171–354
6 Hidalgo JE, Carballo-Zarate A, Verstovsek S, *et al.*: Bone marrow findings in blast phase of polycythemia vera. Ann Hematol 2018;97:425–34
7 Ley TJ, Ding L, Walter MJ, *et al.*: DNMT3A mutations in acute myeloid leukemia. N Engl J Med 2010;363:2424–33
8 McLornan D, Percy M, McMullin MF: JAK2 V617F. A single mutation in the myeloproliferative group of disorders. Ulster Med J 2006;75:112–19
9 James C, Ugo V, Le Couedic JP, *et al.*: A unique clonal JAK2 mutation leading to constitutive signaling causes polycythaemia vera. Nature 2005;434:1144–8
10 Pellegrini S, Dusanter-Fourt I: The structure, regulation and function of the Janus kinases (JAKs) and the signal transducers and activators of transcription (STATs). Eur J Biochem 1997;248:615–33
11 Lister Hill National Center for Biomedical Communications - Genetics Home Reference. https://ghr.nlm.nih.gov/gene/JAK2. [Last accessed 18 September 2018].
12 Giona F, Teofili L, Moleti ML, *et al.*: Thrombocythemia and polycythemia in patients younger than 20 years at diagnosis: clinical and biologic features, treatment and long-term outcome. Blood 2012;119:2219–27
13 Pietra D, Li S, Breisci A, *et al.*: Somatic mutations of JAK2 exon 12 in patients with JAK2 (V617F)-negative myeloproliferative disorders. Blood 2008;111:1686–90
14 Jatiani SS, Baker SJ, Silverman LR, *et al.*: JAK/STAT pathways in cytokine signaling and myeloproliferative

disorders: approaches for targeted therapies. Genes Cancer 2010;1:979–93
15 Levine RL, Wadleigh M, Cools J, *et al.*: Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell 2005;7:387–97
16 Sonbol MB, Firwana B, Zarzour A, *et al.*: Comprehensive review of JAK inhibitors in myeloproliferative neoplasms. Ther Adv Hematol 2012;4:15–35
17 Tefferi A, Lasho TL, Schwager SM, *et al.*: The clinical phenotype of wild-type, heterozygous and homozygous JAK2 V617F in polycythemia vera. Cancer 2006;106:631–35
18 Genetic Testing Registry, National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/gtr/genes/3716/ [accessed 7 September 2020].
19 Godfrey AL, Chen E, Pagano F, *et al.*: Clonal analyses reveal associations of JAK2V617F homozygosity with hematologic features, age and gender in polycythemia vera and essential thrombocythemia. Haematologica 2013;98:718–21
20 Passamonti F, Rumi E: Clinical relevance of JAK2 (V617F) mutant allele burden. Hematologica 2009;94:7–10
21 Tefferi A, Barbui T: Polycythemia vera and essential thrombocythemia. 2019 update on diagnosis, risk stratification and management. Am J Hematol 2018;94:133–43
22 Ortmann CA, Kent DG, Nangalia J, *et al.*: Effect of mutation order on myeloproliferative neoplasms. N Eng J Med 2015;372:601–12
23 Tefferi A, Lasho TL, Guglielmelli P, *et al.*: Targeted deep sequencing in polycythemia vera and essential thrombocythemia. Blood Adv 2016;1:21–30
24 Hemingway TJ:Hyperviscosity syndrome. Emedicine/Medscape. Available at https://emedicine.medscape.com/article/780258-overview [Last accessed 21 October 2018].
25 Tefferi A, Vardiman JW: Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. Leukemia 2008;22:14–22
26 Thiele J, Kvasnicka HM: The 2008 WHO diagnostic criteria for polycythemia vera, essential thrombocythemia, and primary myelofibrosis. Curr Hematol Malig Rep 2009;4:33–40
27 Barbui T, Thiele J, Gisslinger H, *et al.*: Masked polycythemia vera (mPV): results of an international study. Am J Hematol 2014;89:52–4
28 Passamonti F, Maffioli M: Update from the latest WHO classification of MPNs: a user's manual. Hematology 2016;1:534–42
29 Fairbanks VF, Klee GG, Wiseman GA, *et al.*: Measurement of blood volume and red cell mass: re-examination of ^{51}Cr and ^{125}I methods. Blood Cells Mol Dis 1996;22:169–86
30 Shaw G, Berg R: Beyond hemoglobin: when and how to work up possible polycythemia vera. Clin Med Res 2020;18:11–20. https://doi.org/10.3121/cmr.2019.1483.
31 Nagalla S, Besa EC. Polycythemia vera. Emedicine/Medscape. https://emedicine.medscape.com/article/205114-overview [Last accessed 9 December 2019].
32 Alvarez-Larran A, Ancochean A, Angona A, *et al.*: Red cell mass measurement in patients with clinically suspected diagnosis of polycythemia vera or essential thrombocythemia. Haematologica 2012;97:1704–7
33 Kralovics R, Passamonti F, Buser A, *et al.*: A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Eng J Med 2005;352:1779–90
34 Lussana F, Rambaldi A: Inflammation and myeloproliferative neoplasms. J Autoimmun 2017;85:58–63
35 Tefferi A: Primary myelofibrosis: 2012 update on diagnosis, risk stratification, and management. Am J Hematol 2012;2011:1017–26
36 Helbig G: Classical Philadelphia-negative myeloproliferative neoplasms: focus on mutations and JAK2 inhibitors. Med Oncol 2018;35:119
37 Cario H, McMullin MF, Pahl HL: Clinical and hematological presentation of children and adolescents with polycythemia vera. Ann Hematol 2009;88:713–9
38 Cario H, Schwarz K, Herter JM, *et al.*: Clinical and molecular characterisation of a prospectively collected cohort of children and adolescents with polycythemia vera. Br J Haematol 2008;142:622–6
39 Barbui T, Passamonti F, Accorsi P, *et al.*: Evidence- and concensus- based recommendations for phlebotomy in polycythemia vera. Leukemia 2018;32:2077–81
40 Veterans Administration Medical Center San Diego - Personal communication with the infusion service. November 2019.
41 Evers D, Kerkhoffs JL, Egmond LV, *et al.*: The efficiency of therapeutic erythrocytapheresis compared to phlebotomy: A mathematical tool for predicting response in hereditary hemochromatosis, polycythemia vera, and secondary erythrocytoerythrocytasis. J Clin Apher 2014;29:133–8
42 Harrison CN, Koschmieder S, Foltz L, *et al.*: The impact of myeloproliferative neoplasms (MPNs) on patient quality of life and productivity: results from the international MPN Landmark survey. Ann Hematol 2017;96:1653–65
43 Teofili L, Valentini CG, Rossi E, *et al.*: Indications and use of therapeutic phlebotomy in polycythemia vera: which role for erythrocytapheresis? Leukemia 2019;33:279–81
44 Kaboth U, Rumpf KW, Liersch T, *et al.*: Advantages of isovolemic large-volume erythrocytapheresis as a rapidly effective and long-lasting treatment modality for red blood cell depletion in patients with polycythemia vera. Ther Apher 1997;1:131–4
45 Landolfi R, Marchioli R, Kutti J, *et al.*: Efficacy and safety of low dose aspirin in polycythemia vera. N Eng J Med 2004;350:114–24
46 Dillinger JG, Sideris G, Henry P, *et al.*: Twice daily to improve biological asprin efficacy in patients with essential thrombocythemia. Thromb Res 2012;129:91–4
47 HYDREA (hydroxyurea) highlights of prescribing information https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/016295Orig1s047,s048Lbl.pdf [accessed 30 November 2020].
48 Alvarez-Larran A, Martinez-Aviles L, Hernandez-Boluda JC, *et al.*: Busulfan

in patients with polycythemia vera or essential thombocythemia refractory or intolerant to hydroxyurea. Ann Hematol 2014;93:2037–43

49 National Library of Medicine. Pubchem. https://pubchem.ncbi.nlm.nih.gov/compound/busulfan [accessed 30 August 2020.

50 Highlights of prescribing information. Busulfex (busulphan) for injection 18 Jun 2014 https://www.accessdata.fda.gov/drugsatfda_docs/label/2015/020954s014lbl.pdf [accessed 30 August 2020.

51 Tefferi A, Rumi E, Finazzi G, *et al.*: Survival and prognosis among 1545 patients with contemporary polycythemia vera: an international study. Leukemia 2013;27:1874–81

52 PEGASYS (peginterferon alfa-2a) https://www.accessdata.fda.gov/drugsatfda_docs/label/2002/pegihof101602LB.htm [accessed 24 September 2020].

53 Manufacturer's site. Jakafi (ruxolitinib) targets a primary driver of polycythemia vera. Mechanism of action. https://hcp.jakafi.com/polycythemia-vera/pathophysiology-moa [accessed 7 December 2020].

54 Genetic Testing Registry, National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/gtr/genes/3717/ [accessed 16 September 2020].

55 Raedler LA: Jakafi (ruxolitinib). First FDA-approved medication for the treatment of patients with polycythemia vera. Am Health and Drug Benefits 2015;8:75–9

56 Kesarwani M, Huber E, Kincaid Z, *et al.*: Targeting substrate-site in JAK2 kinase prevents emergence of genetic resistance. Nat Sci Rep 2015;5:14538.

57 Blair HA: Fedratinib: first approval. Drugs 2019;79:1719–25

58 Teofili L, Giona F, Martini M, *et al.*: Markers of myeloproliferative disease in childhood polycythemia vera and essential thrombocythemia. J Clin Oncol 2007;25:1048–53

59 Kaboth U, Rumpf KW, Lipp T, *et al.*: Treatment of polycythemia vera by isovolemic large-volume erythrocytapheresis. Wien Klin Wochenschr 1990;68:18–25

60 Valbonesi M, Bruni R: Clinical application of therapeutic erythrocytapheresis (TEA). Transfus Sci 2000;22:183–94

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ORIGINAL PAPER

Complications of blood donation reported to haemovigilance systems: analysis of eleven years of international surveillance

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Vox Sanguinis

Background and Objectives The International Haemovigilance Network collects aggregate data on complications of blood donation from member haemovigilance systems (HVS). We analysed the data collected in 2006–2016 in order to learn from it and consider future improvements.

Materials and Methods National HVS entered annual data on donation complications and on annual whole blood and apheresis donations in the 'ISTARE' (International Surveillance of Transfusion Adverse Reactions and Events) online database. We calculated national and aggregate donation complication rates.

Results Twenty-four HVS provided data for 138 country years (CY; median 7 CY, IQR 2–8), covering 155 M donations. The overall complication rate was 6·3/1000 donations and the median country rate 3·2/1000 (IQR 1·1–10·1). Overall and severe complication rates varied considerably between HVS. Vasovagal reactions (VVR) were most commonly reported: 4·6/1000 donations, median country rate 3·1/1000 donations (IQR 0·6–7·7). Rare complications included generalized allergic reaction (0·10/100 000) and major blood vessel injury (category available since 2015; 0·12/100 000). Eighteen HVS reported complications of whole blood donation (WBD) and apheresis separately (89 CY, 101·6 M WBD and 26·3 M apheresis donations). The median country VVR rate was 3·4/1000 WBD (IQR 1·0–9·1) and 1·5/1000 apheresis donations (1·0–4·2). Rates of venepuncture-related complications tended to be higher for apheresis: the median country rate of reported haematomas was 0·39/1000 WBD (IQR 0·31–1·2) vs. 4·2/1000 apheresis donations (0·69–5·6).

Conclusion International reporting allows HVS to study rates of blood donation complications and capture information about very rare events. The present variability of reporting and severity assessment hampers comparisons between HVS and requires further work.

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Key words: apheresis donation, blood collection, complication, donor health, haemovigilance.

Introduction

Blood donation by volunteer healthy donors is essential to the provision of safe blood components for transfusion, as well as for the production of plasma-derived medicines. A small proportion of blood donors may suffer from a generalized reaction such as dizziness or fainting (commonly termed vasovagal reactions, VVR) or a venepuncture-related complication such as a haematoma or less commonly a nerve or tendon injury. Serious adverse complications can also result, for example compartment syndrome following a haematoma, arteriovenous fistula or thrombosis [1–3].The great majority of blood donors make an uncomplicated recovery from complications with appropriate supportive management. Nevertheless, even experienced donors are less likely to return following a complication, even if this was only mild [1,2].

For practical as well as ethical reasons, it is essential for blood services to record complications of whole blood and apheresis procedures and to develop strategies to minimize their occurrence. In recent years, increasing numbers of publications have highlighted risk factors and demonstrated the effectiveness of mitigation measures. Generally, such work has been conducted within the setting of a single blood establishment.

In response to a desire to benchmark and compare donation complication rates as well as transfusion reactions between countries and haemovigilance systems (HVS), the International Haemovigilance Network (IHN) developed the 'ISTARE' database (International Surveillance of Transfusion Adverse Reactions and Events) for annual capture of national aggregate haemovigilance data [4]. The database includes spreadsheets for collecting data on complications of whole blood and apheresis donations as well as annual numbers of donations. Countries can view automated graphs representing their own data alongside that of other countries. We report here on an analysis of data of reported donation complications, collected from member national HVS since 2006. Our aim was to learn from the data and consider future improvement of data collection.

Materials and methods

Software

Pilot data collection in 2006–2007 was conducted using a spreadsheet (Excel, Microsoft Corp). From 2008, national HVS entered annual aggregate data on donor complications in the password-protected ISTARE online database (programmed and maintained by Steficon S.A., Athens). Annual numbers of whole blood and apheresis donations were also collected. Governance and general functioning of ISTARE have been reported elsewhere [4]. The online donor complication spreadsheet requested entry of separate data for whole blood donation (WBD) and apheresis, but also provided an option for entering pooled data for all donation types. Reactions were captured according to severity level (mild, moderate, severe) but without distinction between male and female donors or first time vs. repeat donation. At the time of data collection, support staff attempted to resolve data inconsistencies by communicating with the reporting HVS. The present analysis was conducted retrospectively and without further attempts to clarify data.

Definitions

The first IHN-ISBT Standard Surveillance definitions for blood donation complications were published in 2008 and were recommended for use for reporting to ISTARE until 2014. Revised ISBT-IHN-AABB harmonized international standard definitions were published in December 2014 and were implemented in the ISTARE spreadsheet for data from 2015 [5]. In the 2014 revision, severity assessment is stated to be optional, and no category-specific criteria are provided for classification of reactions as mild, moderate or severe. The rationale for this was that 'The grading of severity used for adverse reactions in recipients is poorly adapted to donor reactions. It was therefore decided that grading of reactions for severity would be optional. The notion of severity will also be captured by the types and definitions of reactions' [6–8].

Statistical analysis

Country annual submissions were included in the analysis if donation complication data were reported and the number of donations was also available for that year. Extracted data were used to calculate overall aggregate rates of each type of complication (i.e. the grand total rate over all donations and countries) and aggregate national rates of complications (per 1000 or 100 000 donations); we calculated medians and interquartile ranges (IQR) for the country data. Where possible, figures

for complications were analysed separately for whole blood and apheresis donations within a country. Separate analyses of the defined subcategories of VVR were performed to evaluate data homogeneity and limitations of the reported data for comparison purposes because VVR represented the largest group of reported reactions, and because VVR are important for donors as well as for blood services, for research on risk factors and mitigation strategies. The variances in VVR rates within and between countries were compared using the Variance Components command in the General Linear Model procedure of SPSS (IBM SPSS Statistics, Version 25.0. IBM Corp, Armonk, NY, USA).

Results

Twenty-four HVS provided figures for donations and donation complications (at least one complication being reported) for one or more years: the median number of years per system was 7, IQR 2–8. All but one HVS were national, with one (North American) HVS providing data from a large, multi-state-based blood establishment; however, for economy of expression, this region will also be referred to as a 'country' in the remainder of this paper. The total number of country years (CY) was 138, covering 154·8 million (M) donations. The HVS contributing data on donation complications represented 24/34 (70·6%) of all HVS which participated in ISTARE data collection (the other HVS providing only recipient vigilance data) and 138/222 (62·2%) of CY data sets captured in the ISTARE system in 2006–2016. A total of eight CY data sets were submitted by five HVS in Europe and Asia in the pilot years of 2006 and 2007 (12·0 M donations).

With respect to compliance with the international definitions, comments in the annual submissions were examined. Three HVS mentioned deviations in their data owing to inability to distinguish mild vs. moderate VVR or immediate vs. delayed type reactions (total of 7 CY, 8·7 M donations) and two HVS provided partial data (7 CY, 8·7 M donations), omitting cases not classified according to the definitions. It was not possible to adjust the number of donations included in rate calculations in these cases. The remaining data were submitted as compliant (124 CY, 89% of donations covered), and however, the system did not actively obtain confirmation from the correspondents that their data were compliant with definitions.

The overall complication rate was 6·3/1000 donations, and the median country rate was 3·2 complications/1000 donations (IQR 1·1–10·1). Table 1 shows summary data of the contributing HVS and Table 2 gives the calculated aggregate incidences of the different types of complications. Note that rates of severe reactions in Table 2 were calculated based on severity as reported; one or more severe reactions were reported in 113/138 CY (84·1%). Some types of complication which occur frequently in collection centre practice were reported in fewer than half of the CY forms, for example, delayed bleeding in 53/138 CY (38·4%) and infiltration in 3/31 CY (9·7%), the latter being an optional category under apheresis from 2015.

Vasovagal reactions

Vasovagal reactions were the most commonly reported complication with overall incidence 4·6/1000 donations and median country rate 3·1/1000 donations (IQR 0·6–7·7); VVR were reported in 136/138 CY, 98·6%. Immediate reactions comprise all reactions occurring before the donor left the collection site; out of all the reported VVR, 95·5% were immediate type reactions where no accident (injury) was reported. An associated accident was reported in 0·7% of all VVR (114/136 CY, 83·8%), 0·5% of immediate type and 4·6% of delayed VVR. Fig. 1 shows the proportions of immediate vs. delayed reactions and of VVR which were associated with an accident. Regarding severity, 6/136 CY used only the moderate severity level to report VVR, 20 gave reactions as either mild or severe, four only as mild, and one only as severe. Fig. 2 shows the proportions of mild, moderate and severe VVR per HVS. There were considerable differences in VVR rates per HVS, seen not only in the 1000-fold difference in overall reported VVR rate, but also in rates of severe VVR: median 8·7/100 000 donations with a nearly 90-fold range of 1·1 to 98/100 000 donations in countries which reported any severe VVR. Fig. 3 presents the rates of reported mild, moderate and severe VVR per HVS. From the modelling of the variance components of annual total VVR rates, variance within countries was 13% of the variance between countries. Thus, overall VVR rates tended to be relatively consistent within a HVS from year to year but showed more variation between HVS; this was also the case for reactions classed as severe.

Rare complications

Rare complications such as generalized allergic reaction (overall rate 0·10 per 100 000, cases being reported in 40 CY), major blood vessel injury (category available since 2015; overall 0·12 per 100 000, 6 CY) or air embolism associated with apheresis (0·05 per 100 000, 11 CY) were only reported occasionally (Table 2). It should be noted that the data capture spreadsheets do not make it possible to determine whether a particular type of complication would potentially have been reported had it occurred, or whether the HVS did not request blood establishments to submit that complication type.

Table 1 Overview of blood donation complication data sets

Continent:	No. of HVS	No. of country years (CY)	Total donations	Years covered
Europe	17	95	83 898 086	2006–2016
N. America	1	2	1 512 975	2010–2011
S. America	–	–	–	–
Africa	1	7	6 809 157	2010–2016
Asia	4	26	61 194 861	2006–2016
Oceania	1	8	1 377 355	2009–2016

	Total	No. of CY	Median per CY	IQR
No. of donations	154 792 434	138	404 270	180 969–971 781
Whole blood	124 823 991	136[a]	396 497	175 160–942 965
Apheresis	29 968 443	134[a]	24 524	5 584–261 632
Separate analyses of whole blood vs. apheresis complications				
Whole blood	101 592 905	89	469 805	190 563–2 478 810
Apheresis	26 266 241	89	50 677	10 661–442 895

Abbreviations: CY, country year (submitted data for one year); HVS, Haemovigilance system; IQR, interquartile range; WBD, whole blood donation.
[a]Two HVS (2 CY) provided a pooled figure for WBD and apheresis, and one HVS (2CY) provided data only for WBD.

Table 2 Reported complications of whole blood or apheresis donations in 2006-2016

	Total reported	(Total CY[a])	Rate per 100 000[b]	Total severe[c]	% Severe
Haematoma	178 513	(126)	115	6251	3·5
Arterial puncture	1618	(104)	1·0	85	5·3
Delayed bleeding	1830	(53)	1·2	29	1·6
Painful arm (2006–2014)	24 440	(102)	20	2330	9·5
Localized infection	265	(33)	0.17	90	34
Thrombophlebitis	374	(56)	0·24	162	43
VVR (vasovagal reaction) Immediate type	680 644	(136)	442	25 240	3·7
VVR Immediate type, accident	3707	(107)	2·4	1623	44
VVR Delayed type	27 005	(110)	18	2377	8·8
VVR Delayed, accident	1288	(88)	0·84	431	33
Generalized allergic reaction	149	(40)	0·10	55	37
Other[d]	19 281	(90)	NA[d]	2088	11
Citrate reaction (apheresis)	14 002	(117)	47	926	6·6
Haemolysis (apheresis)	43	(16)	0·14	10	23
Air embolism (apheresis)	16	(11)	0·05	6	38
New categories from 2015					
Nerve injury	1625	(23)	5·3	216	13
Other arm pain	5104	(20)	17	353	6·9
Cellulitis	10	(2)	0·03	9	90
Local allergic reaction	105	(10)	0·34	29	28
Blood vessel injury[e]	36	(6)	0·12	24	67
Infiltration (apheresis)	40	(3)	0·71	0	0

[a]CY (country year records) in which this complication was reported.
[b]Rate calculated based on total donations in countries which reported any complications in that year, except for VVR where donations in two CY without reported VVR were not included.
[c]Calculations based on severity as reported; see discussion regarding need for harmonization.
[d]No overall rate calculated for other reaction owing to lack of homogeneity; major blood vessel injury included in Other prior to 2015.
[e]Major blood vessel injury includes but does not distinguish between rare serious events, for example myocardial infarction, TIA or CVA.

The data collection table also comprises a category of other, descriptively presented in Table S1. The category included cases of major blood vessel injury prior to 2015 when a separate category of major blood vessel injury was introduced with the new definitions. 'Other' is used highly variably by reporters. For instance, one country

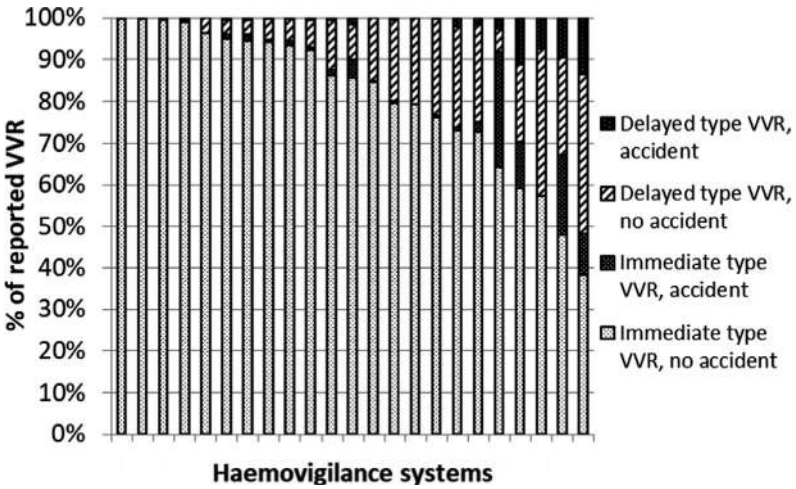

Fig. 1 Breakdown of reported vasovagal reactions (VVR) according to time of reaction and occurrence of accident.

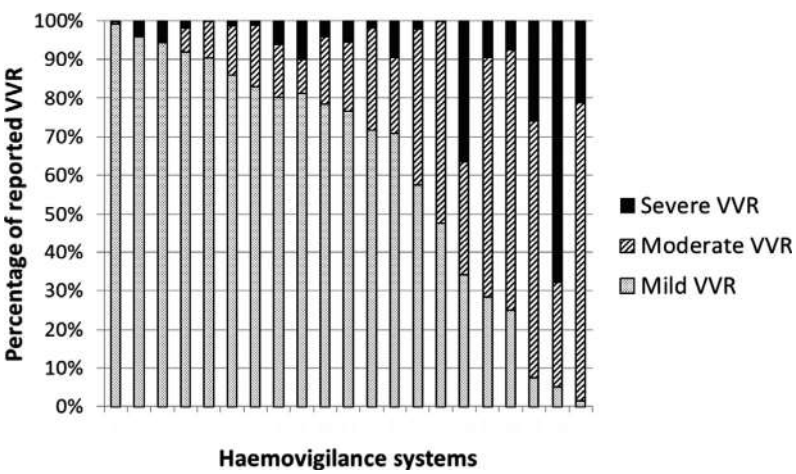

Fig. 2 Breakdown of vasovagal reactions (VVR) by severity.

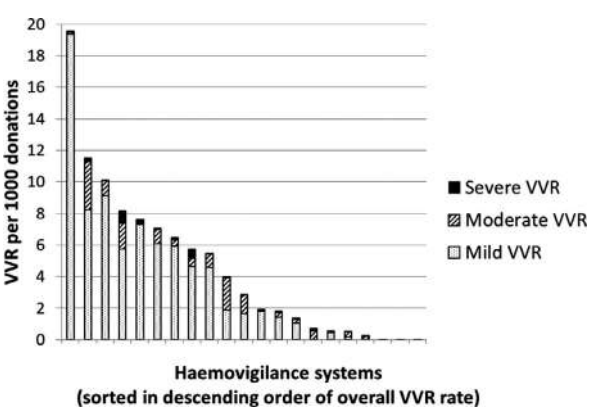

Fig. 3 Country rates of vasovagal reactions (VVR): average over each country's year with data.

recorded reports of red blood cells not being returned to an apheresis donor in this group, not listed as a reportable complication in the definitions. Serious other complications amounted to 1·3 per 100 000 in the total data set.

Complication rates for whole blood vs. apheresis

Eighteen of the HVS provided separate data for complications of both whole blood and apheresis donations in one or more years (total 89 CY, 101·6 million WBD and 26·3 million apheresis donations, total 128 million donations). Out of the 130 CY submissions in 2008 or later, 126 provide the number of apheresis collections which amount to a total of 27·1 million. Therefore, all but 0·8 million apheresis collections were accompanied by specific complication data. Nevertheless, three out of the four CY lacking an apheresis denominator came from countries where it is known that apheresis collections are performed therefore the calculated 0·8 million represents an underestimation of the apheresis collections for which complications could not be analysed separately. In annual submissions mentioning citrate reactions on the apheresis complication spreadsheet but no other types of complications for apheresis (10 CY), this was not counted as provision of separate apheresis complication data.

For the HVS which provided separate data for complications of WBD and apheresis, the median country rate of vasovagal reactions was 3·4/1000 WBD (IQR 1·0–9·1) and 1·5/1000 apheresis procedures (1·0–4·2). Nevertheless, the VVR rate was higher for apheresis than for WBD in some countries: the median country relative risk for VVR with apheresis compared to WBD was 0·64 but the interquartile range ran from 0·43 to 1·15. Reported haematoma rates were higher for apheresis than for WBD: the median per HVS was 39/100 000 WBD (IQR 31–116) vs. 417/100 000 aphereses (IQR 69–557); rates of arm pain and/or nerve injury (captured as a single category in 2006–2014 and separately in 2015–2016) also tended to be higher: median 9/100 000 with WBD, IQR 3–34, vs. 39/100 000 with apheresis, IQR 5–57.

Discussion

The present report is the first to publish information about such a large volume of data and this is only possible because of the voluntary participation by multiple national HVS which are actively monitoring the safety of their blood donors. Such a large body of data has the potential to provide relevant data on rare types of donation complications. Over the observation period, the reporting HVS showed broadly constant rates of complications within systems. Data on delayed bleeding and infiltration, which are relatively frequent types of complications but listed as optional categories in the revised definitions, were not reported by all HVS and this can contribute to variation in overall complication rates between systems. The category Other was used variably by the different HVS, and this revealed a need for additional guidance on reportable donation complications. 'Other' remains relevant for submission of events and complications not captured in other groups. Reports of Other should be accompanied by adequate description and may highlight hazards such as anaemia, for which reporting criteria and definitions should be developed in the future.

There was considerable between-system variation in overall rates of reactions, with a thousandfold difference between the highest and lowest country rates of vasovagal reactions. The ability of HVS to provide separate donation complication data for whole blood and apheresis donations improved from 47% of CY in 2008–2009 to 70% in 2013–2016.

Variation in overall reporting rates of complications is already known to exist between collection centres within one system, where all work under one protocol (authors' experience), but the differences should 'average out' within centres of a large blood establishment. When considering differences between multiple blood establishments in one country or variation between countries, one is interested in the true differences and particularly in what they may reveal about safer and less safe practices. Part of the true variation represents differences associated with the donor population characteristics, and part is related to potentially modifiable factors such as donation parameters, donor care and circumstances (e.g. less experienced staff, poor centre climate control, inadequate equipment or less suitable mobile/stable collection site) [9]. Variation may also result from differences in reporting behaviour, as when staff on busy sessions may forget or not make time to record complications. In addition, there are differences between organizations and between countries in implementation of definitions and reporting instructions. Table 3 summarizes some relevant factors.

Regarding the breakdown of VVR into subgroups of reactions on or off site and according to whether an accident occurred, broadly it appeared that most HVS were able to provide this level of detail since one or more delayed VVR were reported in 82·4% of CY forms and one or more VVR with accident in 83·8% of forms. Nevertheless, there were some countries with over 10 000 donations but without any delayed VVR or reports of injuries. In the present state of data collection, it could not be ascertained whether these HVS were not able to extract data about the place (time) of onset and occurrence of accidents associated with faints; or whether – as is conceivable in smaller organizations – these did not occur. In a country with more than one blood establishment, there may be differences in capturing data.

The higher percentage of the delayed reactions (i.e. offsite) which were associated with accidents might (partly) result from reporting or recall bias. Reporting of delayed reactions is probably incomplete, with donors less likely to inform the blood establishment or mention it at their next attendance unless they had an accident. However, a relatively high rate of accidents in donors who react after leaving the centre is plausible since accidents are less likely if a donor reacts while reclining on the phlebotomy chair. Also, supervision and support in the recovery area should contribute to avoiding accidents in donors who become dizzy or faint at this stage. Delayed reactions remain an important concern.

The subdivision of VVR by severity revealed that there were differences between the use of the levels mild/moderate/severe to submit country data, implying that there were corresponding differences between the labels in use in reporting blood establishments. Indeed, several HVS mentioned difficulties in complying with the severity breakdown in their data submissions, as described in the Results section.

Table 3 Challenges for benchmarking: causes of differences in reported incidence of complications

True differences in rates	Differences in reporting
Donation process, for example	Applied definitions
Whole blood vs. apheresis	Protocol re what is captured
Volume collected	Staff awareness, knowledge, and time to report
Citrate concentration, use of replacement fluid, extracorporeal volume in apheresis	Are donors encouraged to report symptoms?
Care given, for example	Mechanisms (if any) re late complications
Adequate donor screening and information, phlebotomy skills, smooth process, explanation and attention given	Differences in application of definitions (even where definitions are the same)
Environment, recovery area, atmosphere and refreshments	Differences in severity assessment • Between centres within one organization • Between organizations within one country • Between countriesAccessibility of data for monitoring
Donor population and circumstances:	
Male vs. female	
1st donation vs. repeat, number of previous donations	
Age	
Estimated blood volume, haemoglobin levels	
Ambient temperature, etc.	

Restricting the analysis to the reactions which were reported as severe, there was still a 90-fold difference in country rates. There may be differences in severity assessment between organizations submitting data to a single HVS. Lack of guidance in the international definitions doubtless contributed, and this was observed in the validation of the revised definitions [8]. However, even where standard criteria are given, wide variations exist as is seen in the voluntary data on complications of blood donation which are reported annually to the European Commission [10]. The need for additional guidance was recently addressed by a Working Group of the AABB with representation of IHN and ISBT, which developed and validated a tool modelled on the pharmacovigilance Common Terminology Criteria for Adverse Events [11]. The tool is intended as an appendix to the revised definitions and provides complication-specific as well as generic criteria and explanations to support severity classification of blood donation complications into five grades, which broadly correspond to mild, moderate, severe, life-threatening or death. Blood establishments and national HVS (including the national competent authorities within the European Union) may not immediately adopt international definitions or severity grading criteria because of their existing practices and interpretations of criteria. However, if they are able to 'map' their data to such international points of reference or formally align with them in time, this could improve the comparability of data in future.

A limitation of the aggregate country data in the present ISTARE data set is that it does not allow investigation of associations with underlying parameters. If summary data of countries' donation parameters and summary donor population characteristics were available, hypotheses could be formed about possible explanations for differences. In future, more granular (stratified) country data might be collected to permit such analyses. An appendix to the revised definitions makes recommendations for parameters, such as a breakdown into age bands, which could be used in either of these ways [5]. Readers can consult the standard definitions on the ISBT website and obtain more information about relevant denominator attributes in order to examine the impact of these parameters in their own data or the literature. However, even if full information were available in the ISTARE data, this could not explain a 1000-fold difference in complication rates.

In our personal experience, experts (as well as donors) differ in their perception and experience of whether rates of VVR are higher in association with whole blood donation or apheresis. The ISTARE analyses show within-country figures that go either way, with the IQR of the country relative risk for reactions running from 0·43 to 1·15 for apheresis in comparison to WBD. A recent analysis in the Netherlands showed that overall the rate of VVR is lower with apheresis, but that if donors are compared within age bands and stratified according to the number of previous donations, the rate of VVR is higher with apheresis (unpublished data). This again shows the importance of incorporating additional parameters when attempting to compare country rates of complications. Regarding venepuncture-related complications, these are consistently higher in association with apheresis as can be expected because of the return of donors' red blood cells during the procedures.

An important limitation of the data is the inability to verify compliance with the standard definitions or to adequately take account of deviant and incomplete reporting. Solutions for this problem are needed in order for data sharing and comparisons between HVS and countries to become meaningful. For the capture and analysis of very rare complications of blood donation, until 2015 no separation of such cases was possible except by free text comments.

A weakness of the collected data is the lack of information on long-term complications of blood donation. However, this only mirrors the current situation in donor haemovigilance. The awareness of iron deficiency as an important complication of regular blood donation has

increased in recent years, with evidence accumulating for the effectiveness of various preventive or mitigation strategies [12]. Nevertheless, there is not yet an international consensus definition for this complication, nor an established indicator by which it could be monitored by haemovigilance systems. Lack of agreed definitions and indicators also currently prevents meaningful international regular collection of data concerning other relevant issues, such as that of lower albumin levels or immunoglobulin levels in relatively high-frequency plasma donors [13].

Despite the limitations of the data, they draw attention to the occurrence of rare, very serious complications of which the rate is so low that inter-organization and international data exchange is necessary to gain insight into rates, risk factors and preventive measures. Moreover, the analysis has pinpointed differences between countries and organizations in their data capture and underlined a need for tools to support organizations in achieving 'mappability' of data for comparing and sharing data. The International Haemovigilance Network aims to incorporate the lessons from the experience to date in redeveloping its data collection tool to allow HVS to share and compare data in a more robust way in the future. Overall, the data show that reported complication rates are low and that donating blood or blood components can be considered a safe process.

Summary/conclusions

International reporting allows HVS to study rates of different types of blood donation complications. Differences in rates between WBD and apheresis donation procedures can be examined and information captured about very rare, serious events. Differences between donation procedures and donor demographics may account for some of the variation between country data, but the current ISTARE tool does not collect these data. Variability of reporting practices and of severity assessment between countries also impairs the feasibility of comparisons between HVS. Work is needed to improve harmonization of the classification of donation complications and severity assessment for data comparison and research.

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Conflict of interests

The authors declare no conflict of interests.

Author contributions

JCW-O, CP and CR supervised the data collection, and KL also had access to the primary data; JCW-O and CR conducted the data analyses. JCW-O drafted the manuscript. All authors critically reviewed the manuscript and agree to its submission.

References

1 Wiersum-Osselton JC, Marijt-van der Kreek T, Brand A, *et al.*: Risk factors for complications in donors at first and repeat whole blood donation: a cohort study with assessment of the impact on donor return. *Blood Transfus*. 2014; **12**(Suppl 1):S28–S36

2 Newman BH, Newman DT, Ahmad R, *et al.*: The effect of whole-blood donor adverse events on blood donor return rates. *Transfusion* 2006; **46**:1374–1379

3 Newman B, Rajpurkar M, Ozgonenel B, *et al.*: Upper-extremity deep venous thrombosis after whole blood donation: Report of three cases from a single blood center. *Transfusion* 2015; **55**:1290–1293

4 Politis C, Wiersum JC, Richardson C, *et al.*: The International Haemovigilance Network Database for the Surveillance of Adverse Reactions and Events in donors and recipients of blood components: technical issues and results. *Vox Sang* 2016; **111**:409–417

5 Standard for Surveillance of Complications Related to Blood Donation. ISBT working party on haemovigilance in collaboration with the International Haemovigilance Network and the AABB Donor Haemovigilance Working Group. Available at. Available at: http://www.isbtweb.org/fileadmin/user_upload/Donor_Standard_Definitions_

Final_2014.pdf [Last Accessed 1-5-2020]
6 Rationale for changes from 2008 to 2014 classification of adverse donor events. http://www.isbtweb.org/filead min/user_upload/files-2015/haemovi gilance/definitions/Rationale%20cha nges%202008%20to%202014%20cla ssification%20of%20complications% 20of%20donation%20haemovigilance %202014.pdf [Last Accessed 1-5-2020]
7 Goldman M, Land K, Robillard P, *et al.*: Development of standard definitions for surveillance of complications related to blood donation. *Vox Sang* 2016; **110**:185–188
8 Land KJ, Townsend M, Goldman M, *et al.*: International validation of harmonized definitions for complications of blood donations. *Transfusion* 2018; **58**:2589–2595
9 Council of Europe: *Guide to the Preparation, Use and Quality Assurance of Blood Components*, 20th edn.. San Antonio: European Directorate for the Quality of Medicines & HealthCare of the Council of Europe (EDQM). Council of Europe, 2020 ISBN 978-92-871-8644-7.
10 European Commission DG for Health and Food Safety: Summary of the 2018 annual reporting of serious adverse reactions and events for blood and blood components, 2020. Ref. Ares (2020) 147152–10/01/2020 https://ec.europa.eu/health/sites/hea lth/files/blood_tissues_organs/docs/ 2018_sare_blood_summary_en.pdf [Last Accessed 1-5-2020]
11 Townsend M, Kamel H, van Buren N, *et al.* Development and validation of donor adverse reaction severity grading tool: enhancing objective grade assignment to donor adverse events. *Transfusion* 2020; **60**:1231–1242
12 Kiss JE, Vassalo RR: How do we manage iron deficiency after blood donation? *Br J Haem* 2018; **181**:590–603
13 Laub R, Baurin S, Timmerman D, *et al.*: Specific protein content of pools of plasma for fractionation from different sources: impact of frequency of donations. *Vox Sang* 2010; **99**:220–231

Supporting Information

Additional Supporting Information may be found in the online version of this article:
Table S1. Descriptive data, where provided as optional free text comments, on information reported under “Other”.

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Trends in blood donations, blood donors' knowledge, practices and expectations during the COVID-19 pandemic in Cameroon

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Vox Sanguinis

Introduction The COVID-19 pandemic, caused by a novel coronavirus, has already affected over 99 062 people in 53 African countries and killed 3082. The pandemic threatens blood supply but we do not yet know its impact on blood donations or on the perceptions and expectations of donors.

Methods We conducted a cross-sectional study in four hospital-based blood services in Cameroon, using a survey design and focusing on the subjective and cultural aspects of donors. Using a semi-structured questionnaire, we collected the participants' responses as to their understanding of COVID-19 and of current protection measures, and their expectations. Data on trends of blood donations were collected retrospectively for the period from 1st January to 30th April 2019 and the same period in 2020.

Results Of 494 donors included, 432 (87·4%) were enrolled from fixed blood collection sites and 62 (12·6%) were contacted by phone. A total of 464 (93·9%) participants believed that the COVID-19 is a lung disease, but some declared it to be imaginary (7·7%), a foreign disease (8·3%) or a blood-borne disease (3·2%). The participants reported that the distribution of face masks and hydroalcoholic solutions (92·5%), social distancing and hygiene (6·3%) are the most important measures that need to be in place for safe donation. The number of blood donations dropped by 21·5% between 2019 and 2020.

Conclusion Most of the donors know COVID-19, its transmission routes and manifestations. In the absence of barrier measures, they perceive blood donation as a threat to their health. Distribution of masks and hydroalcoholic solution might motivate more donors and improve the blood supply.

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Introduction

At the time this report was written, the COVID-19 pandemic, caused by a novel coronavirus, has already affected over 99 062 people in 53 African countries and killed 3,082 [1]. The number of cases in Africa is currently lower than those reported in the United States,

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China and Europe, although the figures are increasing very fast every day in sub-Saharan countries [1–3]. The global spread of COVID-19 acute respiratory disease continues to grow, and the full extent and severity of this outbreak is yet to be seen. Furthermore, delays in diagnosis and appropriate management and in effective control measures increase the likelihood of spread. These concerns apply particularly to low- and middle-income countries where health systems tend to be weaker. In such settings, laboratory resources may be limited, notification of infectious diseases not timely or incomplete, and public health infrastructures not adaptable [4]. In May 2020, Cameroon registered 4288 cases, the highest number of people affected by the disease in Central Africa [1].

The pandemic threatens the safety of both donors and recipients. The median incubation period of COVID-19 is up to 10 days, suggesting possible infectivity of blood donors even if they appear safe after screening [5,6]. Low concentrations of viral RNA in the plasma or serum from COVID-19 persons probably present only a theoretical or minimal risk of viral transmission. [7,8]. In January 2020, the World Health Organization (WHO) recommended a precautionary deferral of donations of blood and cells for 21 days after possible exposure to a confirmed patient, and at least 28 days after symptom resolution and completion of therapy for those who have the disease. [9].

With an already insufficient blood supply in sub-Saharan Africa (SSA), the pandemic further poses a threat to the number of units donated leading to a lower supply. Home confinement limits the ability of donors to attend blood services and the staff to take part in collection teams as well. The restrictions and limitations of large gatherings also reduce the number of large blood drives and campaigns. Moreover, donors' fear of potential infection may lead them to refrain from donating. Blood banks will need to develop new educational and motivational strategies to attract donors. But the real impact of the pandemic COVID-19 on blood donations is not yet known, nor is the current perceptions and expectations of blood donors. Blood donations have dropped significantly in the principal Cameroonian blood services during the first 4 months of the year 2020. We believe that the voluntary non-remunerated blood donors (VNRBD) are more knowledgeable than family replacement donors (FRD) about the pandemic and that blood donors' perceptions and expectations are mainly related to their personal protection.

Methods

Study design

This was a cross-sectional study conducted during the months of March and April 2020 in four hospital-based blood services located in the two most affected cities in Cameroon (Yaoundé and Douala): The Yaoundé University Teaching Hospital (YUTH), The Douala Laquintinie Hospital (DLH), the Essos Hospital Center of Yaoundé (EHC) and the Yaoundé Central Hospital (YCH). It was a survey design focusing on subjective and cultural aspects of blood donation, highlighting cultural features that emerged in the interviews of the participants. In order to define the impact of the COVID-19 on donations, we conducted a retrospective analysis of data collected on blood donations.

Subjects and sampling

For the prospective phase, we included consenting blood donors aged 18–65 years. A small proportion, who had not turned up for donation within the last 6 months, were also invited to participate through phone calls. Donors with at least two donations in the last twelve months were called repeat blood donors (RBD). We needed at least 246 blood donors to be able to reject the hypothesis that the proportion of blood donors having a good level of knowledge is lower than 80% with confidence level of 95% and a confidence interval of 0·05.

Measurements

A paper-based questionnaire was designed and added to the routine donor screening questionnaire. We enrolled the participants and collected their responses using a semi-structured questionnaire during the month of May 2020. The questionnaires were self-administered at the fixed blood collection sites for all donors. For those benevolent donors who had not come to donate for the last 6 months, the questionnaires were administered by phone. Each questionnaire consisted of multiple -choice questions, structured into 3 categories: (1) Knowledge of COVID-19, (2) Current practices used to protect themselves, and (3) what would motivate them to donate blood. The most likely answers to these questions were set by staff of the blood services. A fourth section allowed for any additional information from the donors to be provided during the same interview. The questionnaire was designed to take 5 min and was tried out on 10 blood donors before use. At the fixed sites, questionnaires were completed in a private room on the facility's premises. Data on trends in blood donation and blood donors' demographic characteristics were collected retrospectively for the period of 1st January to 30th April 2019 and January through April 2020.

Analysis

Data were entered in Excel 6.0 according to the blood donor type (benevolent, family) and blood service. The following outcomes were calculated: number of blood

donations per month, proportion of VNRBD, frequency of donation based on their response to the questions. Data were calculated and distributed according to the four blood centres and the type of donation if applicable. We compared the proportions using chi-squared tests. A difference in the results with a P value <0·05 was considered significant. The data collected were codified, and risks for donors were minimal. All participating blood donors were properly informed and provided a consent form for signature prior to his /her recruitment. The research site institutional review board provided and expedited approval for the study.

Results

Study population

A total of 494 blood donors from 4 blood services in Yaoundé and Douala were enrolled into the study for the survey. Of these, 432 (87·4%) were enrolled from fixed sites. We called 83 donors by phone and reached 75 donors; among these, 13 refused to participate. Thus, 62 (12·6%) were enrolled by phone. A total of 119 (24·1%) were VNRBD and 375 (75·9%) were FRD. The median age was 28·3 years (Interquartile range (IQR) 18–55 years) and 31··2 years (IQR 18–65 years) for VNRBD and FRD, respectively. The sex ratio was 1·7 in the benevolent group and 1·5 in the family replacement group in favour of males. There was a statistical difference (P = 0·001) in the age distribution between the benevolent and the family replacement donors. The RBD (n = 9) were all VNRBD and represented 7·5% of VNRBD. The demographic and social characteristics of the participants are shown in Table 1.

Donors' knowledge and perceptions

Of the 494 participants, 486 (98·3%) had heard about COVID-19. A total of 464 (93·9%) declared that it is a lung disease but 7·7% and 8·3% declared that the COVID-19 was an imaginary disease or a foreign disease, respectively. This was mainly the FRD (P = 0·01 and 0·06, respectively). Among the participants, 16 (3·2%) believed that it is a blood-borne disease. [There's something I don't get here. There can be overlap between the first category, lung disease, and foreign or blood borne. But those who consider COVID imaginary surely can't also consider it a lung disease. 93·9 plus 7·7 exceeds 100%. Perhaps I'm being finicky. DWH] The participants knew that the main manifestations are cough (95·5%) and fever (85·6%). There was no difference (P = 0·47 and P = 0·38) between the VNRBD and FRD. The participants believed that, during the crisis, there is a higher risk of infection during blood donation (99.4%) and that a donor can infect a blood recipient (74·9%) (Table 2).

Donors' practices and expectations

Measures taken by the blood donors to avoid the infection were hand washing (95·9%), the use of a face mask (88·7%) and social distancing (81·2%).

Blood services can undertake certain measures to encourage blood donation during this period. The participants recommended the distribution of face masks and hydroalcoholic solution (92·5%), social distancing and hygiene at the blood service (6·3%), and appropriate welcome and services to the donor (3·6%) as important. (Table 3).

Table 1 Distribution of the 494 donors enrolled for the survey on level of knowledge, practices and expectations in regard to COVID-19 in Cameroon

		EHC (n = 26)	YUTH (n = 137)	YCH (n = 110)	DLH (n = 221)	All blood services (n = 494)
Age range	18–29 n (%)	2 (7·7)	67 (48·9)	18 (16·4)	114 (51·6)	201 (40·7)
	30–39 n (%)	14 (53·9)	41 (29·9)	69 (62·7)	57 (25·8)	181 (36·6)
	40–49 n (%)	8 (30·7)	19 (13·9)	21 (19·1)	32 (14·5)	80 (16·2)
	50 or more n (%)	2 (7·7)	10 (7·3)	2 (1·8)	18 (8·1)	32 (6·4)
Sex	Male n (%)	14 (53·9)	111 (81·0)	87 (79·1)	181 (81·9)	393 (79·5)
	Female n (%)	12 (46·1)	26 (19·0)	23 (20·9)	40 (18·1)	101 (20·5)
Type of recruitment	Recruited at fixed site n (%)	19 (73·1)	109 (79·5)	91 (82·7)	213 (96·3)	432 (87·4)
	Recruited by Phone n (%)	7 (26·9)	28 (20·5)	19 (17·3)	8 (3·7)	62 (12·6)
Type of donor	VNRBD n (%)	11 (42·3)	47 (34·3)	28 (25·4)	33 (14·9)	119 (24·1)
	FRD n (%)	15 (57·7)	90 (65·7)	82 (74·6)	188 (85·1)	375 (75·9)

DLH, Douala Laquintinie Hospital; EHC, Essos Hospital Center of Yaoundé; FRD, Family replacement donors; RBD, repeat blood donors (n = 9) represented 7.5% of VNRBD; VNRBD, Voluntary Non Remunerated blood donors ; YCH, Yaoundé Central Hospital; YUTH, Yaoundé University Teaching Hospital.

Table 2 Level of knowledge and perceptions in regard to COVID-19 among 494 Cameroonian blood donors

		Type of donor			
Questions Knowledge and perceptions		**All donors (n = 494)**	**VNRBD (n = 119)**	**FRD (n = 375)**	***P* value**
Have you heard about the coronavirus disease called COVID-19?	Yes	486 (98·3)	118 (99·1)	368 (98·1)	0·30
Which type of disease is it?	Mystical disease	2 (0·4)	2 (1·6)	0 (0)	-
	Lung disease	464 (93·9)	117 (98·3)	347 (92·5)	0·41
	Imaginary disease	38 (7·7)	2 (1·6)	36 (9·6)	0·004
	Blood disease	19 (3·8)	5 (4·2)	14 (3·7)	0·23
	European and Chinese disease	41 (8·3)	12 (10·1)	29 (7·7)	0·06
	Other (pandemic, viral disease)	5 (1·0)	1 (0·8)	4 (1·1)	0·52
How can somebody get the disease?	Saliva	316 (63·9)	102 (85·7)	214 (57·1)	0·000
	Air	361 (73·1)	74 (62·1)	287 (76·5)	0·04
	Sex	4 (0·8)	2 (1·7)	2 (0·5)	0·01
	Hands	376 (76·1)	98 (82·3)	278 (74·1)	0·34
	Blood	16 (3·2)	2 (1·7)	14 (3·7)	0·000
	Other	10 (2·0)	2 (1·7)	8 (2·1)	0·19
What are the manifestations of the disease?	Cough	472 (95·5)	114 (95·8)	358 (95·5)	0·47
	Fever	408 (85·6)	101 (84·9)	307 (81·9)	0·38
	Weight loss	9 (1·8)	1 (0·8)	8 (2·1)	0·04
	Sexual weakness	6 (1·2)	2 (1·7)	4 (1·0)	0·14
	No manifestation	0 (0)	0 (0)	0 (0)	-
	Other	0 (0)	0 (0)	0 (0)	-
Can a blood donor contaminate a blood recipient?	Yes	370 (74·9)	62 (52·1)	308 (82·1)	0·000
Do you think that there is a risk for you if you come to donate blood these days?	Yes	380(76·9)	88 (73·9)	292 (77·9)	0·14
Which risks?	Contamination by someone or something	491 (99·4)	118 (99·1)	373 (99·4)	0·36
	HIV infection	1 (0·2)	1 (0·8)	0 (0)	-
	Faint	1 (0·2)	0 (0)	1 (0·3)	-
	Road accident	1 (0·2)	0 (0)	1 (0·3)	-

Trends in blood donations

In total, 9318 and 7292 blood units were collected during the first quarter of 2019 and 2020 respectively. The overall VNRBD rates were 4·4% (409/9318) and 3·2% (233/7292) in 2019 and 2020, respectively. The number of blood donations declined by 25.2% (n = 601), 8·9% (n = 199), 16·7% (n = 388) and 35··1% (n = 838) in January, February, March and April 2020, respectively, compared to the same months in 2019 (Figs. 1 and 2).

Discussion

This study aimed at describing the impact of the pandemic on the number of blood donations in Cameroon and evaluating the level of knowledge, the practices and expectations of donors with respect to the pandemic. Overall blood donations in Cameroon diminished by about a quarter between the periods of January to April 2019 and the same period in 2020. Most of the blood donors know COVID-19, its transmission routes and manifestations. The distribution of face masks and hydroalcoholic solutions would greatly mitigate fear from the donor's perspective.

The COVID-19 pandemic may have caused a reduction in the number of blood donations as the global trends show a decrease in donations during the crisis compared with the same period beforehand. Similar observations are reported in China, Iran, India and the USA [10–13]. Unlike the increase in blood donations observed during some disasters such as earthquakes or complex road accidents, people may refrain from donating blood to protect themselves during the pandemic crisis. And in the case of COVID-19, confinement strategies reduce the frequency of blood drives. According to our donors, fear of infection is the main reason not to donate. This study did not analyse the relationship between the type of donation (VNRBD and FRD) and donors' perceptions, but the

Table 3 Practices and expectations in regard to COVID-19 of 494 Cameroonian blood donors

Questions on practices and expectations		Type of donor			
		All donors (n = 494)	VNRBD (n = 119)	FRD (n = 375)	P value
What do you do to protect yourself?	I wash my hands regularly	474 (95·9)	112 (94·1)	362 (96·5)	0·52
	I use a face mask	438 (88·7)	97 (81·5)	341 (90·9)	0·25
	I practise distancing	401 (81·2)	87 (73·1)	314 (83·7)	0·25
	I do nothing	0 (0)	0 (0)	0 (0)	-
	I take some medicine	55 (11·1)	10 (8·4)	45 (12·0)	0·03
	Other	0 (0)	0 (0)	0 (0)	-
What should the blood service do to encourage you to come and donate?	Distribution of face mask and/or hydroalcoolic solution	457 (92·5)	88 (73·9)	369 (98·4)	0·04
	Sensitization, education, information	25 (5·1)	14 (11·8)	11 (2·9)	0·01
	Appropriate welcome and service to donor	19 (3·8)	17 (14·2)	2 (0·5)	0·000
	Protection during transportation from my home to the blood service	8 (1·6)	1 (0·8)	7 (1·8)	0·07
	Payment for the blood donation	7 (1·4)	0 (0)	7 (1·8)	-
	Systematic screening of the COVID-19 prior to donation	26 (5·2)	11 (9·2)	15 (4·0)	0·02
	Blood collection at home	18 (3·6)	3 (2·5)	15 (4·0)	0·22
	Practice of social distancing and environmental hygiene at the blood service	31 (6·3)	18 (15·1)	13 (3·5)	0·009

findings suggest that both VNRBD and FRD are equally afraid of possible infection during blood donation. A significant number of donors have misconceptions based on cultural influences including the erroneous beliefs that COVID-19 is a 'mystical' disease or that COVID-19 is a blood-borne disease. This was especially observed among the family blood donors. The uncertainty and low predictability of COVID-19 affect people's mental health, especially in terms of emotions and cognition. Messages related to death, severity and potential mortality of the pandemic cause cognitive dissonance, insecurity and mental discomfort [14]. Lack of coordination and political commitment and failure to invest in stronger educational and motivational activities through a national blood service exacerbate this situation.

Actions such as blood donor awareness and recruitment campaigns may mitigate the decrease in blood donations as shown in the data obtained from the 'YUTH'. Indeed, donor awareness and education campaigns in a coordinated programme conducted by a National blood service have been reported to significantly increase the proportion of VNRBD and hence the blood supplies [15,16]. Education should focus on misconceptions about the disease and its transmission routes, emphasizing the fact that the transmission of SARS-CoV-2 through blood transfusion is only theoretical [7]. Furthermore, a reduction in the deferral period of donors [9,17] and intensification of the efforts to schedule appointments for donations, or adjusting operating hours [9] may increase the number of donations. However, we found that the level of knowledge and education about COVID-19 among blood donors may not be associated with the type of donor, and may be higher than their general knowledge of blood safety [18,19]. This may be due to the widespread current information and publications on COVID-19 through various channels including social media.

Regarding motivation, protection of the donor during blood donation is a key expectation and even a condition for some donors during this crisis. The protection of the health personnel, volunteers, and donors are recommended measures, including hand hygiene practices, environmental infection control, and personal protective equipment (PPE) [20]. These measures were shown to be effective during the previous SARS pandemic [21]. The provision of face masks and hydroalcoholic solutions seemed to not only mitigate fear, but may even serve as an 'incentive' for blood donors during this pandemic in Cameroon, where numerous people cannot afford to buy a face mask. Routine practices in personnel protection have been reported as satisfactory in China, Iran and the USA [22–24]. Donors appreciated the reduction of the infection risk through appropriate preventive measures as recommended by the health authorities. While waiting for more evidence that such measures are efficient in sub-Saharan Africa, blood services should urgently consider deploying face masks and hydroalcoholic solutions to help prospective blood donors overcome their fears of blood donation. However, the provision of face masks incurs additional costs for the blood services an important

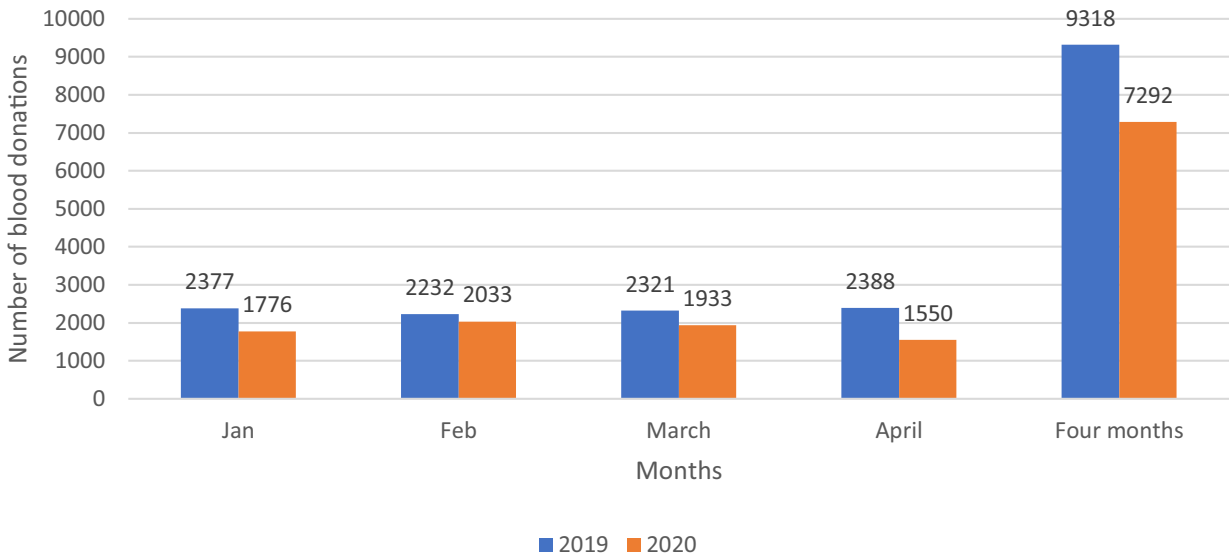

Fig. 1 Trends in total number of blood donations in Cameroonian blood services in the first quarter of 2019 versus the first quarter of 2020.

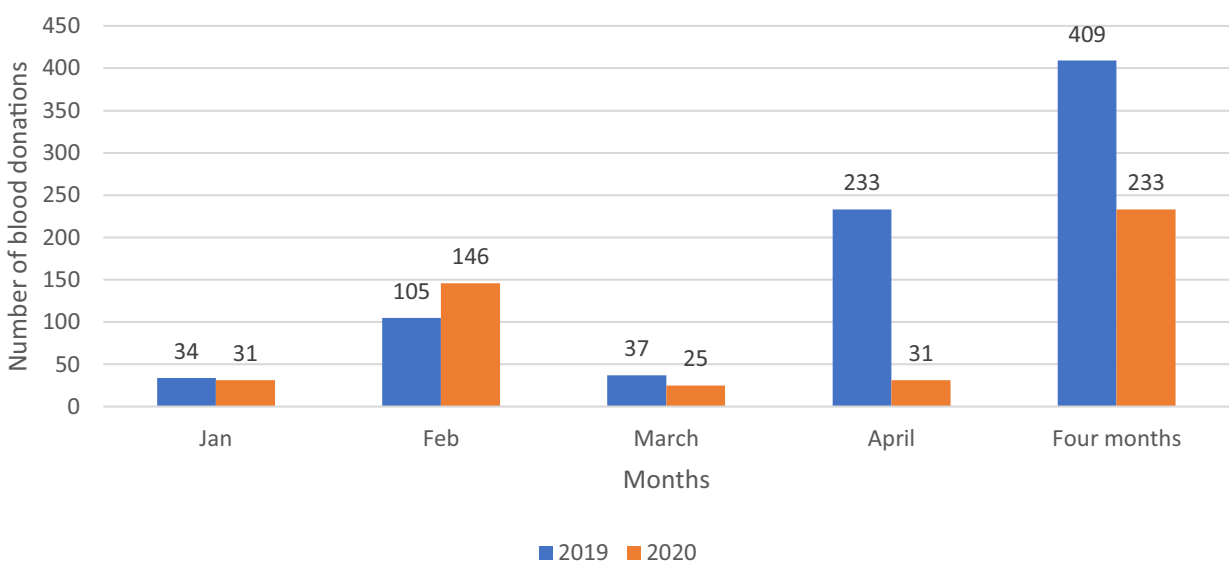

Fig. 2 Trends in number of voluntary non-remunerated blood donations in Cameroonian blood services in the first quarter of 2019 versus the first quarter of 2020.

factor for those with limited budgets. Donors also suggested a warmer welcome by the staff, and quality services, that are often lacking in African blood services, especially in hospital-based blood banks. Other possible motivational messages for the donation of blood, such as solidarity, the need to replenish supplies, the benefits of donating should be reinforced as perceived blood transfusion safety and personal motivations may be stronger determinants of willingness to donate than receiving certain incentives [25–28].

We recognize some limitations in this study. Because of the low numbers of VNRBD available to Cameroonian blood services as a whole, we included very few, thus placing a limitation on the study. Furthermore, the study was not conducted in all the 10 regions in Cameroon. Nevertheless, the two locations of this study are the main cities of the country with a cosmopolitan population, and the selected blood services collect almost half of the blood donations of the two cities. The two cities were also the most affected by the COVID-19 pandemic at the time of the study. These retrospective data were only collected for the first four months because the impact of the pandemic on blood donation was more visible during the early months of the pandemic. Our study is descriptive

and suggests that donors' perceptions might improve the return-to-donate rate. But a future case–control study needs to confirm this.

Despite adequate knowledge about COVID-19, which may have contributed to the reduction of blood donations in Cameroon, we need further studies of blood needs during the pandemic. Distribution of face masks and hydroalcoholic solution to blood donors might provide a motive to improve the blood supply.

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Conflict of interests

The authors declare no conflict of interests.

Author contributions

CT and DM developed the study design. IL, FN, PN, CN, AD and NA provided the data. CT, DM, AN and JBT reviewed the manuscript. CT and MN conducted the interviews.

References

1 CDC Africa Centers for Disease Control: Coronavirus Disease 2019 (COVID-19) Latest updates on the COVID-19 crisis from Africa, 2020
2 John Hopkins Coronavirus Resource Center: John Hopkins University and Medicine. [Internet]. 2020 [cited 2020 May 22]. Available from: https://coronavirus.jhu.edu/map.html [Last accessed 28/05/2020]
3 World Health Organization:WHO Coronavirus Disease (COVID-19) Dashboard [Internet]. 2020. Available from: https://covid19.who.int/ [Last accessed 28/05/2020]
4 Sohrabi C, Alsafi Z, O'Neill N, *et al.*: World Health Organization declares global emergency: a review of the 2019 novel coronavirus (COVID-19). *Int J Surg Lond Engl* 2020; **76**:71–6
5 Rothan HA, Byrareddy SN: The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak. *J Autoimmun* 2020; **109**:102433
6 Singhal T: A review of Coronavirus Disease-2019 (COVID-19). *Indian J Pediatr* 2020; **87**:281–286
7 Chang L, Yan Y, Wang L: Coronavirus Disease 2019: Coronaviruses and blood safety. *Transfus Med Rev* 2020; **34**: 75–80
8 Dodd RY, Stramer SL: COVID-19 and blood safety: help with a dilemma. *Transfus Med Rev* 2020; **34**:73–74
9 World Health Organization. Maintaining a safe and adequate blood supply during the pandemic outbreak of coronavirus disease (COVID-19) Interim guidance 20 March 2020. © World Health Organization 2020. Some rights reserved; 2020
10 Mohammadi S, Tabatabaei Yazdi SM, Eshghi P, *et al.*: Coronavirus disease 2019 (COVID-19) and decrease in blood donation: experience of Iranian Blood Transfusion Organization (IBTO). *Vox Sang* 2020; **115**:595-596
11 Wang Y, Han W, Pan L, *et al.*: Impact of COVID-19 on blood centres in Zhejiang province China. *Vox Sang* 2020; **115**:502-506
12 Raturi M, Kusum A: The blood supply management amid the COVID-19 outbreak. *Transfus Clin Biol J Soc Francaise Transfus Sang* 2020; **27**:147-151
13 Pagano MB, Hess JR, Tsang HC, *et al.*: Prepare to adapt: blood supply and transfusion support during the first 2 weeks of the 2019 novel coronavirus (COVID-19) pandemic affecting Washington State. *Transfusion (Paris)* 2020; **60**:908–911
14 Li S, Wang Y, Xue J, *et al.*: The Impact of COVID-19 epidemic declaration on psychological consequences: a study on active weibo users. *Int J Environ Res Public Health* 2020; **17**:2032
15 Hussein E, Teruya J: Evaluation of blood supply operation and infectious disease markers in blood donors during the Egyptian revolution. *Transfusion (Paris)* 2012; **52**:2321–2328
16 Schmidt PJ: Blood and disaster–supply and demand. *N Engl J Med* 2002; **346**:617–620
17 US Food and Drug Administration. Coronavirus (COVID-19) Update: FDA provides updated guidance to address the urgent need for blood during the pandemic; 2020 Feb 4 [cited 2020 May 22]; Available from: https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-provides-updated-guidance-address-urgent-need-blood-during-pandemic [Last accessed 28/05/2020]
18 Ehimen FA, Osagiede EF, Abah SO, *et al.*: Assessment of the knowledge, attitude and practice of voluntary non-remunerated blood donation among residents of Ekpoma, a peri-urban community in Edo State. *Niger J Med J Natl Assoc Resid Dr Niger* 2016; **25**:282–292
19 Enawgaw B, Yalew A, Shiferaw E: Blood donors' knowledge and attitude towards blood donation at North Gondar district blood bank, Northwest Ethiopia: a cross-sectional study. *BMC Res Notes* 2019; **12**:729
20 Centers for Disease Controls and Prevention. Guidance for blood and plasma facilities interim infection control Guidance on COVID-19 for personnel at blood and plasma collection facilities; 2020 Apr 29; Available from: https://www.cdc.gov/coronavirus/2019-ncov/hcp/blood-and-pla

sma-collection.html [Last accessed 28/05/2020]
21 Lee CK: Impact of Severe Acute Respiratory Syndrome (SARS) on Blood Services and Blood in Hong Kong in 2003. *Transfus Med* 2020; **30**(3):169–171. https://pubmed.ncbi.nlm.nih.gov/32432804/
22 Taghrir MH, Borazjani R, Shiraly R: COVID-19 and Iranian medical students; a survey on their related-knowledge, preventive behaviors and risk perception. *Arch Iran Med* 2020; **23**:249–254
23 Zhong B-L, Luo W, Li H-M, *et al.*: Knowledge, attitudes, and practices towards COVID-19 among Chinese residents during the rapid rise period of the COVID-19 outbreak: a quick online cross-sectional survey. *Int J Biol Sci* 2020; **16**(10):1745–1752
24 Geldsetzer P: Knowledge and perceptions of COVID-19 among the general public in the United States and the United Kingdom: a cross-sectional online survey. *Ann Intern Med* 2020
25 Bednall TC, Bove LL, Cheetham A, *et al.*: A systematic review and meta-analysis of antecedents of blood donation behavior and intentions. *Soc Sci Med* 2013; **1982**:86–94
26 Huis I'nt Veld EMJ, de Kort WLAM, Merz EM: Determinants of blood donation willingness in the European Union: a cross-country perspective on perceived transfusion safety, concerns, and incentives. *Transfusion (Paris)* 2019; **59**:1273–1282
27 Asamoah-Akuoko L, Hassall OW, Bates I, *et al.*: Blood donors' perceptions, motivators and deterrents in Sub-Saharan Africa - a scoping review of evidence. *Br J Haematol.* 2017; **177**:864–877
28 Koster J, Hassall OW: Attitudes towards blood donation and transfusion in Bamenda, Republic of Cameroon. *Transfus Med Oxf Engl.* 2011; **21**:301–307

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Validation of a standardized donor health questionnaire across substances of human origin

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Vox Sanguinis

Abstract

Background and objectives A donor health questionnaire (DHQ) aims to ensure the safety of donors and recipients of transfusions or transplantations with blood components, plasma-derived medicinal products, tissues, haematopoietic stem cells and medically assisted reproduction (in short substances of human origin; SoHO). Currently, many different DHQs exist across countries and SoHO. TRANSPOSE (TRANSfusion and transplantation PrOtection and SElection of donors) developed and validated a standardized DHQ to use across countries and SoHO. We tested whether participants understand the questions and provide honest answers.

Methods For the validation of the standardized DHQ, two demographically representative online surveys were conducted in Germany (N = 3329) and Austria (N = 3432). We surveyed whether participants understood each DHQ question and would answer the questions truthfully. We used experimental settings to test whether there is a difference between mode of administration (print vs. online), the order of the questions (subject vs. chronological order), and the positioning of the *general state of health* question (beginning vs. end) in the DHQ. Using regression models, we tested the DHQ's impact on participant mood after completion and on socially desirable response behaviour.

Results Participants understood the DHQ questions well and would answer them honestly. Nevertheless, the data show different levels of understanding and honesty when responding. Administration mode was the only characteristic that had a significant influence on mood, with the online version resulting in a more favourable mood in comparison to the printed version.

Conclusion The DHQ was well understood and had a low dishonest tendency. Our findings can serve as an impulse for further research on DHQ criteria across other SoHO and countries.

Key words: social desirability, donor health management, standardized questionnaire.

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Introduction

Donor and patient safety can be enhanced by improving strategies for donor pre-donation screening. Donor health questionnaires (DHQ) are important tools to assess whether an individual is suitable as a donor [1,2]. The European consortium project TRANSfusion and transplantation: PrOtection and SElection of donors (TRANSPOSE, transposeproject.eu) aimed to harmonize donor selection and protection policies related to donations of SoHO (blood components, plasma-derived medicinal products, tissues, haematopoietic stem cells and medically assisted reproduction) within Europe. In TRANSPOSE, a harmonized version of the DHQ has been assessed. Several different DHQs currently exist across different Substances of Human Origin (SoHO) within Europe but differ both in content (e.g. addressed risks) and form (e.g. number of questions) [3]. Therefore, it is of utmost importance to have better insight into the development and validation of DHQs regarding donors.

The validity of a DHQ depends on the answering behaviour of potential donors. Invalid answers occur when the question is not well understood or induces a risk of a dishonest answer. This is potentially due to a social desirability bias (e.g. risk behaviour), in which the given answers present the participant more favourably towards others [4]. Sensitivity to social desirability bias differs between individuals (e.g. varying notions of social norms) and within individuals (e.g. perceived sensitivity of different items) [5]. It involves conscious and willing dishonest behaviour from people weighing up the supposed external benefits and costs of said dishonest behaviour [6]. Particularly, participants may answer dishonestly to avoid threatening their self-esteem and to sustain a favourable self-image. However, regarding the DHQ, participants are often unaware of the risk such behaviour entails for the recipient and themselves. Thus, raising awareness of the consequences of such behaviour, as well as a proper way of presenting the questions is crucial. We study socially desirable response behaviour using the balanced inventory of desirable responding (BIDR) [4]. The BIDR stresses notably enhanced affirmations of positive cognitive characteristics. Individuals tend to resist negative self-assessments when they show a high degree of self-deception and thus want to pursue more positive self-assessments. Participants who overly give desirable answers achieve high scores [4].

The aim of our work is to validate and provide a DHQ by analysing whether participants understand the questions and whether they would answer them honestly (Study 1), as well as whether different characteristics of the DHQ (i.e. mode of administration, order of the questions and positioning of the *general state of health* question) have an impact on the participants' mood and socially desirable response behaviour (Study 2). Due to the validation presented here, and further help from medical experts, a standard core version of the DHQ has been developed and proposed. This core version can be found in the appendix (Table S10) and is structured as a construction kit divided into 12 subject areas and consisting of a total of 58 questions. Subject areas include for example donor health, previous donations, and behaviours that might indicate a risk of blood-born infections (i.e. risk behaviour).

Materials and methods

Our work builds on the results of the proceeding work packages (WP) in TRANSPOSE. All reports are available at the EU website [7]. First, an inventory of DHQs used in Europe was compiled. The questionnaires of each SoHO were then reviewed and revised in collaboration with medical experts to make a prototype of the DHQ available for validation. The medical content was based on a list of proposed donor selection criteria that had been assessed by a non-validated risk assessment method build on the Alliance of Blood Operators Risk Decision Making Framework [3]. The focus was to combine and update the different DHQs of the individual countries into one core DHQ covering all potential risks in an acceptable length. To develop this prototype, experts from Austria, Denmark, Germany, and the Netherlands were consulted in personal interviews. Moreover, input of experts from Germany, Finland, Portugal, Sweden, and the UK was obtained, ensuring that each SoHO was involved in the prototype.

Validation was conducted as two online studies in two countries (Germany and Austria), resulting in four data sets. Cultural differences were not included in the validation since research has shown that the variation in dishonesty within countries outweighs the variation between countries [8]. Two EU countries with the same language were used to avoid distortion by language differences during validation.

For our studies, we use the access panel of a German market research institute (i.e. respondi AG). Panel participants are generally recruited by *respondi* through campaigns and various marketing measures. Identity, plausibility, and response behaviour are regularly monitored by *respondi*. For participation in surveys, respondents are credited with points that can be redeemed for cash, vouchers or even a donation to charities. To obtain a demographically representative sample, study invitations were based on age (i.e. 18–75 years – the usual age range of most donor populations in Europe) and gender

(i.e. 50.0% women). We did not explicitly invite blood donors and candidate donors, so the distribution between the two groups occurred randomly. We excluded 1211 candidates based on their response time (processing time less than half of the average), as this eliminates participants who rush through the questionnaire, and based on their self-reported attention ('I fill out this survey carefully', 7-point Likert scale, at least 5 point marked) resulting in a total of 6,490 participants for analysis. Testing for non-responder bias analysis did not reveal substantial differences of our results (appendix, Table S3). All variables, the exact formulation, and answer options are presented in the appendix (Table S1).

Study 1

Research questions

The first study analysed (1) if participants understood each DHQ question and (2) if participants would answer the questions honestly. The following research questions (RQs) were addressed:

- RQ1: Do (potential) donors understand the questions in the DHQ?
- RQ2: How likely are they to respond to the questions honestly?
- RQ3: Which factors influence the level of understanding and honesty?

Study structure and variables

Regarding RQ1, the participants were directly asked per question 'How understandable is the question?' and regarding RQ2 'How honest would you answer the question?'. Each participant was randomly assigned to one of the five conditions (complies with the five SoHO) using a between-subject design. We averaged the responses across all SoHO and defined these as our dependent variables, measuring the overall level of understandability and honesty per respondent (7-point Likert scale, see appendix Table S4). Participants indicated their level of understanding and honesty towards each question; they were not requested to answer the questions.

For RQ3 we included the following variables in the study: Based on the PANAS scale (Positive And Negative Affect Schedule, 5-point Likert scale) we asked the participants about their mood directly before and after completing the DHQ [9]; the calculated difference (mood change = mood after – mood before) served as an influencing factor. After completing the DHQ and the stating their mood, we asked about intention to donate and the level of knowledge regarding blood donation (self-reported [10]). We focused on blood donation as this is the most common type of public donation [11]. Respondents indicated where they get information about donations from (Table S1). Six information sources, such as media (e.g. websites of blood donation services or news sites), official leaflets, healthcare professionals, healthcare insurance, friends, and family could be selected (multiple selection was possible).

In addition, we controlled in this study for social desirability bias using the balanced inventory of desirable responding BIDR [4]. Accordingly, participants with a high score were excluded from the analysis as it could be assumed that they responded in a socially desirable manner and the response is therefore biased, resulting in nine candidates being excluded and 2463 being included in the analysis [4].

Impact of sociodemographics and information sources

To address differences in perception due to different knowledge or sources of information the impact of sociodemographics and use of information sources (0/1) was modelled on the overall level of understanding and honesty using a linear model (estimated via OLS). The means for both *understanding* and *honesty* were calculated jointly for all SoHO. We differentiated the two countries via the dummy variable *country*. We focused on the subject areas (a) risk behaviour, since the appearance of socially desirable response behaviour is most sensitive here [12], and (b) health, as it can be assumed that these questions are the most unclear due to the use of medical terms. The respective models for *understanding* and *honesty* were estimated individually for each subject area resulting in four different models.

$$\hat{Y}_{i,j} = ß_{0,i,j} + ß_{1,i,j}.x_{1,i,j} + ß_{2,ij}.x_{2,i,j} + ß_{3,i,j}.x_{3,i,j} + ß_{4,i,j}.x_{4,i,j} + ß_{5,i,j}.x_{5,i,j}$$

$$+ß_{6,i,j}.x_{6,i,j} + ß_{7,i,j}.x_{7,i,j} + ß_{8,i,j}.x_{8,i,j} + ß_{9,i,j}.x_{9,i,j} + ß_{10,i,j}.x_{10,i,j}$$

$$+ß_{11,i,j}.x_{11,i,j} + ß_{12,i,j}.x_{12,i,j} + ß_{13,i,j}.x_{13,i,j} + \varepsilon_{i,j} \quad (1)$$

where i: 1 = honesty and 2 = understanding
j: 1 = risk behaviour and 2 = health
$\hat{Y}$ = means of the dependent variable i for the subject area j
x_1 = gender (0 = female 1 = male)
x_2 = age
x_3 = donor (0 = no 1 = yes)
x_4 = country (0 = Austria 1 = Germany)
x_5 = mood change
x_6 = intention
x_7 = level of knowledge

x_8 = media as a source of information (0 = no 1 = yes)

x_9 = official leaflets as a source of information (0 = no 1 = yes)

x_{10} = healthcare professionals as a source of information (0 = no 1 = yes)

x_{11} = healthcare insurance as a source of information (0 = no 1 = yes)

x_{12} = friends as a source of information (0 = no 1 = yes)

x_{13} = family as a source of information (0 = no 1 = yes)

Study 2

Research questions

The aim of Study 2 was to analyse, based on experimental settings, whether different characteristics of the DHQ have an influence (a) on mood after completion and (b) on social response behaviour (all questions in this study referred to blood donations). This resulted in the following research questions being addressed:

- RQ4: Does the administration mode of the DHQ (print vs. online) influence (a) mood after completion of the DHQ or (b) socially desired response behaviour?
- RQ5: Does the question order (subject area vs. chronologically) influence (a) mood after completion of the DHQ or (b) socially desired response behaviour?
- RQ6: Does the positioning of the *general state of health* question (beginning vs. end) influence (a) mood after completion of the DHQ or (b) socially desired response behaviour?

Study structure and variables

RQ4: We varied the potential online version of the DHQ from the potential printed version. This means a printed version was included in an online environment as a scenario. The introductory text was adapted, and filters were set for age and gender such that some questions being omitted (e.g. about pregnancy) and fewer questions being displayed on one page for the online version.

RQ5: We manipulated the order in which the questions were displayed. The questions were either sorted by subject area (e.g. health, risk behaviour) or chronologically from the near to the far past (e.g. last week, last 4 weeks, ever).

RQ6: The manipulation of the *general state of health* question ('Do you feel fit and well enough to donate?') was based on the work by Shu et al. [13], who have shown that signing at the beginning of a form reduces the probability of dishonesty within tax refund and insurance context. We applied this to the general health question as signing can be a means of awakening the focus on the self, therefore potentially increasing the honesty level [6]. Asking the question at the beginning may cause self-reflection on the donor's feelings regarding their own health status, whereas answering the question at the end may be influenced by previous responses to the DHQ.

Each participant was randomly assigned to one of eight experimental conditions (2 (print vs. online) × 2 (subject area vs. chronologically) × 2 (beginning vs. end) between-subject design). First, the DHQ was given to the participants to fill out truthfully. Then, candidates were asked about their mood directly before and after filling out the DHQ [9]. The mood after answering the DHQ served as the first dependent variable since the feelings after filling it out contributes to the overall experience of the donation process, which reciprocally can affect the return rate [14]. The second dependent variable was the socially desirable response behaviour measured by BIDR.

We obtained measures representing effort when filling out the DHQ, and the overall attitude towards the DHQ by measuring the following statements: 'It takes a lot of effort to fill out the DHQ', 'It is easy to fill out the DHQ', 'I felt good while filling out the donor questionnaire', and 'What is your general attitude towards the DHQ?'. The questions on attitude were analysed descriptively.

Participants had the opportunity to make remarks about the DHQ in an open text field (an overview is provided in Table S5 in the appendix). In total, 12.6% of participants expressed criticism, particularly of the questions regarding men having sex with other men (MSM). Since no other question received so many comments, we decided to code a binary variable *MSM* (=1 if criticized, 0 else) and included it in the subsequent analyses (equations (2), predicting mood after filling in the DHQ, and (3) predicting social desirability and answering behaviour). Finally, we measured participants' intention to donate.

Impact of DHQ characteristics

We estimated two linear models to test the impact of different DHQ characteristics on both the mood afterwards (equation 2) and social desirability (BIDR, equation 3).

$$\hat{Y} = ß_0 + ß_1 x_1 + ß_2 x_2 + ß_3 x_3 + ß_4 x_4 + ß_5 x_5 + ß_6 x_6 + ß_7 x_7 + ß_8 x_8 + ß_9 x_9 + ß_{10} x_{10} + ß_{11} x_{11} + \varepsilon \quad (2)$$

where = $\hat{Y}$ mood afterwards

x_1 = mode of administration (0 = online 1 = print)

x_2 = question order (0 = subject 1 = chronological)

x_3 = position of the general state of health questions (0 = end 1 = beginning)
x_4 = gender (0 = female 1 = male)
x_5 = age
x_6 = donor (0 = no 1 = yes)
x_7 = intention
x_8 = MSM (0 = no 1 = yes)
x_9 = country (0 = Austria 1 = Germany)
x_{10} = mood before
x_{11} = BIDR

$$\hat{Y} = ß_0 + ß_1 x_1 + ß_2 x_2 + ß_3 x_3 + ß_4 x_4 + ß_5 x_5 + ß_6 x_6 + ß_7 x_7 + ß_8 x_8 + ß_9 x_9 + ß_{10} x_{10} + \varepsilon \quad (3)$$

where = $\hat{Y}$BIDR
x_1 = mode of administration (0 = online 1 = print)
x_2 = question order (0 = subject 1 = chronological)
x_3 = position of the general state of health questions (0 = end 1 = beginning)
x_4 = gender (0 = female 1 = male)
x_5 = age
x_6 = donor (0 = no 1 = yes)
x_7 = intention
x_8 = MSM (0 = no 1 = yes)
x_9 = country (0 = Austria 1 = Germany)
x_{10} = mood before.

Results

The sociodemographic characteristics of both studies in the two countries are shown in Table 1.

Study 1

Finalizing the DHQ

We analysed the means and standard deviations of each question in the DHQ for each SoHO (see appendix, Table S4). The questions with the lowest means for understanding were reconsidered and revised with the help of medical experts, resulting in the standard core version of the DHQ. Apart from the time periods, questions were therefore split if they contained too many different aspects or reformulated if it was not clear enough before (e.g. 'Have you consulted a healthcare professional or have you had an illness (such as fever and diarrhoea) in the last four weeks?' was reformulated into two separated questions: 'Have you consulted a healthcare professional in the last 3 months?' and 'Have you experienced fever, diarrhoea or vomiting in the last 2 weeks?', all changes can be found in the appendix, Table S7). This was done during on-site meetings within

Table 1 Sociodemographic characteristics.

	Study 1						Study 2					
	Germany (n = 1223)		Austria (n = 1240)		Both Countries (n = 2463)		Germany (n = 2016)		Austria (n = 2002)		Both Countries (n = 4018)	
	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
Age (in years)												
18–29	132	10.8	408	32.9	540	21.9	229	11.4	496	24.8	725	18.0
30–39	179	14.6	272	22.9	451	18.3	319	15.8	417	20.8	736	18.3
40–49	261	21.3	202	16.3	463	18.8	421	20.9	399	19.9	820	20.4
50–59	358	29.3	205	16.5	563	22.9	555	27.5	438	21.9	993	24.7
>=60	293	24.0	153	12.3	446	18.1	492	24.4	252	12.6	744	18.5
Gender												
Male	549	44.9	431	34.8	980	39.8	988	49.0	792	39.6	1780	44.3
Female	674	55.1	809	65.2	1483	60.2	1028	51.0	1210	60.4	2238	55.7
Blood Donor												
Yes	605	49.5	710	42.7	1315	53.4	954	47.8	1209	60.4	2173	54.1
No	618	50.5	530	57.3	1148	46.6	1052	52.2	793	39.6	1845	45.9

the TRANSPOSE project as well as by personal communication with medical experts and involved several phases of revision and adjustment until full consensus among participants. The added and revised questions were then tested again for understanding in a survey (N = 27; see appendix, Table S8). Overall, the respondents understood the new questions very well ('I understand the question totally', mean 6.40, SD 1.09) and perceived them as easy to answer ('The question is easy to answer', mean 6.61, SD 0.49).

Research questions

Overall, participants *understood* the questions across all SoHO very well (RQ1, overall mean 6.23, SD 1.25). The means indicated that health-related questions are less well understood due to the use of medical terms (see appendix, Table S6). It must be noted though, that the differences were small and that the mean levels were nevertheless high. To verify the reasons for the lowest mean values in the original questions, a validation test was conducted (Appendix, Table S9). This survey revealed that 67% of the respondents did not know what the medical terms meant. However, individuals having a specific health problem are, due to learning effects, generally more likely to be familiar with the associated medical terminology.

Analysing the *honesty* of the questions, we found lower values for risk behaviour (appendix, Table S6). Generally, the values indicated that participants answered the questions truthfully (RQ2, overall mean 6.44, SD 1.11).

Comparing participants' sociodemographic characteristics there were significant differences in the level of *understanding* and *honesty* (RQ3). First, females (Mean 6.35, SD 1.14) had a higher level of understanding than males (Mean 6.05, SD 1.39), $t(2461) = 6.072$, $p < 0.001$. Second, younger participants (Mean 6.32, SD 1.11) have a higher level of understanding than older ones (Mean 6.15, SD 1.37), $t(2461) = 3.477$, $p = 0.001$. There were no significant differences between donors and non-donors. Third, the analysis showed that female participants (Mean 6.53, SD 0.998) would answer the questions more honestly than males (Mean 6.31, SD 1.25), $t(2461) = 4.863$, $p < 0.001$. In terms of age and previous donations, there was no significant difference in the level of honest answers.

The results of the estimated regression (Table 2) support the univariate findings. *Gender* effects indicate that women have a higher level of understanding and honesty than men. This applies to questions about risk behaviour, as well as to questions about health. *Age* had a significant negative effect on the level of understanding. For both risk behaviour and health questions, it seemed that the older the participants were, the less they understood the questions. However, age had no significant effect on the level of honesty. *Country* had no significant influence on questions of risk behaviour, neither in terms of understanding nor honesty. Contrary to this was the effect related to health questions. These were significantly understood better by participants in Austria than in Germany. However, we did not find any effects on *honesty*.

The level of knowledge was positively related to the level of understanding concerning questions about health. Addressing the questions on risk behaviour, we found that the level of knowledge had a significant positive effect on the level of honesty, but not on the level of understanding. The more participants knew about blood donation, the more honest they responded. Although, the level of understanding remained unchanged. The classification as blood donor (or not) had no influence on *honesty* and *understanding*. This suggested that the DHQ questions are understood and answered honestly by both potential and experienced donors.

Participants understood the questions better, both on risk behaviour and health, when they obtained their information through the media and answered both types of questions more honestly. Official leaflets also helped the participants to better understand questions about health and risk behaviour. Moreover, they encouraged participants to answer questions about health more honestly. In contrast, receiving information through a health insurance company was the only source that had a significant negative impact. For both risk behaviour and health-related questions, receiving information from a health insurance company was associated with a lower level of understanding and honesty.

Study 2

Overall, the general attitude towards the DHQ was fairly positive (Mean 5.81, SD 1.26) and it did not require much effort to fill out the DHQ (Mean 2.07, SD 1.44). The donors found it easy to answer the DHQ (Mean 6.21, SD 1.29) and felt rather good while doing so (Mean 5.62, SD 1.51).

The online version leads to a more favourable mood after completion than the printed version (RQ4, Table 3). However, this had no significant effect on the socially desirable response behaviour.

Neither the order of the questions (RQ5) and the positioning of the *general state of health* question (RQ6) had a significant effect on mood after filling out the DHQ, nor on the socially desirable response behaviour.

In Model 1 (Table 3), donation intention had a significant positive effect on the mood after completing the DHQ. The variable whether participants complained about the MSM question had a significant negative influence on the mood. Furthermore, gender had a significant influence on the mood afterwards such that males felt worse

Table 2 Results estimated regression Study 1.

	Model 1 (DV: Understanding – Risk Behaviour, R^2 = 0.028)		Model 2 (DV: Honesty – Risk Behaviour, R^2 = 0.017)		Model 3 (DV: Understanding – Health, R^2 = 0.042)		Model 4 (DV: Honesty – Health, R^2 = 0.022)	
	Regression Coefficient (SE)	Significance	Regression Coefficient (SE)	Significance	Regression Coefficient (SE)	Significance	Regression Coefficient (SE)	Significance
Constant	6.362 (0.165)	0.000	5.787 (0.157)	0.000	5.967 (0.143)	0.000	6.472 (0.128)	0.000
Age	**−0.009 (0.003)**	**0.001**	0.003 (0.003)	0.252	**−0.005 (0.002)**	**0.042**	−0.003 (0.002)	0.142
Gender (0 = female 1 = male)	**−0.266 (0.069)**	**0.000**	**−0.289 (0.069)**	**0.000**	**−0.282 (0.060)**	**0.000**	**−0.234 (0.054)**	**0.000**
Blood Donor (0 = no 1 = yes)	0.045 (0.078)	0.559	−0.050 (0.076)	0.520	−0.004 (0.067)	0.951	−0.030 (0.060)	0.613
Country (0 = Austria 1 = Germany)	−0.058 (0.070)	0.405	−0.072 (0.070)	0.304	**−0.192 (0.060)**	**0.002**	−0.042 (0.054)	0.434
Mood change	0.074 (0.119)	0.534	0.097 (0.118)	0.412	0.108 (0.103)	0.291	0.049 (0.092)	0.594
Intention	0.008 (0.018)	0.671	0.017 (0.018)	0.348	−0.009 (0.016)	0.554	0.006 (0.014)	0.644
Knowledge	0.031 (0.023)	0.174	**0.052 (0.023)**	**0.021**	**0.071 (0.020)**	**0.000**	0.015 (0.017)	0.402
Media (0 = no 1 = yes)	**0.263 (0.070)**	**0.000**	**0.185 (0.070)**	**0.008**	**0.233 (0.061)**	**0.000**	**0.166 (0.054)**	**0.002**
Official leaflets (0 = no 1 = yes)	**0.122 (0.068)**	**0.072**	0.023 (0.068)	0.737	**0.189 (0.059)**	**0.001**	**0.099 (0.052)**	**0.057**
Healthcare professionals (0 = no 1 = yes)	−0.028 (0.076)	0.718	0.109 (0.077)	0.177	−0.049 (0.066)	0.463	0.008 (0.059)	0.890
Healthcare insurance (0 = no 1 = yes)	**−0.291 (0.109)**	**0.008**	**−0.109 (0.109)**	**0.044**	**−0.305 (0.095)**	**0.001**	**−0.210 (0.084)**	**0.013**
Friends (0 = no 1 = yes)	0.043 (0.079)	0.589	0.079 (0.079)	0.599	0.077 (0.068)	0.260	0.078 (0.061)	0.202
Family (0 = no 1 = yes)	−0.071 (0.083)	0.393	−0.083 (0.083)	0.453	−0.105 (0.072)	0.145	−0.084 (0.064)	0.193
F(13)	5.445	0.000	3.179	0.000	8.279	0.000	4.195	0.000

Significant results are marked in bold.

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Table 3 Results estimated regression Study 2

	Model 1 (DV: PANAS After, $R^2 = 0.753$)		Model 2 (DV: BIDR, $R^2 = 0.116$)	
	Regression Coefficient (SE)	**Significance**	**Regression Coefficient (SE)**	**Significance**
Constant	0.520 (0.034)	0.000	35.085 (1.489)	0.000
Online (0) – Print (1)	**−0.018 (0.008)**	**0.030**	−0.405 (0.383)	0.290
Subject (0) – Chronological Order (1)	−0.002 (0.008)	0.761	−0.211 (0.383)	0.582
End (0) – Beginning (1)	0.000 (0.008)	0.992	0.290 (0.383)	0.448
Intention	**0.021 (0.002)**	**0.000**	**0.193 (0.109)**	**0.076**
MSM (0 = no 1 = yes)	**−0.089 (0.032)**	**0.006**	−0.520 (1.512)	0.731
Age	**0.001 (0.000)**	**0.034**	**0.198 (0.015)**	**0.000**
Gender (0 = female 1 = male)	**−0.026 (0.008)**	**0.002**	−0.194 (0.395)	0.624
Blood Donor (0 = no 1 = yes)	−0.001 (0.009)	0.939	**−1.465 (.443)**	**0.001**
Country (0 = Austria 1 = Germany)	−0.011 (0.009)	0.198	**−0.898 (0.399)**	**0.024**
PANAS before	**0.816 (0.008)**	**0.000**	**6.196 (0.363)**	**0.000**
BIDR	**0.001 (0.000)**	**0.000**	/	/
F(10)	/	/	52.557	0.000
F(11)	11080.394	0.000	/	/

Significant results are marked in bold.

than females. The results show that the DHQ did not deteriorate the mood. The better one felt before, the better one felt after completing the DHQ. The mood after filling out the DHQ was also affected by the socially desired response behaviour (BIDR). Although this effect was highly significant ($p < 0.001$), the effect size was very small ($ß = 0.001$, $SE = 0.000$), meaning that the mood only slightly improved when participants gave more desirable answers.

In Model 2 (Table 3) we find that donation intention had a significant positive effect on the socially desirable response behaviour. An increase of donation intention leads to an increase in desirable answers. The participants' age had a significant positive influence on socially desirable response behaviour. Being a blood donor additionally had a significant negative influence on the socially desirable response behaviour. Thus, blood donors do not tend to give desirable answers nor defend themselves against negative self-assessments. *Country* had a significant effect as well. Participants from Austria tended to answer more desirably than those from Germany. The prior mood of the participants exerts a significant positive influence on the socially desired response behaviour as well.

Discussion

Validation of the DHQ is of utmost importance to make sure that both regular and candidate donors understand the questions, answer honestly, and to ensure the safety of both donors and recipients [15–17].

Previous literature has focused on current donors or individuals who have already arrived at the blood donation centre [15,18], but ignored the large number of potential new donors. This is crucial since attitudes and behaviour of individuals who have not been confronted with donation yet is also essential. Therefore, we studied both donors and candidate donors and can provide broader insights. Additionally, and unlike previous research, we considered five SoHO and not only one type of donation [15,16,18]. Furthermore, we extended traditional evaluation methods which were either focus groups [15,19], cognitive interviews [15] or paper surveys [17,18,20], by running two large online studies to generate a representative sample from two countries, firstly and secondly, to test three new manipulations regarding different variants of the DHQ.

In Study 1, we found that both regular and candidate donors, understood the DHQ well and would answer honestly. Our results are consistent with previous studies in other countries [20,21]. However, a distinction needs to be made between the reasons for the non-understanding. It may be due to the medical terminology and thus the content of the question (i.e. questions regarding health) [21] or participants may not understand the necessity of the question and why an honest answer is important (i.e. questions regarding risk behaviour) [21,22]. Hence, it is not only important that the content is properly clarified, but also that the participants are informed of the reason for certain questions [21,22]. Our results show that the level of knowledge significantly influenced the level of honesty of the questions on risk behaviour, highlighting

the need for careful information of the donors from the donation establishments. Previous studies have shown that although the screening educational materials are read by the donors, they are often skimmed or not read with sufficient concentration to actually understand the risks that can arise from dishonest answers [21,23,24]. A suitable alternative could be short information videos on the donation service website, which can either be viewed as preparation at home or at the collection centre [21,23]. The latter has already been implemented in Sweden [21].

Although participants who felt better informed have a higher level of understanding, this only concerned questions about health. The cause for this might be that participants with higher levels of information are more familiar with the medical terminology of the questions and thus understand these questions better. In contrast, questions on risk behaviour contained almost no medical terminology so the level of information was not associated with the level of understanding. However, the level of information did influence the level of honesty regarding risk behaviour questions. This could be justified by the notion that participants with a higher level of knowledge are more likely to know why these questions are asked, and why it is important that these types of questions are answered honestly. Previous research has also stated that additional explanations about the reason of certain questions can boost donor's motivation to answer honestly [21]. The level of information had no influence on the level of honesty on questions concerning health. This was probably because participants knew why these questions are important and why they needed to be answered honestly regardless of their information level. Our results also confirmed previous findings [21] that information of the procedures and possible risks in the donation process are important to both regular and candidate donors.

Furthermore, we were also able to show that the use of media has the strongest positive influence – among different sources of information - on honesty and understanding. These results clearly encourage the use of media to strengthen honesty and understanding and to further enhance donor and patient safety.

In Study 2 we showed that participants who completed the online DHQ version were in a more favourable mood afterwards than participants who answered the printed version. Since the DHQ is an essential part of the overall donation experience, and this experience is an important driver for the return rate [14], it is crucial that the DHQ experience is favourable and that the donors finish the DHQ feeling good. The stated mood in our study is based on a hypothetical scenario and rules out other influences from a real donation experience (e.g. nervousness and excitement before a real donation, interaction with staff). Thus, the reported mood is directly related to the DHQ. Interestingly, our data also show that there is no difference in socially desirable response behaviour and mood when the questions are sorted by subject area or chronologically, nor when the question on general health is asked at the beginning or at the end. Previous research has stated that both subject area and chronological sorting are possible as a logical order [22] but this was not clear from our analysis. Further research should investigate this matter.

However, both studies are based on hypothetical and not on real donation situations. This poses a new and unusual scenario, especially for candidate donors and they may respond differently in a real donation situation. In our analysis, we further rely on self-stated and not on assessed knowledge. Future research should therefore include verified and assessed answers to gain deeper insights. We directly asked whether participants understood and would honestly answer the question. No further validation tests of whether they indeed did understand the questions or answered honestly were performed. We could demonstrate, though, with a small sample that for most medical terms there is a lower level of understanding, which is in line with previous research [21].

Overall, the self-reported data reflects the general degree of understanding and honesty. Nevertheless, we note the limitations that we cannot directly report the exact level and reasons of misunderstanding or dishonesty. The issue of blood donation and social desirability is addressed in the literature [25], but much more research is needed, especially on the effect of a confidential environment on the honesty of answers. While, based on previous research [8,20,21], we have not focused on culturally different countries, deviating results in other countries cannot be excluded and a generalization of our results needs to be further investigated.

Conclusion

Our analysis revealed underlying factors that may influence understanding and honesty. This made it possible to develop a donor health questionnaire that is well received by (potential) donors and can be completed without major difficulty. Questions concerning risk behaviour remains an issue which requires a higher level of information to ensure honest answers. Donations establishments should carefully include this in their donor assessment to ensure the best effect of their DHQ.

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Conflicts of interest

The authors declare no conflicts of interest.

References

1 Germain M, Goldman M: Blood donor selection and screening: Strategies to reduce recipient risk. *Am J Ther* 2002; **9**:406–410
2 Kleinman S, Williams AE: Donor selection procedures: Is it possible to improve them? *Trans Med Rev* 1998; **12**:288–302
3 Mikkelsen C, Mori G, van Walraven SM, *et al.*: How donor selection criteria can be evaluated with limited scientific evidence: lessons learned from the TRANSPOSE project. *VOX Sang* 2020. online first
4 Paulhus DL: Assessing self-deception and impression management inself-reports: The Balanced Inventory of Desirable Responding. University of British Columbia 1988, Vancouver Canada.
5 Wlömert N, Pellenwessel D, Fox JP, *et al.*: Multidimensional assessment of social desirability bias: An application of multiscale item randomized response theory to measure academic misconduct. *J Surv Methodol* 2019; **7**:365–397
6 Mazar N, Amir O, Ariely D: The dishonesty of honest people: A theory of self-concept maintenance. *JMR* 2008; **45**:633–644
7 European Commission, CHAFEA Health Programmes Database. https://webgate.ec.europa.eu/chafea_pdb/health/projects/738145/summary (last access on September 29 2020).
8 Mann H, Garcia-Rada X, Hornuf L, *et al.*: Cut from the same cloth: Similarly dishonest individuals across countries. *J Cross-Cult Psych* 2016; **47**:858–874
9 Watson D, Clark LA, Tellegen A: Development and validation of brief measures of positive and negative affect: The PANAS scales. *J Pers Soc Psychol* 1988; **54**:1063–1070
10 Lemmens KPH, Abraham C, Hoekstra T, *et al.*: Why don't young people volunteer to give blood? An investigation of the correlates of donation intentions among young nondonors. *Transfusion* 2005; **45**:945–955
11 The Blood Connection: Types of Donations. https://thebloodconnection.org/about-blood/donation/ (last access on September 29 2020).
12 Tourangeau R, Yan T: Sensitive questions in surveys. *Psychol Bull* 2007; **133**:859–83
13 Shu LL, Mazar N, Gino F, *et al.*: Signing at the beginning makes ethics salient and decreases dishonest self-reports in comparison to signing at the end. *PNAS* 2012; **109**:15197–15200
14 Sargeant A: Managing donor defection: Why should donors stop giving? *New Dir Phil Fund* 2001; **32**:59–74
15 Offergeld R, Heiden M: Selecting the right donors – still a challenge: Development of a uniform donor questionnaire in Germany. *Transfus Med Hemother* 2017; **44**:1–8
16 Houareau C, Deitenbeck R, Sümnig A, *et al.*: Good feasibility of the new German blood donor questionnaire. *Transfus Med Hemother* 2017; **44**:1–8
17 Goldman M, Ram SS, Yi QL, *et al.*: The Canadian donor health assessment questionnaire: Can it be improved? *Transfusion* 2006; **46**:2169–2175
18 Weidmann C, Müller-Steinhardt M, Schneider S, *et al.*: Donor satisfaction with a new German blood donor questionnaire and intention of the donor to return to further donations. *Transfus Med Hemother* 2013; **40**:356–361
19 Orton SL, Virvos VJ, Williams AE: Validation of selected donor-screening questions: Structure, content, and comprehension. *Transfusion* 2000; **40**: 1407–1413
20 Sümnig A, Lembcke H, Weber H, *et al.*: Evaluation of a new German blood donor questionnaire. *Vox Sang* 2014; **106**:55–60
21 Öhrner C, Kvist M: How do blood donors interpret the Swedish donor history questionnaire? *ISBT Sci Ser* 2018; **13**:123–130
22 O'Brien S, Ram SS, Vamvakas EC, *et al.*: The Canadian blood donor health assessment questionnaire: Lessons from history, application of cognitive science principles, and recommendations for change. *Transfus Med Rev* 2007; **21**:205–222
23 Rugege-Hakiza SE, Glynn SA, Hutching ST, *et al.*: Do blood donors read and understand screening educational materials? *Transfusion* 2003; **43**: 1075–1083
24 O'Brien S, Osmond L, Choquet K, *et al.*: Donor attention to reading materials. *Vox Sang* 2015; **109**: 336–342
25 Clement M, Sandner S: Blood safety considerations in the developing world—the problem of social desirability biases. *Transfusion* 2020; **60**: 225–226

Supporting Information

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

Appendix S1
Table S1 Variables & Scales.
Table S2 Classification of Quotas.
Table S3 Non-responder analysis.
Table S4 Means of all Questions.
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Enabling accurate measurement of activated factor XI (FXIa) in therapeutic immunoglobulin products

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Vox Sanguinis

Background and objectives In 2010, an intravenous immunoglobulin (IVIG) product was removed from the market due to an association with serious thromboembolic events. Investigations revealed that factor XIa (FXIa) was present as a process-related impurity. This study investigated the ability of two commercial FXIa assays to measure FXIa in immunoglobulin preparations and conducted a survey of FXIa activity in marketed immunoglobulin products.

Materials and methods Factor XIa assays were modified to include spiking of samples with FXIa before testing. An immunoglobulin product and its excipient were used to assess the ability of the assays to recover the spiked FXIa levels.

Results The Biophen FXIa assay required a high pre-dilution of the sample to obtain statistically valid results and complete FXIa recovery. The ROX FXIa assay was more sensitive, giving statistically valid results at a lower sample pre-dilution and FXIa spike level. This modified ROX FXIa assay was used to assay 17 lots of immunoglobulin products for FXIa. Two product lots had measurable FXIa levels without the need for spiking. A further 3 lots produced detectable but not statistically valid FXIa results when left unspiked. Spiking produced statistically valid assays and recoveries above 100%, demonstrating inherent FXIa.

Conclusion This study shows marketed immunoglobulin products can contain detectable levels of FXIa. Spiking brings the FXIa levels into the quantifiable range of the assay, allowing measurement of inherent FXIa. Accurate measurement is important to inform on 'safe' levels of FXIa in these products and allow future safety guidelines to be set.

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Key words: IVIG, immunoglobulins, plasma derivatives.

Introduction

Human plasma-derived immunoglobulin (IG) products are licensed to treat primary and secondary immunodeficiencies and conditions such as idiopathic thrombocytopenia purpura and Kawasaki disease. Off-label use is also common for a wide range of conditions [1,2]. Products are fractionated from human plasma pooled from thousands of healthy donors and contain mainly IgG antibodies. They can be given either as intravenous immunoglobulins (IVIG) or subcutaneous immunoglobulins (SCIG) and are generally safe and well tolerated; however, adverse events are numerous and range from mild (headaches, skin conditions) [3] to severe (renal impairment, thromboembolic events) [4,5].

In 2010, the IVIG Octagam® was temporarily removed from the market due to its association with a sudden increase in thromboembolic events (TE) [6]. This was

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followed by a withdrawal of the product Omr-IgG-am® from the market and the association of the SCIG Vivaglobin® with TE [7,8]. Investigative work on Octagam® identified the problem was the presence of a process-related impurity, activated factor XI (FXIa), in the final product [9,10]. A change in the manufacturing process improved the safety of the product [11] and the market suspension was lifted subject to additional testing being required [12]. Analysis of implicated lots of Octagam® in a rabbit thrombogenicity model suggested that a FXIa content of ≥7 mU/ml FXIa was enough to cause thrombosis [9]; however, the Octagam® study pre-dated the establishment of an International Unit (IU) for FXIa in 2014 [13]. The unity of activity in the Octagam® study is therefore not comparable to the IU.

In response to the adverse events, the European Pharmacopeia monographs for IVIG and SCIG were updated to state that 'the method of preparation also includes a step or steps that have been shown to remove thrombosis-generating agents' [14–15]. In order to show the absence or removal of procoagulant factors such as FXIa, accurate assay methods must be available. Methods such as the non-activated partial thromboplastin time, thrombin generation assays and non-specific FXIa chromogenic assays have been used for investigating procoagulant activity in IG products or their manufacturing intermediates [9,16–19]. At the time, specific FXIa chromogenic assays were not available, but two FXIa chromogenic assays are now commercially available and have been used by manufacturers to demonstrate the reduction or removal of FXIa during their manufacturing process [20–22]. However, lack of detection in an assay is not the same as showing an absence of activity, because it depends upon the limits of detection and quantification of the assays. Although there have not been reported increases in TE in IG products since the new monograph guidance has been introduced, there still needs to be an assessment of the suitability of the assays for determining procoagulant activity (or its absence) in IG preparations and some guidance on acceptable levels (if any) of FXIa may be helpful for manufacturers. This study therefore assesses the ability of both chromogenic kits to measure FXIa in IG products and uses marketed lots of various IG products to determine FXIa levels in products that have been used in patients.

Methods

Assays were run on an ACL TOP 550 automated coagulometer (Werfen, Bedford, USA). Each sample was tested at a minimum of 3 dilutions, in duplicate, per assay.

Development of spiking methods for FXIa quantification in IG samples

Factor XIa kits used in the study were the Biophen FXIa kit (Hyphen-BioMed) and the ROX FXIa kit (Rossix AB) (both Quadratech, Cambridge, UK). Briefly, both kits rely on FXIa present in the sample to activate factor IX and subsequently factor X. Activated factor X cleaves a chromogenic substrate, causing a colour change that can be determined kinetically or by an end-point method. This study employed the kinetic method.

Samples used for development of the spiking methods were IG-A, a preparation of immunoglobulin (5% Flebogamma) freeze-dried at NIBSC and its corresponding frozen excipient. Two marketed lots of 5% Flebogamma were used for proof of concept testing. The 1st International Standard (IS) for FXIa, 13/100 (NIBSC, Potters Bar, UK) was used as the quantitative standard in the assays and for spiking experiments. FXIa spiking volume was incorporated into a minimum of a 1/2 pre-dilution of the IG in kit buffer.

Parallel line analysis was used for calculation of activity estimates (CombiStats™ Version 6.0, Council of Europe). Equivalence testing in CombiStats® with an acceptance criterion of 0·9–1·1 was used to compare the ratio of slopes between the standard and the sample dose–response to demonstrate parallelism. Linearity was assessed visually. Examples are shown in Fig. 1.

Assay of marketed lots of IG products

The modified ROX FXIa method developed in this study was used to assay 17 lots of IG samples from 5 manufacturers (listed in Tables 3 and 4). All IVIG samples were initially tested with and without FXIa spiking at 1 mIU/ml. If the first assay of an unspiked product showed no dose–response (indicating no detectable FXIa), then the subsequent assays were performed on spiked samples only. SCIG products were also tested spiked and unspiked, but if unspiked samples produced a valid result against the standard, then spiking was deemed unnecessary. All spiked samples were pre-diluted at least 1/2 during spiking. The minimum dilution necessary to achieve linearity and parallelism in the assay was used for each product and was usually 1/2, apart from Subgam®, which was assayed at pre-dilutions of 1/4 or 1/6. Assays and analyses were carried out as described above, with three independent assays for each product. Some unspiked products showed a dose–response over 2 or 3 dilutions but were not parallel to the standard and, in some cases, were below the quantification limit of the assay. In these cases, the results are

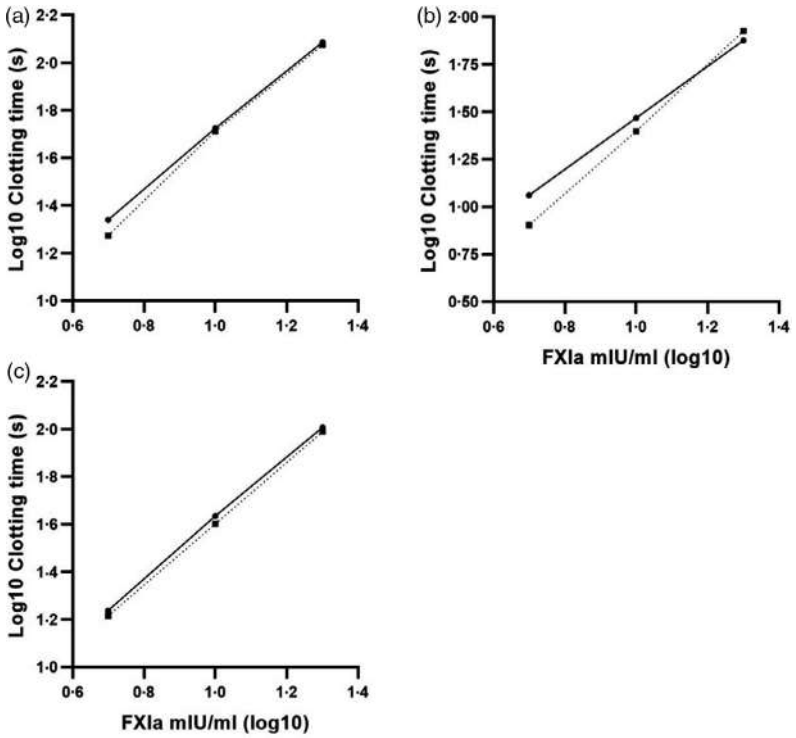

Fig. 1 Example graphs plotting $\log_{10}$ clotting time (s) against $\log_{10}$ FXIa concentration for a test sample (dotted line) and FXIa standard (solid line). Parallelism was deemed acceptable when the ratio of the test slope to the standard as determined using Combistats® was between 0.9 and 1.1 and the linearity was assessed visually from the graphs. The test sample is non-linear in (A), linear but non-parallel to the standard in (B), and linear and parallel to the standard in (C).

reported as 95% confidence limits (CL) only, to indicate that FXIa content was detectable but not accurately quantifiable.

A paired *t*-test was used to compare differences between results, where appropriate.

Results

Development of methods to measure FXIa in immunoglobulin preparations – Biophen FXIa assay

Preparation IG-A was loaded for assay at pre-dilutions of 1/1, 1/2 and 1/4 in duplicate, followed by 1/1, 1/2, 1/4 and 1/8 dilutions within the assay on the analyser. No dose–response was observed in any of the assays (data not shown), and changes in absorbances were small, indicating that any FXIa present was below the level of quantification (LoQ). The IG-A preparation (dilutions 1/2 to 1/35) and its corresponding excipient (dilutions 1/10 to 1/35) were then spiked with 20 mIU/ml purified FXIa to determine recovery. This pre-dilution also allowed for the necessary volume of FXIa to be added to the IG sample and corresponds to the resulting dilution of the IG matrix. The results (Table 1) for both were assessed on 3 conditions: recovery of the expected levels of FXIa (taken as 95% confidence limits overlapping 20 mIU/ml), parallelism and linearity. A dilution of 1/35 ($n = 3$) was required to meet all three conditions for both the immunoglobulin preparation and its corresponding excipient. This suggests that a minimum of a 1/35 dilution allows for valid assay of immunoglobulins in the Biophen FXIa assay.

Based on a dilution of 1/35, this spiking method was used to test 2 marketed batches (Lot 1 and Lot 2) of 5% Flebogamma®, the same product type as IG-A. In these assays ($n = 3$), there was acceptable FXIa recovery for the 2 lots (Table 1). All assays were valid for linearity and parallelism. This indicated that the 1/35 dilution worked well for therapeutic preparations of this product. No additional FXIa was recovered indicating that the assay did not detect inherent FXIa in these IG lots.

Development of methods to measure FXIa in immunoglobulin preparations – ROX FXIa assay

Table 2 shows results from similar spiking experiments using the ROX FXIa assay. The kit insert suggests that the

Table 1 Effect of sample pre-dilution on the validity of the Biophen FXIa assay used to measure FXIa-spiked immunoglobulin samples. Shaded boxes represent acceptable results for recovery of FXIa (95% confidence limits overlapping 20 mIU/ml), parallelism and linearity

Sample and pre-dilution	Recovery in mIU/ml (95% confidence limits)	Parallelism OK?	Linearity OK?
IG-A 1/2	25.4 (22.3–28.9)	✗	✓
IG-A 1/4	19.9 (17.5–22.7)	✗	✓
IG-A 1/8	20.5 (18.3–22.9)	✗	✓
IG-A 1/10	20.0 (17.3–23.2)	✓	✗
IG-A Excipient 1/10	18.9 (16.7–21.5)	✓	✗
IG-A 1/20	19.9 (17.2–23.1)	✗	✗
IG-A Excipient 1/20	19.0 (16.7–21.5)	✓	✗
IG-A 1/25	19.1 (17.8–20.4)	✓	✗
IG-A Excipient 1/25	18.6 (17.3–20.0)	✓	✗
IG-A 1/30	18.4 (17.5–19.5)	✓	✓
IG-A Excipient 1/30	18.9 (18.3–19.5)	✓	✓
IG-A 1/35	19.7 (18.8–20.7)	✓	✓
IG-A Excipient 1/35	19.6 (18.7–20.5)	✓	✓
5% Flebogamma Lot 1 1/35	19.3 (17.7–20.9)	✓	✓
5% Flebogamma Lot 2 1/35	19.2 (18.2–20.2)	✓	✓

standard curve can range between 0·04 and 10 mIU/ml FXIa, with the option to use a narrower range of 0·04–1·4 mIU/ml if required. When the higher part of the standard curve was used, the responses for samples IG-A spiked with 20 mIU/ml FXIa did not show acceptable parallelism or linearity. When a narrower range of the standard curve was used (0·04–0·31), the 20 mIU/ml spiked IG-A samples required a dilution of 1/64 (a 1/16 on-bench followed by doubling dilution from 1/4 on the analyser) to bring the FXIa levels into the range of the FXIa standard. This improved the statistical validity of the assays though the recovery was not quite 100%. When manufacturer lots of the same IG product were tested (5% Flebogamma® Lots 1 and 2), the recovery for these was 100%. Recovery was very slightly lower than 100% for the spiked excipient (Table 2).

The use of the narrow standard curve for the Rossix FXIa assay meant that, at a spike level of 20 mIU/ml, a high dilution was required to bring the FXIa concentration into the range of the standard. A spike level of 1 mIU/ml was therefore investigated. At 1 mIU/ml, the analyser dilutions of 1/4 to 1/16 were sufficient to bring the FXIa content of the spiked sample into the range of the standard, without the need for additional dilutions beforehand. The results for IG-A and its excipient (Table 2) showed that 100% recovery was obtained (n = 3) and the assays were valid for both parallelism and linearity. A 100% recovery with 95% CL overlapping 1 mIU/ml and valid assays (n = 3) were also obtained for the two 5% Flebogamma® lots. No additional recovery of FXIa was observed, and neither IG lot had any measurable FXIa when tested unspiked, shown by a lack of dose–response across the dilutions (data not shown). By comparison with the 20 mIU/ml spike (95% CL approximately ± 1 mIU/ml), better sensitivity was obtained spiking with 1 mIU/ml, with 95% CL spanning approximately ± 0·1 mIU/ml of the measured value. The ROX FXIa assay using the 1 mIU/ml spiking method was therefore suitable for generating valid assays for therapeutic lots of IG and more likely to detect small amounts of inherent FXIa activity.

Testing of marketed IG lots

Using the modified ROX FXIa assay with a spike level of 1 mIU/ml, 10 marketed IG products from 5 manufacturers (=17 lots) were evaluated for their FXIa content. Results are split into IVIG products (Table 3) and SCIG products (Table 4). The 5% Flebogamma® results generated previously (Table 2) using the same method are replicated for comparison. Seven IVIG products (12 lots) were tested, and only the two lots of Vigam® had detectable FXIa without spiking; however, due to non-parallelism, the 95% CL only are reported. Spiking meant that valid assays were obtained and showed that the recovery after spiking increased by an amount corresponding to the 95% CL of the unspiked product. All IVIG samples were tested with a pre-dilution of 1/2 to allow for spiking. The additional recovery of 0·30 and 0·26 mIU/ml FXIa for Vigam® lots 1 and 2, respectively, translates to 0·60 and 0·52 mIU/ml FXIa in the undiluted product. All other products had a recovery of

Table 2 Effect of sample pre-dilution on the validity of the ROX FXIa assay used to measure FXIa-spiked immunoglobulin samples. Shaded boxes represent acceptable results for FXIa recovery (95% confidence limits overlapping original spike levels), parallelism and linearity.

Sample and pre-dilution	High or narrow standard curve?	Assay dilutions	FXIa spike level (mIU/ml)	Recovery in mIU/ml (95% confidence limits)	Parallelism OK?	Linearity OK?
IG-A 1/2.5	High	1/4–1/16	20	17.9 (17.5–18.3)	✓	✗
IG-A Excipient 1/2.5	High	1/4–1/16	20	17.5 (17.1–17.8)	✓	✗
IG-A 1/5	High	1/4–1/16	20	17.5 (17.2–17.9)	✓	✗
IG-A Excipient 1/5	High	1/4–1/16	20	17.4 (17.1–17.8)	✓	✗
IG-A 1/2	Narrow	1/16 then 1/4–1/16	20	18.9 (18.3–19.4)	✓	✓
IG-A Excipient 1/2	Narrow	1/16 then 1/4–1/16	20	18.7 (18.2–19.3)	✓	✓
IG Lot 1 1/2	Narrow	1/16 then 1/4–1/16	20	19.8 (19.0–20.7)	✓	✓
IG Lot 2 1/2	Narrow	1/16 then 1/4–1/16	20	19.7 (19.0–20.6)	✓	✓
IG-A Excipient 1/2	Narrow	1/16 then 1/4–1/16	20	18.9 (18.1–19.8)	✓	✓
IG-A 1/2	Narrow	1/4–1/16	1	0.98 (0.95–1.02)	✓	✓
Excipient 1/2	Narrow	1/4–1/16	1	0.96 (0.84–1.09)	✓	✓
5% Flebogamma Lot 1 1/2	Narrow	1/4–1/16	1	0.99 (0.90–1.08)	✓	✓
5% Flebogamma Lot 2 1/2	Narrow	1/4–1/16	1	0.96 (0.87–1.07)	✓	✓

100% of FXIa (taken as 95% confidence limits overlapping 1 mIU/ml), apart from Privigen® and one lot of 10% Flebogamma®, which both had recovery slightly less than 100%. The assays were all statistically valid, so it is possible that there was a small amount of inhibitory activity in these samples.

Three different SCIG products from 3 different manufacturers (5 lots in total) were tested (Table 4) using the same method as for the IVIGs, except that one product (Subgam®) was found not to require spiking with FXIa. Both lots of this product had enough inherent FXIa (approximately 4 and 1·5 mIU/ml) such that the dilution required to bring the FXIa levels into the range of the standard was sufficient to remove any matrix effect and produce valid assays. This dilution has been accounted for in the data. Hizentra® showed no additional FXIa activity when spiked, and the less than 100% recovery suggests some FXIa inhibitory activity in the sample. The 2 lots of unspiked Gammanorm® had low levels of FXIa (0·1 and 0·4 mIU/ml for Lots 1 and 2, respectively), but assays were non-parallel. When spiked, recovery of Lot 1 was 1·1 mIU/ml, but with 95% CL overlapping 1 mIU/ml, this increase in recovery was not significant. For Lot 2, the spiked sample showed a corresponding increase in recovery above 1 mIU/ml FXIa, without 95% CL overlapping 1 mIU/ml. Taking into account the 1/2 pre-dilution, this equated to a FXIa level in the product of around 0·4 mIU/ml. This demonstrates that the modified ROX FXIa assay is sensitive to very small levels of inherent FXIa in IG samples.

Retesting of Subgam®

During the testing of the Subgam® Lot 1 in the ROX FXIa assay, it was noted that the FXIa activity increased over time. After initial assays, activity was measured again 5 and 8 months later. The results (Table 5) showed that initial FXIa levels of around 4·2 mIU/ml (as reported in Table 4) increased to 5·7 and 7·1 mIU/ml, respectively, after 5 and 8 months. The difference in activity was significant ($P = 0·034$) between 0 and 5 months and between 0 and 8 months ($P = 0·016$). The 95% CL for the 5- and 8-month testing overlapped, with no statistical difference between the results ($P = 0·115$). This indicated that the FXIa activity increased over time but then plateaued. The two lots of IG with the next highest FXIa activity, Subgam® Lot 2 and Vigam® Lot 1, were also retested for any change in FXIa activity after 8 months. Subgam® Lot 2 showed a significant ($P = 0·02$) increase from 1·53 mIU/ml (Table 4) to 3·18 mIU/ml (95% CL 2·65–3·82) after 8 months, and spiked samples of Vigam® Lot 1 showed a slight decrease in activity from 1·30 mIU/ml, to 1·17 mIU/ml

Table 3 Measurement of FXIa in marketed intravenous immunoglobulin samples using the modified ROX FXIa assay.

			FXIa level in mIU/ml (95% confidence limits)		
Manufacturer	**Product (IVIG)**	**Pre-dilution**	**Unspiked sample**	**Sample spiked with 1 mIU/ml FXIa**	**Estimated in product**
BPL	Gammaplex Lot 1	1/2	<LOQ	1.02 (0.88–1.19)	-
	Gammaplex Lot 2	1/2	<LOQ	1.00 (0.98–1.02)	-
	Vigam Lot 1	1/2	NP (0.26–0.31)	1.30 (1.29–1.31)	0.60 (0.58–0.62)
	Vigam Lot 2	1/2	NP (0.23–0.34)	1.26 (1.21–1.31)	0.52 (0.42–0.62)
CSL Behring	Privigen	1/2	<LOQ	0.94 (0.94–0.95)	-
Grifols	5% Flebogamma Lot 1	1/2	<LOQ	0.99 (0.90–1.08)	-
	5% Flebogamma Lot 2	1/2	<LOQ	0.96 (0.87–1.07)	-
	Gamunex	1/2	<LOQ	0.96 (0.92–1.00)	-
	10% Flebogamma Lot 1	1/2	<LOQ	0.93 (0.82–1.05)	-
	10% Flebogamma Lot 2	1/2	<LOQ	0.94 (0.91–0.97)	-
Kedrion	IG Vena Lot 1	1/2	<LOQ	0.97 (0.91–1.04)	-
	IG Vena Lot 2	1/2	<LOQ	1.00 (0.98–1.01)	-

NP, non-parallel; <LOQ, below the limit of quantification

(95% CL of 1·06–1·28; $P = 0·04$) after 8 months. Testing of Gammanorm® Lot 2 after 3 months showed no change in activity, at 1·19 mIU/ml (95% confidence limits of 1·16–1·23; $P > 0·05$). The cause of the changes in activity over time is unknown, however could be due to autoactivation of FXI in the product to FXIa, and decreases in activity are most likely due to degradation of FXIa.

With Subgam® lot 1 having an increased activity of 7 mIU/ml, there was a possibility that the activity could be detected using the Biophen assay. A sample was therefore tested unspiked at a pre-dilution of 1/2 in the assay, and also using a 1/35 dilution spiked with 20 mIU/ml FXIa, as recommended earlier. The results showed that there was a dose–response for the unspiked Subgam® in the assay, however only for the first two dilutions. The response was also not parallel to the standard. The 95% CL estimated the FXIa content to be 6·5–8·8 mIU/ml (Table 5). This is in agreement with the ROX FXIa assay performed at the same time (7·1 mIU/ml), however cannot be considered to be accurate because of the non-validity of the results. The spiked sample, though producing valid results, did not show any increase in recovery of spiked FXIa, since the 95% CL overlapped 20 mIU/ml (Table 5). This shows that, owing to the high dilution needed, the modified Biophen assay was not sensitive enough to measure FXIa in this sample, despite it having a relatively high content.

Conclusions

This study has developed methods for valid measurement of FXIa in IG products. Both commercially available FXIa chromogenic kits, ROX and Biophen, were investigated. The Biophen assay has a detection limit of ~2·5 mIU/ml, according to the package insert [23]. Using parallel line assay with a minimum of 3 doubling dilutions, with 2·5 mIU/ml being the lowest concentration tested, equates to a suggested limit of quantification (LOQ) of 10 mIU/ml. In practice, when assaying the spiked IG samples, the dilution corresponding to 2·5 mIU/ml was often not linear, even for the standard. Loading the appropriately diluted samples with a spike level of 20 mIU/ml allowed for testing at 20, 10 and 5 mIU/ml (1/1, 1/2 and 1/4) and gave statistically valid assays, once matrix interference had been removed. Development work with 5% Flebogamma® samples suggested that a pre-dilution of 1/35 removed any matrix interference and allowed statistically valid assays to be obtained. Therefore, for measurement of FXIa in spiked IG samples, we have found the Biophen FXIa assay to have an LOQ of 20 mIU/ml using parallel line assay, with a suggested pre-spike dilution of 1/35.

The ROX FXIa states the single-point limit of detection to be 0·03 mIU/ml [24], which would equate to a quantification limit of around 0·12 mIU/ml by parallel line assay using 3 doubling dilutions. In this study, the LoQ using parallel line assay was 0·25 mIU/ml FXIa, based on 3 doubling dilutions corresponding to concentrations of 0·25, 0·125 and 0·0625 mIU/ml. The next doubling dilution (0·03125 mIU/ml), though detectable and showing a dose–response, often led to some non-linearity. With the increased sensitivity, a lower spike level of 1 mIU/ml FXIa could be used, and a lower overall dilution was required to remove matrix interference. For all spiked samples, a pre-dilution of 1/2 followed by in-assay dilutions of 1/4 onwards was sufficient to obtain statistically

Table 4 Measurement of FXIa in marketed subcutaneous immunoglobulin samples using the modified ROX FXIa assay.

			FXIa level in mIU/ml (95% confidence limits)		
Manufacturer	**Product (subcutaneous immunoglobulin)**	**Pre-dilution**	**Unspiked sample**	**Sample spiked with 1 mIU/ml FXIa**	**Estimated in product**
BPL	Subgam Lot 1	Accounted for within results	4.13 (3.32–5.22)	Not required	4.13 (3.32–5.22)
	Subgam Lot 2	Accounted for within results	1.53 (1.45–1.62)	Not required	1.53 (1.45–1.62)
CSL Behring	Hizentra	1/2	<LOQ	0.94 (0.90–0.97)	-
Octapharma	Gammanorm Lot 1	1/2	<LOQ but −0.1 (0.11–0.15)	1.08 (0.96–1.21)	-
	Gammanorm Lot 2	1/2	NP (0.16–0.37)	1.18 (1.05–1.33)	0.36 (0.1–0.66)

NP, non-parallel; <LOQ, below the limit of quantification.

valid assays. For the samples that had a higher level of inherent FXIa (>1 mIU/ml), no spiking was required, and the pre-dilution could be adjusted to bring the levels into the range of the standard without interference from the sample matrix.

Overall, the spiking of immunoglobulin with FXIa reduced the effect of the matrix on the linearity and parallelism of both assays and allowed statistically valid assays to be obtained. The ROX assay had a higher sensitivity and was therefore chosen for measurement of the marketed IG products. Of the 10 products studied, only 3 (Vigam®, Subgam® and Gammanorm®) had detectable levels of FXIa. Without the spiking modification to the assay, only Subgam® could have validly been measured by the ROX FXIa assay using parallel line analysis. To our knowledge, this is the first study that has reported measurable levels of FXIa in identified IG products. The manufacturer of Vigam® has ceased production of the product; however, vials may still be being used until the expiry date (usually 3 years from manufacture date) has passed. Vigam® is an IVIG product, and no patient safety issues have been raised, therefore is it reasonable to assume that the low levels of FXIa measured in these lots have not caused excess adverse effects in patients. The other two products with measurable FXIa were SCIG products. It is thought that the increase in serum viscosity after administration makes TE more likely to occur with IVIG products [25]. Therefore, despite the presence of FXIa, the subcutaneous administration route of Subgam® and Gammanorm® makes TE less likely. Patient safety may therefore be uncompromised by higher levels of FXIa in SCIG compared to IVIG products.

In this study, the levels of FXIa in the Subgam® lots increased over time. An increase in FXIa in IG preparations upon storage has been observed previously [26] and is most likely due to the ability of FXIa to autoactivate FXI [27]. Once all FXI in the product has been activated, it is likely that the FXIa levels will decrease as the FXIa degrades. This may explain the reduction in activity in Vigam® Lot 1 observed in this study.

Despite the relatively high amount of FXIa present in Subgam® Lot 1, the Biophen assay was unable to detect this when the spiking method was used. This is because high dilutions of IG samples are required to produce valid results in this assay. Caution should therefore be used if using this assay to assess FXIa in IG samples, since lack of a positive result could still mean the content is as high as 7 mIU/ml or more. When Subgam® Lot 1 was tested unspiked, only the two lowest dilutions showed a dose–response and the results were not statistically valid due to non-parallelism with the standard. The 95% CL suggested a value of 6·5–8·8 mIU/ml. If

Table 5 Testing of Subgam Lot 1 in the ROX FXIa assay (unspiked) over time, and in the Biophen FXIa assay (unspiked and spiked with 20 mIU/ml FXIa).

Assay type	Spike level (mIU/ml)	Subgam Lot 1 FXIa level in mIU/ml (95% confidence limits)
ROX time 0 months	Not required	4.16 (3.31–5.22)
ROX + 5 months	Not required	6.57 (5.67–7.61)
ROX + 8 months	Not required	7.08 (6.54–7.66)
Biophen + 8 months	None	NP (6.48–8.80)
Biophen + 8 months	20	19.1 (18.2–20.1)

NP, non-parallel.

single-point analysis was used, the results would have been 7·3 and 8·8 mIU/ml, respectively, for the 1/2 and 1/4 total dilutions of the product. Whilst these values agree reasonably well with the FXIa content as measured using the ROX FXIa assay (7·1 mIU/ml), the lack of assay validity by parallel line assay means the results cannot be considered accurate. Use of single-point analysis only would not have indicated that the assay results were statistically invalid and demonstrates the importance of using multiple dose–responses with parallel line analysis. The detection of FXIa in the unspiked sample suggests that this assay could be used to screen IG preparations for high levels of FXIa (7 mIU/ml or more), but it may not be suitable for accurate determination of FXIa levels. However, spiking FXIa in the samples at different levels may help to improve the sensitivity and validity of this assay.

There is a European Pharmacopeial requirement for IG manufacturers to demonstrate the reduction or removal of procoagulant activity from their products [14,15], and therefore, sensitive and accurate assays are required. This study has shown that both the ROX and Biophen FXIa chromogenic kits can be used to validly assay FXIa content in IG preparations, with the ROX kit having greater sensitivity. The ability to measure low levels of FXIa in products may assist manufacturers in demonstrating the reduction of FXIa in their products, as required by the relevant monographs [14,15], even though the measured levels may be below that associated with adverse events. We recommend that measurement of FXIa in IG preparations should be optimized within each laboratory, since suitable conditions may vary according to whether the assays are run manually or using a coagulometer, the samples being tested, and the method of data collection (kinetic or end-point). Assays should use multiple dilutions and attention should be paid to linearity and parallelism of the test responses relative to the standard. Spiking of FXIa should be used in the case of samples that do not exhibit a dose–response when tested unspiked, or where the response is not parallel to the standard.

The European Pharmacopoeia is considering the introduction of a test for FXIa content in final IG products and the suitability of the commerical kits may depend on the specifications set. By assaying marketed lots of 10 different IG products, this study has informed on the FXIa content that may be considered safe, since no additional adverse effects have been associated with their use. For SCIG, the highest level observed during initial testing was 4·2 mIU/ml FXIa, and 0·6 mIU/ml FXIa for IVIG. The increase in FXIa content in Subgam® over time indicates that there may be a need to monitor these products during the shelf life of IG products. None of the IG lot numbers used in this study are known to be associated with adverse events; however, the observations made in this study may be useful in setting future guidelines and acceptable limits for FXIa content in these medically important products.

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Conflict of interests

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References

1 Looney RJ, Huggins J: Use of intravenous immunoglobulin G (IVIG). *Best Pract Res Clin Haematol* 2006; **19**:3–25

2 Perez EE, Orange JS, Bonilla F, *et al.*: Update on the use of immunoglobulin in human disease: A review of evidence. *J Allergy Clin Immunol* 2017; **139**:S1–46

3 Palabrica FR, Kwong SL, Padua FR: Adverse events of intravenous immunoglobulin infusions: a ten-year retrospective study. *Asia Pac Allergy* 2019; **3**:249–256

4 Orbach H, Katz U, Sherer Y, *et al.*: Intravenous Immunoglobulin. Adverse

effects and safe administration. *Clin Rev Allergy Immunol* 2005; **29**:173–184
5 Cherin P, Marie I, Michallet M, *et al.*: Management of adverse events in the treatment of patients with immunoglobulin therapy: A review of evidence. *Autoimmun Rev* 2016; **15**:71–81
6 European Medicines Agency recommends suspension of Octagam in all EU member states (2010). Available at: https://www.ema.europa.eu/en/news/european-medicines-agency-recommends-suspension-octagam-all-eu-member-states. Accessed 14 July 2020.
7 Daniel GW, Menis M, Sridhar G, *et al.*: Immune globulins and thrombotic adverse events as recorded in a large administrative database in 2008 through 2010. *Transfusion* 2012; **52**:2113–2121
8 European Medicines Agency (2012). Referral assessment report for Vivaglobin and associated names (human normal immunoglobulin solution for injection – subcutaneous use). Available at: https://www.ema.europa.eu/en/documents/referral/vivaglobin-article-36-referral-assessment-report_en.pdf. Accessed 22 July 2020.
9 Roemisch JR, Kaar W, Zoechling A, *et al.*: Identification of activated FXI as the major biochemical root cause in IVIG batches associated with thromboembolic events. Analytical and experimental approaches resulting in corrective and preventative measures implemented into the Octagam® manufacturing process. *WebmedCentral Immunother* 2011; **2**(6):WMC002002.
10 Ovanesov MV, Shibeko AM, Woodle SA, *et al.*: Association of factor XIa with intravenous immune globulin products implicated in thrombotic adverse events: biochemical root cause investigation. *J Thromb Haemost* 2011: **9**(Suppl 2):272,O-TU-039.
11 Frenzel W, Wietek S, Svae T, *et al.*: Tolerability and safety of Octagam® a post-authorization safety analysis of four non-interventional phase IV trials. *Int J Clin Pharmacol Ther* 2016; **54**:847–855
12 Octagam intravenous immunoglobulin 5% and 10%: licence suspensions lifted. Available at: https://www.gov.uk/drug-safety-update/octagam-intravenous-immunoglobulin-5-and-10-licence-suspensions-lifted. Accessed 22 July 2020.
13 Gray E, Hogwood J, Wilmot H, *et al.*: Value assignment of the candidate 1st international standard for activated blood coagulation factor XI (FXIa), human, NIBSC code (13/100). Available at: https://www.who.int/biologicals/BS_2245_FXIa_report_20140702.pdf. Accessed 15 Oct 2020.
14 European Pharmacopeia: Human normal immunoglobulin for intravenous administration. Monograph 01/2012:0918. Available at: https://pheur.edqm.eu/subhome/10-2. Accessed 22 July 2020.
15 European Pharmacopeia. Human normal immunoglobulin for subcutaneous administration. Monograph 01/2015:2788. Available at: https://pheur.edqm.eu/subhome/10-2. Accessed 22 July 2020.
16 Etscheid M, Breitner-Ruddock S, Gross S, *et al.*: Identification of kallikrein and FXIa as impurities in therapeutic immunoglobulins: implications for the safety and control of intravenous blood products. *Vox Sang* 2012; **102**:40–46
17 Ma L, Lin FZ, Diao G, *et al.*: Quantitative determination of activated coagulation factor XI as an impurity in therapeutic immunoglobulins from Chinese blood fractionation companies. *Genet Mol Res* 2013; **12**:2556–2561
18 Seifner A, Beck G, Bayer P, *et al.*: Assessment of immunoglobulin concentrates on thrombogenic activity by thrombin generation assay, prekallikrein activator assay, and size-exclusion chromatography. *Transfusion* 2014; **54**:376–383
19 Marzo N, Jose M, Lopez L, *et al.*: Quantitative determination of relevant amounts of blood coagulation factor XI activity in a specific brand of intravenous immunoglobulin. *WebmedCentral Immunother* 2010: **2**(5):WMC001922.
20 Park DH, Kang GB, Kang DE, *et al.*: A new manufacturing process to remove thrombogenic factors (II, VII, IX, X and XI) from intravenous immunoglobulin gamma preparations. *Biologicals* 2017; **45**:1–8
21 Jose M, Marzo N, Pons B, *et al.*: Flebogamma® DIF (intravenous immunoglobulin) purification process effectively eliminates procoagulant activities. *Biologicals* 2013; **41**:393–399
22 Komenda M, Stadlet D, Malinas T, *et al.*: Assessment of the ability of the Privigen® purification process to deplete thrombogenic factor XIa from plasma. *Vox Sang* 2014; **107**:26–36
23 Biophen FXIa chromogenic assay package insert. Available at: https://www.coachrom.com/fileadmin/docs/hbm/en/220412.pdf. Accessed 22 July 2020.
24 ROX FXIa chromogenic assay information available at: https://www.rossix.com/products/rox-factor-xia/. Accessed 21 July 2020.
25 Dalakas MC: High-dose intravenous immunoglobulin and serum viscosity: risk of precipitating thromboembolic events. *Neurology* 1994; **44**:223–226
26 Wolberg AS, Kon RH, Monroe DM, *et al.*: Coagulation factor XI is a contaminant in intravenous immunoglobulin preparations. *Am J Hematol* 2000: **65**:30–34
27 Mohammed BS, Matafonov A, Ivanov I, *et al.*: An update on factor XI structure and function. *Thromb Res* 2018; **161**:94–105

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Impact of pathogen reduction methods on immunological properties of the COVID-19 convalescent plasma

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Background and objectives COVID-19 convalescent plasma is an experimental treatment against SARS-CoV-2. The aim of this study is to assess the impact of different pathogen reduction methods on the levels and virus neutralizing activity of the specific antibodies against SARS-CoV2 in convalescent plasma.

Materials and methods A total of 140 plasma doses collected by plasmapheresis from COVID-19 convalescent donors were subjected to pathogen reduction by three methods: methylene blue (M)/visible light, riboflavin (R)/UVB and amotosalen (A)/UVA. To conduct a paired comparison, individual plasma doses were divided into 2 samples that were subjected to one of these methods. The titres of SARS-CoV2 neutralizing antibodies (NtAbs) and levels of specific immunoglobulins to RBD, S- and N-proteins of SARS-CoV-2 were measured before and after pathogen reduction.

Results The methods reduced NtAbs titres differently: among units with the initial titre 80 or above, 81% of units remained unchanged and 19% decreased by one step after methylene blue; 60% were unchanged and 40% decreased by one step after amotosalen; after riboflavin 43% were unchanged and 50% (7%, respectively) had a one-step (two-step, respectively) decrease. Paired two-sample comparisons (M vs. A, M vs. R and A vs. R) revealed that the largest statistically significant decrease in quantity and activity of the specific antibodies resulted from the riboflavin treatment.

Conclusion Pathogen reduction with methylene blue or with amotosalen provides the greater likelihood of preserving the immunological properties of the COVID-19 convalescent plasma compared to riboflavin.

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Key words: COVID-19 convalescent plasma, NtAbs, pathogen reduction, amotosalen, riboflavin, methylene blue.

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Introduction

The new coronavirus infection (COVID-19) caused by the SARS-CoV-2 virus continues its march around the world, causing a global crisis as the number of new cases and

deaths continues to rise. The treatment is supportive care mostly aimed at relieving symptoms. Candidate vaccines are still going through different stages of clinical trials, and different classes of drugs are being tested to inhibit virus replication and reduce inflammation [1–3].

Passive immunotherapy with polyclonal antibodies from the blood plasma of convalescents was tested earlier in the outbreaks of SARS-CoV, influenza and other dangerous infections of the twentieth century [4–6]. Experts from different countries came to consider the possibility of using COVID-19 convalescent plasma (CCP) for therapeutic purposes in patients with COVID-19 [7–9]. In many countries, national campaigns have been launched to collect CCP. The use of CCP is still exploratory at this time as proof of efficacy has not been demonstrated through RCT even if many RCTs on CCP are ongoing worldwide.

Various mechanisms have been suggested as responsible for the therapeutic effect of CCP such as virus neutralization and immunomodulation [10]. Virus neutralizing antibodies (NAbs) of IgG, IgM and IgA classes bind to different parts of glycoprotein S, including the region of the receptor-binding domain (RBD), spatially blocking its interaction with the membrane protein ACE2 of host cells, which limits the penetration of the virus into the cell, thereby limiting viral replication [11–13]. Along with antibodies against different fragments of S-protein antibodies against nucleocapsid (N-protein) are detected in the course of COVID-19 infection. These antibodies are used as additional diagnostic markers, but do not correlate with virus neutralizing activity *in vitro* [14].

Every plasma transfusion is associated, however, with risks of virus transferral such as HIV, HBV, HCV, etc. [15]. Donor studies of Cappy et al [16] showed that viraemia was extremely rare in asymptomatic blood donors, viral RNA levels were very low when detected, and the corresponding plasma was not infectious in cell culture. At the moment, there are no scientific publications reporting on the transmission of SARS-CoV-2 through the transfusion of blood components [17]. The Working Party on Global Blood Safety of the International Society of Blood Transfusion (ISBT) recommended the use of pathogen reduction (PR) of convalescent plasma to minimize the residual risk of blood-borne infections and to address the problem of possible superinfection with the SARS-CoV-2 virus [8].

Until recently, there was surprisingly very little information on the effect of PR treatment of plasma on the functional properties of immunoglobulins. This issue has been raised previously for Ebola convalescent plasma regarding possible impact of PR by Intercept technology on the neutralizing activity of EBOV IgG, potentially affecting clinical outcomes [18, 19]. Tonn *et al* [20] found that PR did not impair the stability and neutralizing capacity of SARS-CoV-2 specific antibodies in 5 CCP units treated with psoralen/UVA (Intercept).

To date, there are no sufficient data on how pathogen reduction affects the immunological properties of CCP and what PR technologies are preferable to use to maintain its quality and effectiveness.

The objective of this study is to assess the effect of various methods for pathogen reduction on the levels and virus neutralizing activity of the specific antibodies against SARS-CoV2 in CCP.

Materials and methods

The COVID-19 convalescent plasma procurement program in Russia was launched on 2 April 2020 at the Department of Transfusion Medicine of the Sklifosovsky Research Institute of Emergency Medicine, Moscow. At present, this programme involves many hospitals in several regions and has more than 6,500 donations and about 4,500 transfusions of CCP in Moscow alone. According to the adopted regulations, donors of convalescent plasma were recruited among the individuals with prior diagnosis of COVID-19 infection documented by a positive RT-PCR-test who received treatment either in a hospital setting or on an outpatient basis. Donors fulfilled the standard blood donor selection criteria. Plasma was collected at least 2 weeks after the complete disappearance of clinical symptoms.

Ethical approval was granted for this study by the Independent Moscow City Research Ethical Committee in accordance with national regulations. Informed consent was obtained in writing from all donors prior to donation.

Plasmapheresis procedures were performed using Auto-C (Fresenius Kabi), Aurora (Fresenius Kabi) and PCS2 (Haemonetics) machines. Plasmapheresis was carried out in accordance with standard protocols collecting an amount of 650 ml plasma. During collection, the same anticoagulant (ACD-A) was added in a ratio of 1:12 in all machines.

Pathogen reduction procedures were carried out immediately after the end of plasmapheresis. For comparison, three systems for PR were selected:

(1) Intercept (Cerus): 15 ml of 6 mmol/l amotosalen hydrochloride solution were mixed with plasma to a final concentration 150 μmol/l and exposed to UVA light (3 J/cm^2), thereafter residual amotosalen and free photoproducts were adsorbed in a compound adsorption device (CAD).
(2) Mirasol (Terumo, BCT): 35 ml of 500 μmol/l riboflavin solution was mixed with 200 ± 5 ml plasma and exposed to UVB light (6·24 J/ml).
(3) Maco-Tronic (MacoPharma): plasma was transferred to the THERAFLEX MB-Plasma illumination bag, passing through a chamber containing the anhydrous

MB pill, resulting in a minimum MB concentration of at least 0·8 μmol/l in 315 ml of plasma, subsequent illumination (120 J/cm^2) and removal of MB using the Blueflex MB removal filter.

All three PR technologies were validated at our blood bank for routine use, prior to pathogen reduction of CCP for this study. All plasma units were treated under the specific PR manufacturer's instructions and met the required specifications for each pathogen reduction technology. The temperature of plasma units during illumination were maintained at ≤22°C.

After PR plasma was frozen at −40°C and became available for clinical use after receiving negative results of all serology/virology tests for transfusion-transmitted diseases.

The study included 140 doses of plasma obtained by plasmapheresis from 140 COVID-19 convalescent donors. From each plasma unit, samples were collected before and after pathogen reduction for the determination of the titres of SARS-CoV-2 neutralizing antibodies (NtAbs), as well as quantitative determination of specific IgG to the receptor-binding domain (RBD) of the glycoprotein S of the SARS-CoV-2 virus, and specific IgM and IgG to S- and N-proteins of this virus.

To conduct a paired two-sample comparison in order to assess the effect of each of these methods on the immunological parameters of CCP, the plasma dose from each donor was divided into 2 parts, and each part was then simultaneously subjected to a pathogen reduction procedure by one of the two methods according to the following scheme:

- pair 1: methylene blue (M) vs. riboflavin - 48 pairs;
- pair 2: amotosalen (A) vs. riboflavin (R) - 36 pairs;
- pair 3: methylene blue (M) vs. amotosalen (A) - 56 pairs;

Since there were 140 samples *before* treatments, in total 420 (=48*2 + 36*2 + 56*2 + 140) samples were analysed. Virus neutralization assay and ELISA were performed at a day of biomaterial collection at the Gamaleya National Research Center of Epidemiology and Microbiology, Moscow. Samples for chemiluminescent immunoassay (CLIA) were frozen immediately after the collection in Eppendorf tubes in aliquots of 200 μl in −35°C and thawed and analysed later all at the same time.

NtAb titre was determined by microneutralization test [21]. Vero E6 were cultured in DMEM – Dulbecco's Modified Eagle Medium (Gibco) containing 10% heat-inactivated fetal bovine serum (Gibco), 3,7 g/l sodium bicarbonate (PanEco), 1 mM glutamine (PanEco), 100 μg/ml streptomycin (PanEco), 100 IU/ml penicillin (PanEco) in 5% CO_2 humidified incubator at 37°C.

The SARS-CoV-2 (hCoV-19/Russia/Moscow_PMVL-1/2020) virus was obtained from the State Virus Collection of Gamaleya NRCEM. The infectious virus titre was determined on Vero E6 cells using a 50% tissue culture infectious dose (TCID50) assay. Serial 10-fold dilutions of the virus stock were prepared in DMEM with 2% heat-inactivated FBS and in volume of 100 μl were added to Vero E6 cells in a 96-well plate in 8 repeats. The cells were incubated at 37°C in 5% CO_2 for 96 h and scored visually for cytopathic effect. The TCID50 titre was calculated by Reed and Muench method. Neutralization activity of plasma was determined by microneutralization test using SARS-CoV-2 virus in a 96-well plate. Plasma samples were inactivated by incubation at 56°C for 30 min and serial two-fold dilutions in DMEM containing 2% heat-inactivated FBS at a range 1:20 – 1:1280 were made. Then 100 $TCID_{50}$ was added to each sample. The samples were incubated at 37°C for 1 h in a 5% CO_2 incubator. After incubation samples were added to Vero E6 cells and incubated in a 5% CO_2 incubator at 37°C for 96 h. Neutralization titre was defined as the highest serum/plasma dilution without cytopathic effect in two of three replicable wells.

Anti-SARS-CoV-2 IgG semi-quantitative ELISA test-system developed in Gamaleya NRCEM and registered for clinical use in Russian Federation (P3H 2020/10393 2020-05-18) was used for the determination of the IgG specific to the receptor-binding domain of SARS-CoV-2 glycoprotein S [20].

Briefly, the RBD-pre-coated plates (100 ng per well) were washed 5× with 0·1% wash solution and then blocked with blocking solution. Plasma samples were diluted 1/200 in blocking solution and added in wells, and then plates were incubated at 37°C for 1 h. After washing the plates 5× the peroxidase-conjugated anti-human IgG detection antibodies diluted in blocking solution were added and plates were incubated at 37°C for 1 h. After washing 5×, the substrate TMB was added and plates were incubated at 20–25°C for 15 min, the reaction was stopped with the stop solution. The OD signals were determined with a spectrophotometer Multiskan FC (Thermo Fisher Scientific Inc, USA) at 450 nm.

Sensitivity of the test is 96%, specificity 100% and the limit of detection is 0·2 AU (OD450 nm).

The CL-series SARS-CoV-2 IgG and IgM assays are a two-step chemiluminescent immunoassays for detection of IgG and IgM SARS-CoV-2 antibodies in human serum or plasma, performed on the fully automated Mindray CL 1200i analytical system (Shenzhen Mindray Bio-Medical Electronics Co., Shenzhen, China). Samples react with paramagnetic microparticles coated with SARS-CoV-2-specific antigens – recombinant N-Protein and Spike (S)

Protein. Alkaline phosphatase-labelled anti-human IgG or IgM monoclonal antibodies are added to the reaction to form a sandwich with microparticles captured anti-SARS-CoV-2 antibodies. Finally, a substrate solution is added, resulting in a chemiluminescent reaction measured as relative light units by a photomultiplier built into the system. The amount of SARS-CoV-2 IgG antibodies present in the sample is proportional to the relative light units (RLUs) generated during the reaction. The SARS-CoV-2 IgG and IgM antibodies concentration can be determined via a calibration curve, which is built on an encoded Master Calibration Curve and three level product calibrators. Cut-off values are as follows: IgG positive > 10·0 U/ml and IgM positive > 1·0 COI, Positive Percent Agreement 81·7% and 82·2%, Negative Percent Agreement 91·6% and 94·9% for IgM and IgG, respectively, according to the manufacturer's specifications.

Since the data for NtAbs are presented in the format 10 multiplied by an integer power of two (i.e 20, 40, 80, 160 etc.), we log-2-transformed the data: $y = \log^2(x/10)$, where x is the reported value of NtAbs.

To identify the methods of pathogen reduction which have the least negative effect on NtAbs levels, we applied the two-sample paired t-tests (M vs. A, M vs. R and A vs. R) to the difference in reduction in titres of NtAbs, anti-RBD IgG, and anti-S + N IgG and IgM titres, respectively, after pathogen reduction by different methods. The *P*-values < 0·05 were considered to be significant. No corrections were made for multi-significance. Confidence intervals were obtained using the standard methods for estimation of proportions. The software used for the analysis was Maple™.

Results

The assessment of the impact of various methods of pathogen reduction on the titres of SARS-CoV2 neutralizing antibodies (NtAbs) showed a statistically significant decrease in antibody titres after all pathogen reduction processes (Fig. 1; Table 1).

If all plasma units, regardless of the initial titre were included in the analysis, it was shown that in 88% (n = 104; confidence interval 81%–94%) of units NtAbs titres did not decrease after pathogen reduction with methylene blue whereas a one-step titre reduction was observed in remaining 12%. In 70% (n = 88; 95% confidence interval 61%–80%) of units treated with amotosalen, the NtAbs titre did not change, and in 30% it decreased by 1 step. Pathogen reduction with riboflavin left NtAbs titres unchanged in 61% (n = 83; confidence interval 51%–72%) of the units, in 35% decreased by one step and in 4% by two steps.

To compare the impact of different methods of pathogen reduction, we used the data collected on paired data: the plasma units from the same donor were treated using for example method A and M, and then, the resulting

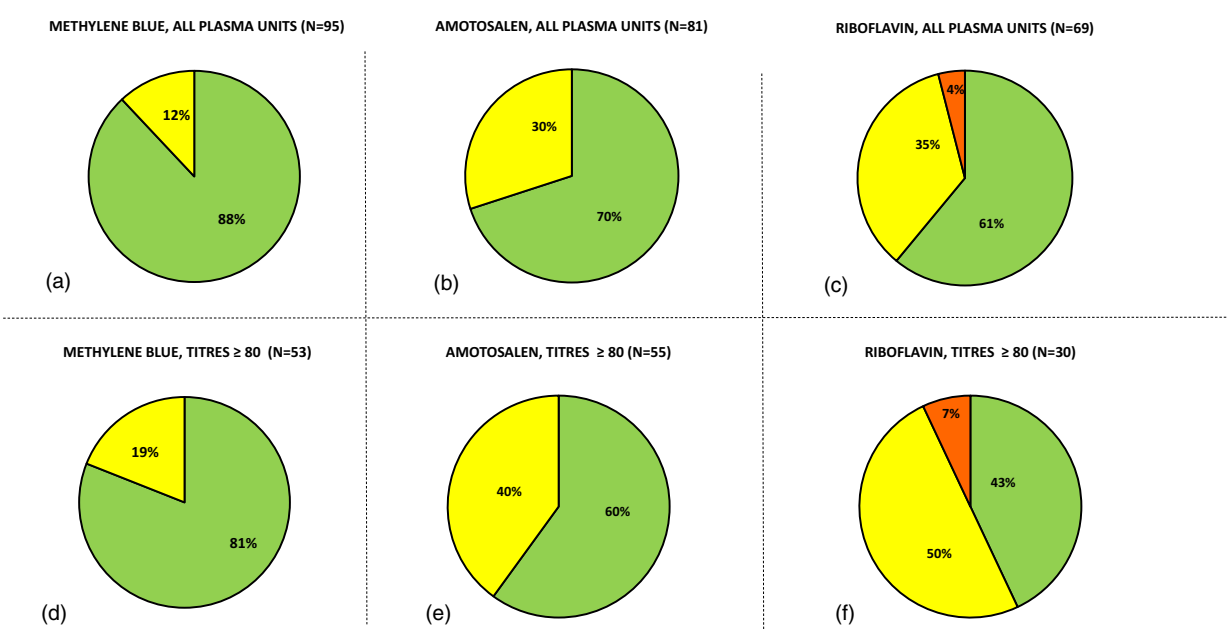

Fig. 1 Percentage of COVID-19 convalescent plasma units that had no decrease in NtAbs titre (■), one-step titre decrease (■) or two-step titre decrease (■) after pathogen reduction with methylene blue (A, D), amotosalen (B, E) and riboflavin (C, F) among all plasma units (A, B, C) or only units with initial NtAbs titre 80 or above (D, E, F).

Table 1 Individual methods of pathogen reduction, comparison of the initial values vs. post-treatment values using two sample paired *t*-test

Method (sample size)	NtAbs*	Anti-RBD IgG (AU)	Anti-S + N IgG (U/ml)	Anti-S + N IgM (COI)
Methylene blue (n = 104)	**0·10** (0·01)	**0·03** (0·03)	1·1 (0·23)	0·007 (0·59)
Amotosalen (n = 88)	**0·23** (0·0003)	0·0 (0·99)	**1·7** (0·008)	0·01 (0·53)
Riboflavin (n = 83)	**0·40** (<0·0001)	**0·07** (0·001)	**8·9** (<0·0001)	**0·19** (<0·0001)

The numbers show the average decline of values of NtAbs, anti-RBD IgG, anti-S + N IgG and anti-S + N IgM after treatments by each of the three methods (M/A/R, respectively). The *P*-values in the parentheses below indicate how statistically different from zero these values are; those with $P < 0.05$ are in bold font.

*The shown values are $\log_2$-transformed and divided by 10.

Table 2 Comparison of different methods of pathogen reduction using two sample paired *t*-test

Method (sample size)	NtAbs*	Anti-RBD IgG (AU)	Anti-S + N IgG (U/ml)	Anti-S + N IgM (COI)
Methylene blue vs Amotosalen (n = 56)	**0·27** (0·001)	-0·02 (0·60)	3·1 (0·21)	0·045 (0·29)
Amotosalen vs Riboflavin (n = 36)	**0·33** (0·0002)	0·06** (0·27)	**6·7** (<0·0001)	**0·20** (<0·0001)
Methylene blue vs Riboflavin (n = 48)	**0·42** (<0·0001)	0·007 (0·82)	**6·6** (<0·0001)	**0·16** (<0·0001)

The numbers show the average differences of declines of values of NtAbs, anti-RBD IgG, anti-S + N IgG and anti-S + N IgM corresponding to the respective pair of methods. The *P*-values in parentheses below indicate how statistically different from zero these values are; those with $P < 0.05$ are in bold font.

*The shown values are $\log_2$-transformed and divided by 10.

**One data point is missing, so n = 35 here.

NtAbs were noted for both methods. We had three different datasets: one compared A vs. R, another dataset M vs. R and the third M vs. A. The results are the following: M is better than R (*P*-value = 0·00002, n = 48), A is better than R (*P*-value = 0·0002, n = 36) and M is better than A (*P*-value = 0·0012, n = 56).

When only units with the initial NtAbs titre 80 or above were chosen (the level considered to be suitable for therapeutic purposes), the distribution was similar: after treatment with methylene blue, 81% of plasma samples had unchanged NtAbs titres (n = 53; confidence interval 71%–92%), while in the remaining 19% of samples the titres decreased by 1 step. Pathogen reduction with amotosalen gave worse results: 60% of samples had the same NtAbs after the reduction (n = 55; confidence interval 47%–73%), while in the remaining 40% samples, the titres decreased by 1 step. Finally, after treatment with riboflavin, only 43% of the samples preserved the level of NtAbs titres (n = 30; confidence interval 26%–61%), whereas a one-step decrease was observed in 50% samples, and a two-step decrease in 7% of samples.

The decrease in anti-RBD IgG in paired comparison with baseline values was most pronounced after pathogen reduction with riboflavin followed by methylene blue whereas after amotosalen there was no significant difference (Table 1).

Plasma pathogen inactivation with methylene blue did not lead to a significant decrease in anti-S + N IgG and IgM, whereas the use of amotosalen significantly reduced only the level of anti-S + N IgG (Table 1). In the study of 83 pairs of samples before and after pathogen reduction with riboflavin, the differences were significant in the anti-S + N levels of both IgG and IgM (Table 1).

Paired two-sample comparisons (M vs. A, M vs. R and A vs. R) revealed the most prominent and statistically significant decline in titres of NtAbs, anti-S + N IgG and IgM (with the exception of anti-RBD IgG titres) resulted from pathogen reduction by riboflavin compared with two other PR technologies (Table 2).

As the riboflavin is not removed after the illumination phase in the Mirasol technique, the residual amount of riboflavin or its by-products may possibly affect the *in vitro* assessment of the NtAbs. To rule out this possibility, we have conducted a series of tests showing that the viability of the cells used to assess the neutralization was not affected by riboflavin itself nor its derivatives. We also measured NtAbs titres in several plasma samples taken after the addition of riboflavin but before the

illumination – the titres did not differ from those in samples before PR. Thus, it seems that the reducing effect of PR with riboflavin on the level and activity of antibodies against SARS-CoV-2 requires both photosensitizing agent and UVB illumination as this PR technology requires.

Discussion

The key safety issue of using convalescent plasma is played by the choice of a PR technology that minimizes the residual transfusion risk of transmissible viruses in the final product, while maintaining a high titre of antibodies to the SARS-Cov-2 virus. A number of different PR technologies are available today [22]. Ultraviolet (UV) A [23, 24] and UVB radiation [25], in combination with amotosalen and riboflavin, respectively, makes it possible to inactivate nucleic acids of pathogenic organisms. These systems can reduce the activity of SARS and MERS viruses in plasma or platelet concentrates to varying degrees. Methylene blue is a phenothiazine compound that, in combination with visible light, is also capable of inactivating coronaviruses in plasma [26, 27]. The photoactive agents used in these methods have different chemical structures and are activated at different wavelengths of radiation (visible light with the peak wavelength of 590 nm, UVA from 400 to 315 nm and UVB from 315 to 280 nm). Consequently, various mechanisms are involved in ensuring pathogen reduction. The amotosalen intercalates into DNA and RNA and, when activated by UVA light, causes covalent cross-linking of those nucleic acids thus preventing the replication. The riboflavin binds to nucleic acids and, when activated by the illumination step, alters guanine residues via type I and type II redox reactions. MB can intercalate into DNA or bind to the DNA helix, depending on the concentration and ionic strength of Mg^{2+}. When exposed to light type I (redox) or type II (photo-oxidative) reactions occur, with most of the PR activity resulting from type II reactions [22, 28].

In earlier studies on Ebola convalescent plasma [18, 19] it was shown that PR with amotosalen/UVA only slightly reduced anti-Ebola virus IgG titres and Ebola-specific neutralizing antibodies. Tonn *et al* [20] found that PR did not impair the stability and neutralizing capacity of SARS-CoV-2 specific antibodies in 5 CCP units treated with psoralen/UVA.

The current study is the first to compare the impact of different PR technologies on SARS-CoV-2 antibody levels and activity in convalescent plasma.

The hypothesis tested in this study is that different types of photo-chemical reactions used in standard PR technologies can have a different effect on the amount and neutralizing activity of SARS-CoV2 specific antibodies in the final product – convalescent plasma. The results obtained indicate a lesser effect on the immunological quality of CCP of pathogen reduction with methylene blue or with amotosalen, possibly due to the lesser amount of energy used for illumination and lesser amount of reactive oxygen species releasing after photoactivation compared with riboflavin [28, 29]. More research is needed to elucidate the exact mechanisms for the oxidative damage of proteins and particularly immunoglobulins in course of different PR technologies.

Based on the study, we can recommend using pathogen reduction with methylene blue or with amotosalen to ensure the safety and quality of CCP, due to the greater likelihood of preserving the immunological properties of the final product. Since even these technologies are associated with a risk of reducing the quantity and quality of antibodies against SARS-CoV-2, it is recommended to transfuse at least 2 units of convalescent plasma (200–300 ml) from different donors to one patient, especially in those medical institutions where the routine measurement of NtAbs titres is not possible.

In those blood establishments where pathogen reduction with riboflavin is traditionally used, it may be worth to consider increasing the dose of transfused convalescent plasma in order to compensate for the decrease in the baseline neutralizing antibody titres after this method of pathogen reduction.

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Conflict of interest

The authors declare no conflict of interests.

Authors' contribution

Design of the study – A. Kostin; convalescent plasma donor recruiting – V. Ganchin, E. Dombrovskiy; collection of data – E. Ladygina, K. Chirkova, A. Kamalova; development of test systems for NtAbs and anti-RBD – A. Gintsburg, D. Shcheblyakov, I. Dolzhikova, D. Logunov;

experimental work M. Godkov, A. Bazhenov, A. Bogdanova, V. Shustov; organization of the working processes for collection of convalescent plasma – S.Petrikov, A. Bulanov, N. Drozdova, N. Borovkova; statistical analysis and interpretation of the data and drafting of the manuscript – A. Kostin, M. Lundgren, S.Volkov. All co-authors critically reviewed and approved the manuscript.

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References

1 Dos Santos WG. Natural history of COVID-19 and current knowledge on treatment therapeutic options. Biomed Pharmacother 2020;129:110493.
2 Lamontagne F, Agoritsas T, Macdonald H, et al. A living WHO guideline on drugs for covid-19. BMJ 2020;370: m3379.
3 Pennica A, Conforti G, Falangone F, et al. Clinical management of adult coronavirus infection disease 2019 (COVID-19) positive in the setting of low and medium intensity of care: a short practical review. SN Compr. Clin Med 2020;2:694–9.
4 Mair-Jenkins J, Saavedra-Campos M, Baillie JK, et al. The effectiveness of convalescent plasma and hyperimmune immunoglobulin for the treatment of severe acute respiratory infections of viral etiology: a systematic review and exploratory meta-analysis. J Infect Dis 2015;211:80–90.
5 Luke TC, Kilbane EM, Jackson JL, et al. Meta-analysis: convalescent blood products for Spanish influenza pneumonia: a future H5N1 treatment? Ann Intern Med 2006;145:599–609.
6 Hung IF, To KK, Lee CK, et al. Convalescent plasma treatment reduced mortality in patients with severe pandemic influenza A (H1N1) 2009 virus infection. Clin Infect Dis 2011;52:447–56.
7 Chen L, Xiong J, Bao L, et al. Convalescent plasma as a potential therapy for COVID-19. Lancet Infect Dis 2020;20:398–400.
8 Epstein J, Burnouf T. Points to consider in the preparation and transfusion of COVID-19 convalescent plasma. Vox Sang 2020;115:485–7.
9 Tiberghien P, de Lamballerie X, Morel P, et al. Collecting and evaluating convalescent plasma for COVID-19 treatment: why and how? Vox Sang 2020;115:488-94.
10 Rojas M, Rodríguez Y, Monsalve DM, et al. Convalescent plasma in Covid-19: Possible mechanisms of action. Autoimmun Rev 2020;19:102554.
11 Jiang S, Hillyer C, Du L. Neutralizing Antibodies against SARS-CoV-2 and Other Human Coronaviruses. Trends Immunol 2020;41:355–9.
12 Shang J, Wan Y, Luo C, et al. Cell entry mechanisms of SARS-CoV-2. Proc Natl Acad Sci USA. 2020;117: 11727–34.
13 Hoffmann M, Kleine-Weber H, Schroeder S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell 2020;181:271–80.
14 Ni L, Ye F, Cheng ML, et al. Detection of SARS-CoV-2-Specific Humoral and Cellular Immunity in COVID-19 Convalescent Individuals. Immunity 2020;52:971–7.
15 Bihl F, Castelli D, Marincola F, et al. Transfusion-transmitted infections. J Transl Med 2007;5:25.
16 Cappy P, Candotti D, Sauvage V, et al. No evidence of SARS-CoV-2 transfusion transmission despite RNA detection in blood donors showing symptoms after donation. Blood 2020;136:1888–91.
17 Leblanc JF, Germain M, Delage G, et al. Risk of transmission of severe acute respiratory syndrome coronavirus 2 by transfusion: A literature review. Transfusion 2020;60:3046-54.
18 Geisen C, Kann G, Strecker T, et al. Pathogen-reduced Ebola virus convalescent plasma: first steps towards standardization of manufacturing and quality control including assessment of Ebola-specific neutralizing antibodies. Vox Sang 2016;110:329–35.
19 Dean CL, Hooper JW, Dye JM, et al. Characterization of Ebola convalescent plasma donor immune response and psoralen treated plasma in the United States. Transfusion 2020;60:1024–31.
20 Tonn T, Corman VM, Johnsen M, et al. Stability and neutralising capacity of SARS-CoV-2-specific antibodies in convalescent plasma. Lancet Microbe 2020;1:e63.
21 Logunov D, Dolzhikova I, Zubkova O, et al. Safety and immunogenicity of an rAd26 and rAd5 vector-based heterologous prime-boost COVID-19 vaccine in two formulations: two open, non-randomised phase 1/2 studies from Russia. Lancet 2020;396:887–97.
22 Gehrie EA, Rutter SJ, Snyder EL. Pathogen reduction: the state of the science in 2019. Hematol Oncol Clin North Am 2019;33:749–66.
23 Hashem AM, Hassan AM, Tolah AM, et al. Amotosalen and ultraviolet A light efficiently inactivate MERS coronavirus in human platelet concentrates. Transfus Med 2019;29:434–41.
24 Hindawi SI, Hashem AM, Damanhouri GA, et al. Inactivation of Middle East respiratory syndrome-coronavirus in human plasma using amotosalen and ultraviolet A light. Transfusion 2018;58:52–9.
25 Ragan I, Hartson L, Pidcoke H, et al. Pathogen reduction of SARS-CoV-2 virus in plasma and whole blood using riboflavin and UV light. PLoS One 2020;15:e0233947.
26 Eickmann M, Gravemann U, Handke W, et al. Inactivation of three emerging viruses—severe acute respiratory syndrome coronavirus, Crimean-Congo haemorrhagic fever virus and Nipah virus—in platelet concentrates by ultraviolet C light and in plasma by

methylene blue plus visible light. Vox Sang 2020;115:146–51.
27 Eickmann M, Gravemann U, Handke W, et al. Inactivation of Ebola virus and Middle East respiratory syndrome coronavirus in platelet concentrates and plasma by ultraviolet C light and methylene blue plus visible light, respectively. Transfusion 2018;58: 2202–7.
28 Mundt JM, Rouse L, Van den Bossche J, et al. Chemical and biological mechanisms of pathogen reduction technologies. Photochem Photobiol 2014;90:957–64.
29 Prudent M, Sonego G, Abonnenc M, et al. LC-MS/MS analysis and comparison of oxidative damages on peptides induced by pathogen reduction technologies for platelets. J Am Soc Mass Spectrom 2014;25:651–61.

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Amotosalen and ultraviolet A light treatment efficiently inactivates severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in human plasma

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Abstract

Background and objectives During the ongoing pandemic of COVID-19, SARS-CoV-2 RNA was detected in plasma and platelet products from asymptomatic blood donors, raising concerns about potential risk of transfusion transmission, also in the context of the current therapeutic approach utilizing plasma from convalescent donors. The objective of this study was to assess the efficacy of amotosalen/UVA light treatment to inactivate SARS-CoV-2 in human plasma to reduce the risk of potential transmission through blood transfusion.

Methods Pools of three whole-blood-derived human plasma units (630–650 ml) were inoculated with a clinical SARS-CoV-2 isolate. Spiked units were treated with amotosalen/UVA light (INTERCEPT Blood System™) to inactivate SARS-CoV-2. Infectious titres and genomic viral load were assessed by plaque assay and real-time quantitative PCR. Inactivated samples were subject to three successive passages on permissive tissue culture to exclude the presence of replication-competent viral particles.

Results Inactivation of infectious viral particles in spiked plasma units below the limit of detection was achieved by amotosalen/UVA light treatment with a mean log reduction of >3·32 ± 0·2. Passaging of inactivated samples on permissive tissue showed no viral replication even after 9 days of incubation and three passages, confirming complete inactivation. The treatment also inhibited NAT detection by nucleic acid modification with a mean log reduction of 2·92 ± 0·87 PFU genomic equivalents.

Conclusion Amotosalen/UVA light treatment of SARS-CoV-2 spiked human plasma units efficiently and completely inactivated >3·32 ± 0·2 log of SARS-CoV-2 infectivity, showing that such treatment could minimize the risk of transfusion-related SARS-CoV-2 transmission.

Key words: amotosalen/UVA, pathogen inactivation, SARS-CoV-2, plasma.

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Introduction

A key element of blood safety is the prevention of transfusion-transmitted infections. Multiple safety measures were introduced in the last decades, strongly accelerated by the recognition of HIV transfusion transmission in the early 1980s, significantly decreasing the risk for infectious adverse events. However, there is still a remaining risk for the recipients of labile blood components, considering the limitations of existing safety measures [1]. Newly emerging pathogens, particularly viruses, may pose a challenge to the safety of plasma transfusion, especially if subclinical infection occurs in combination with blood viremia. The majority of individuals infected by one of the recently emerging arboviruses develop no symptoms but may carry a high titre viral load in the blood [1,2]. The emergence and risk for the blood supply caused by emerging infectious diseases are unpredictable [3], as demonstrated by the current COVID-19 pandemic.

The first cases of COVID-19 disease, an infection of the lower respiratory tract, were reported in late 2019, spreading rapidly in and from Wuhan, China [4]. The highly virulent pathogen causing the disease was identified as a beta-Coronavirus (SARS-CoV-2) [5]. On 11 March 2020, the WHO declared a global COVID-19 pandemic, up to date more than 47 million confirmed infections and more than 1·2 million disease-related deaths have been reported globally, with highest disease burden in Europe and the Americas, followed by the Middle East and Asia. In Saudi Arabia, more than 348 000 confirmed cases and more than 5000 disease-related deaths have been reported up to date according to the WHO COVID-19 Dashboard [6]. Respiratory droplets were quickly identified as the main route of infection, other ways of transmission, especially smear infection/oral intake, may also play a role [7]. Not every infected individual develops the COVID-19 disease, the prevalence of asymptomatic infected individuals has been reported by several studies [7,8] including a study from South Korea that reported a prevalence of 19·2% [9].

SARS-CoV-2 is closely related to other human coronaviruses, especially SARS-CoV which caused an outbreak of the Severe Acute Respiratory Syndrome in Singapore and Korea in 2000–2002. By decision of the International Committee for the Taxonomy of Viruses, SARS-CoV and SARS-CoV-2 are different strains of the same species [5]. Transmission by blood transfusion was not shown for SARS-CoV, however, due to the detection of viral genomes in blood in the symptomatic phase, and the detection of SARS-CoV genomes in leucocytes in the convalescent phase together with evidence for subclinical infection, transfusion transmission of SARS-CoV is of concern and considered a theoretical risk [10]. Evidence for subclinical infection was also shown for SARS-CoV-2 [7]. Low viral load SARS-CoV-2 RNAemia in blood was detected frequently in symptomatic patients [11,12]. A recent study from China reported SARS-CoV RNAemia also in asymptomatic blood donors; viral nucleic acids were detected retrospectively in frozen plasma products, a platelet product and donor samples for pre-screening [13]. Although transfusion transmission of SARS-CoV-2 is not documented to date, the detection of viral nucleic acids in blood products from asymptomatic donors raises concerns about potential transfusion transmission of SARS-CoV-2, especially because blood screening procedures for that pathogen are not established yet. Another potential risk became evident in light of the recent approaches to use convalescent donors' plasma to treat COVID-19 patients, an idea that seems to be one of the most promising therapeutic approach to date [14].

The safety of blood donations has been of major concern for transfusion medicine practitioners and researchers, this has led to the introduction of certain measures to reduce this risk including risk factor questionnaires, screening assay to detect transfusion-transmitted pathogens in blood donations [15]. These measures have led to decreased prevalence of some of the transfusion-transmitted infections such as HIV, HCV and HBV [16]. The increased improvement in the performance of screening assays was very helpful in reducing transmission risks. These assays are pathogen specific and are of no value to detect newly emerging and re-emerging pathogens. Therefore, it is worthwhile to consider treating the donated plasma with a pathogen inactivation technology proven to be efficient against a large panel of pathogens including SARS-CoV-2 [17].

The INTERCEPT technology inactivates a broad spectrum of viruses, bacteria and protozoa in plasma prepared for transfusion [18]. During a photochemical reaction involving amotosalen (the photoactive compound) and UVA light, the pathogens' genomes are modified in a targeted and specific manner by cross-linking the genomic strands, preventing transcription and replication without affecting the clinical outcome of the plasma transfusion [19,20]. This was also shown in a cohort of thrombotic thrombocytopenic purpura (TTP) patients requiring therapeutic plasma exchange [21,22]. As an additional effect, residual white blood cells of the donor are inactivated more efficiently than by gamma-irradiation [23], reducing the risk for immunological transfusion reactions and transfusion-associated graft-versus-host disease (TA-GvHD). Efficient inactivation by amotosalen/UVA treatment of the very closely related SARS-CoV was reported in human plasma units and platelet concentrates [24,25]. We also recently have shown that amotosalen/UVA

treatment can efficiently inactivate the closely related Middle East Respiratory Syndrome Coronavirus (MERS-CoV) in human plasma [26] and platelet concentrates [27], and now extending this work to evaluate the inactivation of SARS-CoV-2 in human therapeutic plasma units.

Materials and methods

Cell line and SARS-CoV-2 culture

Vero E6 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% foetal bovine serum (FBS) as previously described (Al-Amri et al, 2017). A human SARS-CoV-2 clinical patient isolate (SARS-CoV-2/human/SAU/85791C/2020, gene bank accession number: MT630432) was used in all experiments in the Special Infectious Agents Unit (SIAU) Biosafety level 3 facility at King Fahd Medical Research Center (KFMRC), King Abdulaziz University (KAU), Jeddah, Saudi Arabia.

Preparation of SARS-CoV-2 stock

Virus was inoculated on 90%–95% confluent Vero E6 cells (ATCC# CRL-1586) in a T175 tissue culture flask at multiplicity of infection (MOI) of 1 and incubated in a humidified incubator at 5% CO_2 and 37°C for one hour with gentle shaking every 15–20 min. Subsequently, the inoculum was replaced by 25 ml of viral inoculation medium (DMEM with 2% FBS, 1% penicillin/streptomycin and 10 mmol/l HEPES [pH 7·2]) and the cells were incubated in a humidified incubator at 5% CO_2 and 37°C until 80%–90% of cells showed a cytopathic effect (CPE), typically three days post-infection. Supernatant was collected and centrifuged to remove cellular debris for 5 min at 500× *g* at room temperature. Virus was then aliquoted and stored at –80°C, and the titre was determined by plaque assay.

Plasma preparation

Whole blood units (450 ml ± 10%) were collected and prepared at King Abdulaziz University Hospital, Transfusion Services, Jeddah, Kingdom of Saudi Arabia, from voluntary donors. Briefly, blood units were centrifuged at 544× *g* for 10 min to separate the platelet (PLT)-rich plasma. The PLT-rich plasma was then centrifuged at 230× *g* at 20°C for 10 min. The plasma was then transferred into the plasma bag and kept at –30°C. All blood units were screened routinely for HCV antibody, HBsAg, HBc antibody, HIV (1/2) antibody, HTLV (1/2) antibody, Syphilis as well as HCV, HBV and HIV by NAT.

SARS-CoV-2 inactivation

Pools of three single-donor plasma units were used in this study (volume 630–650 ml). All plasma pools were tested for the presence of anti-SARS-CoV-2 neutralizing antibodies using an in-house neutralization assay. Each plasma pool was inoculated with SARS-CoV-2 stock in a 1:100 dilution. Plasma units were treated with amotosalen/UVA using the INTERCEPT Processing Set for Plasma and an INTERCEPT Illuminator INT-100 (Cerus Corporation, U.S.A.). The following samples were collected for testing: a positive control sample from the virus stock; a negative control sample from the plasma before inoculation with the virus; a pretreatment sample after the addition of amotosalen and a sample from the treated plasma units after INTERCEPT illumination (Fig. 1). All samples were stored at –80°C until testing.

Detection of replicating SARS-CoV-2

Detection of replicating SARS-CoV-2 in amotosalen/UVA-treated plasma was performed as previously described for SARS-CoV-1 and MERS-CoV with minor modifications [25,26]. Briefly, collected pretreatment and inactivated samples were diluted at 1:10 dilution in DMEM with 10% FBS, inoculated on Vero E6 cells in 6 well plates in duplicates and incubated for 1 h at 37°C. Inoculum was then removed and replaced with 2 ml DMEM with 10% FBS and incubated for 3 days at 37°C. Then, supernatants were collected, diluted 1:10 in DMEM with 10% FBS and re-inoculated on Vero E6 cells for two more successive passages. Supernatants collected from each passage were also used for viral load quantification by RT-qPCR.

Plaque assay

Plaque assays were performed as previously described [26] with minor modifications. Briefly, samples were serially diluted in DMEM with 10% FBS starting from 1:10 and 1 ml from each dilution was inoculated on confluent Vero E6 cell monolayers and incubated for 1 h at 37°C. Then, the inoculum was removed and overlaid with DMEM containing 0·8% agarose and incubated for 3 days at 37°C. Cells were then stained with crystal violet for 4 h at 37°C, and plaques were counted to determine the viral titre as plaque forming unit (PFU)/ml.

RT-qPCR quantitation

Viral RNA was extracted from all samples collected directly from the plasma units (positive, negative,

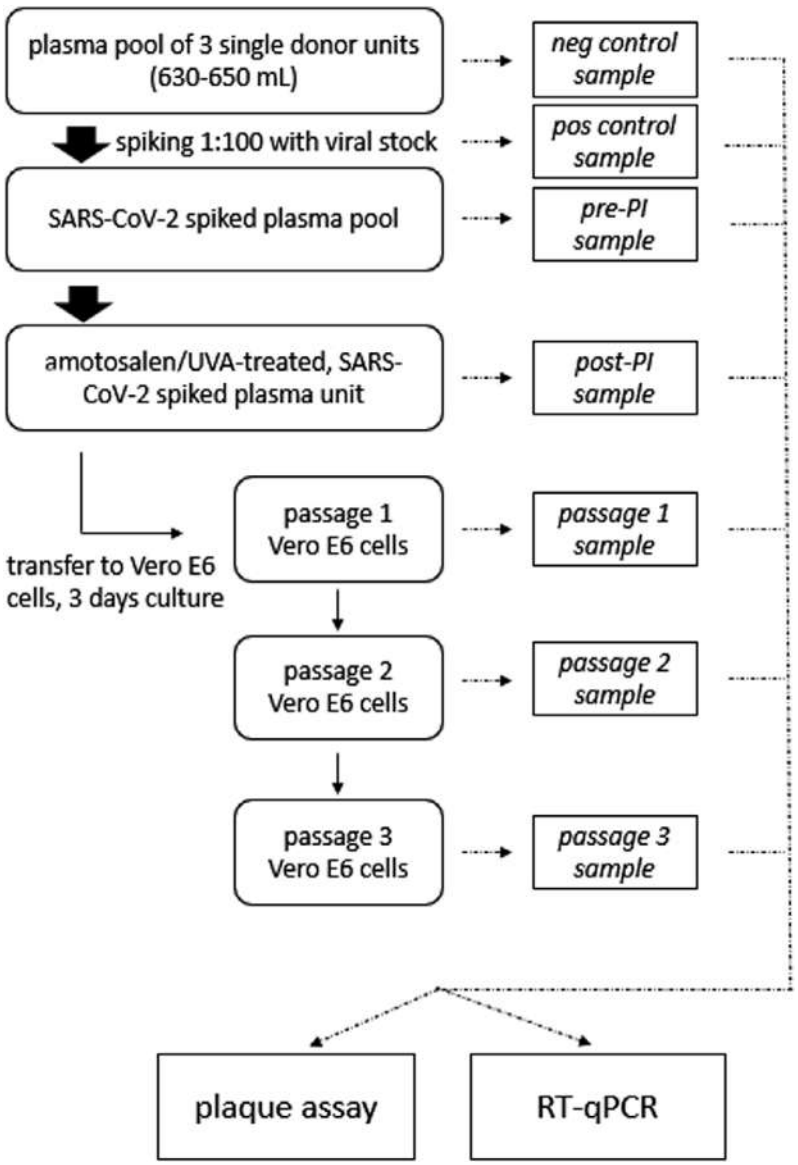

Figure 1 Experimental design. Schematic view of the experimental design. Briefly, pools of 3 plasma units were spiked in a 1:100 dilution with a SARS-CoV-2 viral stock. The pools were treated with amotosalen/UVA pathogen inactivation and post-inactivation subject to 3 consecutive passages of 3 days respectively on Vero E6 cells. Samples were taken at various stages and assessed for replicating virus by plaque assay and viral genomes by RT-qPCR.

pretreatment and post-treatment samples) using the QIAmp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Relative quantification of the SARS-CoV-2 viral load was performed by one-step dual-target real-time RT-PCR (RealStar SARS-CoV-2 RT-PCR Kit 1.0, Altona Diagnostics, Germany) according to the manufacturer's instructions using a 7500 Fast Real-Time PCR System (Applied Biosystems, U.S.A.). The PCR detects a beta-coronavirus specific target (E-gene), a SARS-CoV-2 specific target (S-gene) and an internal control. The decrease in viral load was expressed by comparing the cycle threshold (CT) values from each sample relative to the CT values of the pretreatment inoculated sample (with the SARS-CoV-2 specific primers). The SARS-CoV-2 titres were expressed as PFU equivalents per ml (PEq/ml) using a standard curve (standard: serial dilutions of the viral stock) and choosing dilutions of the original sample (10^{-1} to 10^{-8}) with CT values in the exponential phase. Each run included a positive viral template control and no-template negative control. Each sample was tested in duplicate, and the mean is reported as PEq/ml.

IRB approval

The study was approved by the Unit of Biomedical Ethics of the King Abdulaziz University Hospital (approval # 285-20).

Results

Inactivation of SARS-CoV-2 in human plasma units

Five pools (A-E) of human plasma collected from healthy donors were spiked with SARS-CoV-2. Spiked units were then treated with amotosalen and UVA light. The mean infectious viral titre in pretreatment samples was $3{\cdot}32 \pm 0{\cdot}19$ $\log_{10}$ PFU/ml (range: 3·1–3·6 $\log_{10}$ PFU/ml) (Table 1). Treatment of spiked units resulted in a mean reduction of $>3{\cdot}32 \pm 0{\cdot}19$ $\log_{10}$ PFU/ml as no infectious virus was detected by plaque assay (Table 1). Figure 2 shows a representative plaque assay result for all tested units. As expected, testing of negative control samples collected before spiking with SARS-CoV-2 showed no replication-competent virus. Of note, the viral infectivity titre in viral stock was $5{\cdot}6 \pm 0{\cdot}2$ $\log_{10}$ PFU/ml and it was reduced to a mean infectivity of $3{\cdot}32 \pm 0{\cdot}19$ $\log_{10}$ PFU/ml after spiking in a 1:100 dilution, which is close to an expected post-dilution titre of 3·6 $\log_{10}$ PFU/ml. Considering additional dilution of the spiked plasma by the addition of amotosalen solution, the dilution of the viral stock in plasma did not lead to unexpected loss of viral infectivity.

The impact of pathogen inactivation treatment on the genomic viral load

To further confirm these results, the viral genomic viral load was determined for all collected samples. The median CT preinactivation was 25·6 (18·3–29·1). The pretreatment samples mean genomic viral load was $2{\cdot}92 \pm 0{\cdot}87$ $\log_{10}$ PEq/ml, which was in the same range as the infectious titre ($3{\cdot}32 \pm 0{\cdot}19$ $\log_{10}$ PFU/ml). After treatment, no viral genomes were detectable by PCR (Table 2). The internal PCR control was always positive indicating no PCR inhibition and confirming the decrease in the signal from the SARS-CoV-2 specific primers is due to a mean minimum inactivation of $2{\cdot}92 \pm 0{\cdot}87$ $\log_{10}$ PEq/ml.

Passaging of INTERCEPT treated plasma to confirm complete inactivation

To exclude the possibility of any remaining replicating SARS-CoV-2 associated with the presence of viral genomic load in the treated plasma units (Table 2), we inoculated the collected samples on Vero E6 cells and evaluated infectivity over three successive passages. While culture of all pretreatment samples showed viral replication and complete CPE within 3 days post-inoculation similar to that of positive control, neither viral replication nor CPE was observed in cells inoculated with inactivated samples similar to negative controls (Fig. 3) even after 9 days of incubation in all three passages. For further confirmation, we determined the genomic viral load from supernatants collected from all passages inoculated with either pretreatment or post-treatment samples. As shown in Table 3, passaging of pretreatment samples showed viral replication as evident by CPE. On the other hand, viral genomes in cells inoculated with inactivated samples in culture supernatants were not detectable. Together, these data confirm the complete inactivation of SARS-CoV-2 in the tested platelets units and the absence of replication-competent virus post-inactivation.

Discussion

Newly emerging pathogens are an ongoing potential threat for safety of the blood supply. Pathogen inactivation technology may provide immediate enhanced safety in an outbreak setting, in contrast to diagnostic tools which, even if they already exist, need to be developed and validated for multiple weeks to months [28].

We were able to show complete inactivation of $>3{\cdot}32 \pm 0{\cdot}19$ log PFU/ml and $2{\cdot}92 \pm 0{\cdot}87$ log PEq/ml SARS-CoV-2 in human plasma pools of 630–650 ml in the present study, corresponding to volumes usually collected by plasmapheresis for the production of convalescent plasma (CP). Since a clinical isolate was used, the maximum titre obtained in vitro was not very high. Higher in vitro infectivity titres could likely show higher minimum inactivation efficacy. No CPE and no genomic viral load were detectable after 3 consecutive rounds of passaging on Vero E6 cells, pointing towards complete inactivation. The reported genomic viral load in the blood of symptomatic patients and asymptomatic donors is relatively low, often close to the limit of detection [11–13,29], pointing towards sufficient inactivation efficacy of amotosalen/UVA to prevent potential transfusion transmission (Table 2).

Recently, the inactivation of a different SARS-CoV-2 isolate (USA-WA1-2020) from the USA with Riboflavin/UVB in single-donor plasma units (>3·4 log PFU/ml) and platelets (>4·53 log PFU/ml) was reported [30]. Another study using Riboflavin/UVB technology with the same isolate reported an inactivation efficacy of $>4{\cdot}59 \pm 0{\cdot}15$ log/PFU in single-donor plasma units and $>3{\cdot}30 \pm 0{\cdot}26$

Table 1 Reduction of infectious SARS-CoV-2 titres in human plasma units after amotosalen/UVA treatment

	Viral infectivity titre, log_{10} PFU/ml				
Experiment	**Positive control**	**Negative control**	**Pretreatment sample***	**Inactivated sample**	**Log reduction**
A	5·5	ND	3·4	ND	>3·4
B	5·7	ND	3·2	ND	>3·2
C	5·6	ND	3·6	ND	>3·6
D	5·9	ND	3·3	ND	>3·3
E	5·3	ND	3·1	ND	>3·1
Mean ± SD	5·6 ± 0·20	ND	3·32 ± 0·19	ND	>3·32 ± 0·19[$]

ND indicates not detected.

*After addition of amotosalen.

[$]This indicates complete inactivation of the virus.

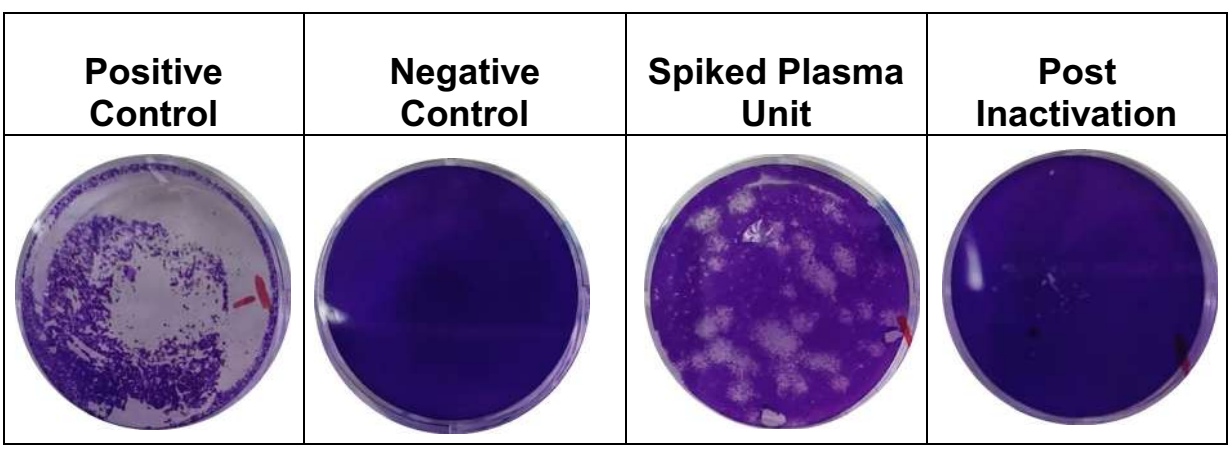

Figure 2 Inactivation of SARS-CoV-2 in plasma by amotosalen and UVA treatment assessed by a plaque assay. Vero E6 cells were inoculated for 1 h with the following samples in a 1:10 dilution in DMEM: the SARS-CoV-2 viral stock (Positive Control), human plasma (Negative Control), plasma from a SARS-CoV-2 spiked pretreatment sample (Spiked Plasma Unit) and amotosalen/UVA-treated, SARS-CoV-2 spikes plasma (Post-iInactivation). The cells were overlaid with agarose, incubated for three more days followed by neutral red staining. Experiments were conducted in serial dilutions. Photographs (4×) are shown from one of five representative experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 2 SARS-CoV-2 genomic load in plasma before and after amotosalen/UVA treatment*,†

Experiment	**Positive control**	**Negative control**	**Pretreatment sample**	**Inactivated sample**
A	5·99	ND	3·60	ND
B	5·88	ND	2·82	ND
C	5·53	ND	3·96	ND
D	5·36	ND	2·44	ND
E	5·18	ND	1·79	0·24
Mean ± SD	5·59 ± 0·34	ND	2·92 ± 0·87	0·04 ± 0·11

ND indicates not detected.

*Data are shown as log_{10} PEq/ml.

[†]Titres were determined from the same samples used in Table 1.

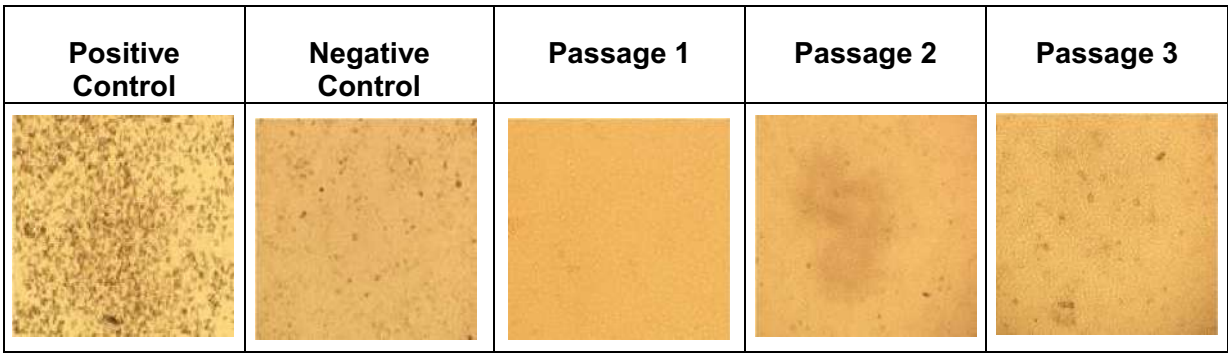

Figure 3 Assessment of complete inactivation of replicative SARS-CoV-2 post-amotosalen/UVA treatment by passaging experiments. Vero E6 cells were inoculated for 1 h with the following samples in a 1:10 dilution in DMEM: plasma from a SARS-CoV-2 spiked pretreatment sample (Positive Control), human plasma (Negative Control) and amotosalen/UVA-treated, SARS-CoV-2 spikes plasma (Passage 1–3) and passaged for three consecutive passages. Both the positive control and the pretreatment sample caused extensive CPE by day 3 post-inoculation in all three passages. Negative control and inactivated sample did not show any CPE in Vero E6 cells. Photographs (4×) are shown from one of five representative experiments on day 3 post-inoculation in each passage. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 3 Results of passaging experiments of SARS-CoV-2 in Vero E6 cells before and after inactivation of spiked plasma*,†

Experiment		Passage 1	Passage 2	Passage 3
A	Pretreatment sample	5·05	4·54	4·14
	Inactivated sample	ND	ND	ND
B	Pretreatment sample	5·12	4·61	4·31
	Inactivated sample	ND	ND	ND
C	Pretreatment sample	5·24	4·05	4·01
	Inactivated sample	ND	ND	ND
D	Pretreatment sample	4·96	4·01	3·95
	Inactivated sample	ND	ND	ND
E	Pretreatment sample	5·16	4·21	3·97
	Inactivated sample	ND	ND	ND

ND indicates not detected.

*Data are shown as $\log_{10}$ PEq/ml.

†Samples in Table 1 were used in this experiment. Samples were used at 1:10 dilution, and titre was determined on day 3 post-inoculation.

log PFU/ml in whole blood [31]. Both Riboflavin/UVB studies did not report passaging experiments or used sensitive NAT testing to confirm the findings of the plaque assay used in these studies.

The current study also supports the use of amotosalen/UVA for mitigating the risk of potential superinfection with SARS-CoV-2 through convalescent plasma.

The use of pathogen inactivation technology for CP also raises the question of the impact on product quality, hence the neutralizing activity. A study with Ebolavirus CP (EBOV CP) showed no significant impact on the neutralizing activity by amotosalen/UVA treatment [32]. A single case study showed a loss of 2–4% total IgG after amotosalen/UVA treatment of EBOV CP without assessing the consequences for the neutralizing activity [33]. Recently, no significant impact of amotosalen/UVA treatment on the neutralizing activity and neutralizing antibody quantity of COVID-19 convalescent plasma (CCP) was reported [34]. These reports point towards the preservation of neutralizing activity during amotosalen/UVA treatment.

Conclusion

In the present study, we showed efficient, complete inactivation of SARS-CoV-2 in large human plasma units by amotosalen/UVA using a local clinical isolate, which may serve as an additional layer of safety in plasma transfusion during the COVID-19 pandemic. These finding are in line with former findings of efficient inactivation of human coronaviruses in plasma by amotosalen/UVA pathogen inactivation [24,26].

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Conflict of interest

There is no conflict of interest identified.

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References

1 Busch MP, Bloch EM, Kleinman S: Prevention of transfusion-transmitted infections. *Blood* 2019; **133**:1854–64

2 Kleinman S: Pathogen inactivation: emerging indications. *Curr Opin Hematol* 2015; **22**:547–53

3 Glynn SA, Busch MP, Dodd RY, *et al.*: Emerging infectious agents and the nation's blood supply: responding to potential threats in the 21st century. *Transfusion* 2013; **53**:438–54

4 Zhu N, Zhang D, Wang W, *et al.*: A Novel Coronavirus from Patients with Pneumonia in China, 2019. *New England Journal of Medicine* 2020; **382**(8):727–33. http://dx.doi.org/10.1056/nejmoa2001017

5 Coronaviridae Study Group of the International Committee on Taxonomy of V: The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat Microbiol* 2020; **5**:536–44

6 WHO: WHO COVID-19 Dashboard. WHO, 2020. (Last accessed November 5 2020).

7 Lotfı M, Hamblin MR, Rezaei N: COVID-19: Transmission, prevention, and potential therapeutic opportunities. *Clin Chim Acta* 2020; **508**:254–66

8 Gao Z, Xu Y, Sun C, *et al.*: A systematic review of asymptomatic infections with COVID-19. *J Microbiol Immunol Infect* = Wei mian yu gan ran za zhi 2020: https://doi.org/10.1016/j.jmii.2020.05.001.

9 Kim GU, Kim MJ, Ra SH, *et al.*: Clinical characteristics of asymptomatic and symptomatic patients with mild COVID-19. *Clin microbiol infect* 2020; **26**:948.e1-e3.

10 Chang L, Yan Y, Wang L: Coronavirus Disease 2019: Coronaviruses and Blood Safety. *Transfus Med Rev* 2020; **34**(2):75–80.

11 Huang C, Wang Y, Li X, *et al.*: Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; **395**(10223):497–506.

12 Chen W, Lan Y, Yuan X, *et al.*: Detectable 2019-nCoV viral RNA in blood is a strong indicator for the further clinical severity. *Emerg Microbes Infect* 2020; **9**:469–73

13 Chang L, Zhao L, Gong H, *et al.*: Severe acute respiratory syndrome Coronavirus 2 RNA detected in blood donations. *Emerg Infect Dis* 2020; **26**(7):1631–3.

14 Casadevall A, Pirofski LA: The convalescent sera option for containing COVID-19. *J Clin Invest* 2020; **130**:1545–8

15 WHO: WHO Guidelines Approved by the Guidelines Review Committee; Blood Donor Counselling: Implementation Guidelines. Geneva, World Health Organization Copyright © World Health Organization 2014., 2014.

16 Epstein JS: Alternative strategies in assuring blood safety: An overview. *Biologicals* 2010; **38**:31–5

17 Epstein J, Burnouf T: Points to consider in the preparation and transfusion of COVID-19 convalescent plasma. *Vox Sang* 2020; **115**:485–7

18 Lanteri MC, Santa-Maria F, Laughhunn A, *et al.*: Inactivation of a broad spectrum of viruses and parasites by photochemical treatment of plasma and platelets using amotosalen and ultraviolet A light. *Transfusion* 2020; **60**:1319–31

19 Prowse CV: Component pathogen inactivation: a critical review. *Vox Sang* 2013; **104**:183–99

20 Mintz PD, Bass NM, Petz LD, *et al.*: Photochemically treated fresh frozen plasma for transfusion of patients with acquired coagulopathy of liver disease. *Blood* 2006; **107**:3753–60

21 Bost V, Chavarin P, Boussoulade F, *et al.*: Independent evaluation of tolerance of therapeutic plasma inactivated by amotosalen-HCl-UVA (Intercept) over a 5-year period of extensive delivery. *Vox Sang* 2015; **109**:414–6

22 Guignier C, Benamara A, Oriol P, *et al.*: Amotosalen-inactivated plasma is as equally well tolerated as quarantine plasma in patients undergoing large volume therapeutic plasma exchange. *Transfus Clin Biol* 2018; **25**:73–7

23 Castro G, Merkel PA, Giclas HE, *et al.*: Amotosalen/UVA treatment inactivates T cells more effectively than the recommended gamma dose for prevention of transfusion-associated graft-versus-host disease. *Transfusion* 2018; **58**:1506–15

24 Singh Y, Sawyer LS, Pinkoski LS, *et al.*: Photochemical treatment of plasma with amotosalen and long-wavelength ultraviolet light inactivates pathogens while retaining coagulation function. *Transfusion* 2006; **46**:1168–77

25 Pinna D, Sampson-Johannes A, Clementi M, *et al.*: Amotosalen photochemical inactivation of severe acute respiratory syndrome coronavirus in human platelet concentrates. *Transfus Med* 2005; **15**:269–76

26 Hindawi SI, Hashem AM, Damanhouri GA, *et al.*: Inactivation of Middle East respiratory syndrome-coronavirus in human plasma using amotosalen and ultraviolet A light. *Transfusion* 2018; **58**:52–9

27 Hashem AM, Hassan AM, Tolah AM, *et al.*: Amotosalen and ultraviolet A light efficiently inactivate MERS-coronavirus in human platelet concentrates. *Transfus Med* 2019; **29**:434–41

28 Domanovic D, Ushiro-Lumb I, Compernolle V, *et al.*: Pathogen reduction of blood components during outbreaks of infectious diseases in the European Union: an expert opinion from the European Centre for Disease Prevention and Control consultation meeting. *Blood Transfus* = Trasfusione del sangue 2019; **17**:433-48.

29 Young BE, Ong SWX, Kalimuddin S, *et al.*: Epidemiologic features and clinical course of patients infected with SARS-CoV-2 in Singapore. *JAMA* 2020; **323**(15):1488–94.

30 Keil SD, Ragan I, Yonemura S, *et al.*: Inactivation of severe acute respiratory syndrome coronavirus 2 in plasma and platelet products using a riboflavin and ultraviolet light-based photochemical treatment. *Vox Sanguinis* 2020; **115**(6):495–501. http://dx.doi.org/10.1111/vox.12937.

31 Ragan I, Hartson L, Pidcoke H, *et al.*: Pathogen reduction of SARS-CoV-2 virus in plasma and whole blood using riboflavin and UV light. *PLoS One* 2020; **15**:e0233947

32 Dean CL, Hooper JW, Dye JM, *et al.*: Characterization of Ebola convalescent plasma donor immune response and psoralen treated plasma in the United States. *Transfusion* 2020; **60**:1024–31

33 Geisen C, Kann G, Strecker T, *et al.*: Pathogen-reduced Ebola virus convalescent plasma: first steps towards standardization of manufacturing and quality control including assessment of Ebola-specific neutralizing antibodies. *Vox Sang* 2016; **110**:329–35

34 Tonn T, Corman VM, Johnsen M, *et al.*: Stability and neutralising capacity of SARS-CoV-2-specific antibodies in convalescent plasma. *The Lancet Microbe* 2020; **1**(2):e63. http://dx.doi.org/10.1016/s2666-5247(20)30037-9.

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A survey of laboratory biosafety and protective measures in blood transfusion departments during the COVID-19 pandemic

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Vox Sanguinis

Abstract

Background and objectives Thousands of healthcare workers (HCWs) have been infected with 2019 novel coronavirus pneumonia (COVID-19) during the COVID-19 pandemic. Laboratory personnel in blood transfusion departments may be infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) if laboratory biosafety protection is insufficient. Therefore, we investigated the current situation of laboratory biosafety protection in blood transfusion departments to determine how to improve the safety of laboratory processes.

Materials and methods An online survey was conducted in blood transfusion departments from 1st to 6th May 2020 in China. A total of 653 individuals completed the questionnaire. The questionnaire was designed with reference to COVID-19 laboratory biosafety summarized in Annex II. All responses were summarized using only descriptive statistics and expressed as frequencies and ratios [*n* (%)].

Results Most participants were concerned about COVID-19. Some participants had inadequate knowledge of COVID-19. Two participants stated that there were laboratory personnel infected with SARS-CoV-2 in their departments. A total of 31 (4.7%) participants did not receive any safety and security training. In terms of laboratory biosafety protection practices, the major challenges were suboptimal laboratory safety practices and insufficient laboratory conditions.

Conclusion The major deficiencies were insufficient security and safety training, and a lack of personal protective equipment, automatic cap removal centrifuges and biosafety cabinets. Consequently, we should enhance the security and safety training of laboratory personnel to improve their laboratory biosafety protection practices and ensure that laboratory conditions are sufficient to improve the safety of laboratory processes.

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Key words: laboratory biosafety protection, COVID-19, SARS-CoV-2, blood transfusion department.

Introduction

In December 2019, pneumonia of an unknown aetiology was reported in Wuhan, China [1]. The disease was subsequently named COVID-19 by the World Health Organization (WHO) [2]. On 11th March 2020, the WHO declared COVID-19 to be a pandemic as the highly infectious severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) rapidly spread worldwide [3].

Healthcare workers (HCWs) are essential to fight this pandemic. However, HCWs involved in the treatment of COVID-19 patients are at high risk of infection, including laboratory personnel in medical technology departments.

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Thousands of HCWs have been infected with SARS-CoV-2 during the pandemic, and some have even died [4–6]. As a type of medical technology department, blood transfusion departments in China perform pretransfusion testing, including pretransfusion compatibility testing and infectious disease screening. Pretransfusion compatibility testing can be performed with the traditional tube method or automated and semi-automated testing platforms that use column agglutination. Enzyme immunosorbent assays and chemiluminescent immunoassays are used to detect infectious diseases. Nucleic acid testing has also been performed in a few hospitals. Van Doremalen et al. reported that aerosol transmission of SARS-CoV-2 is plausible [7]. Moreover, there is growing evidence that aerosol transmission of SARS-CoV-2 is possible in confined settings with poor ventilation and upon long exposure to high concentrations of aerosols [8]. The WHO acknowledged that some medical procedures can produce very small droplets (called aerosolized droplet nuclei or aerosols) containing SARS-CoV-2 [9], which may pose an infectious risk to medical staff. Although most COVID-19 patients had detectable viral RNA in respiratory swabs, some studies have shown that a small proportion of them had detectable SARS-CoV-2 RNA in blood [10–11], which may aerosolize to generate infectious virus-containing droplets or particles. Hence, exposure to other personnel who may be asymptomatically infected with SARS-CoV-2, aerosols generated during specimen pretreatment (e.g. centrifugation) and nosocomial airborne transmission may be the main causes of COVID-19 infection among laboratory workers during outbreaks. In addition, various serological tests performed in blood transfusion laboratories, such as those that use test tubes and microcolumn gels, involve centrifugation, which increases the risk of producing aerosols containing viruses. Therefore, staff in blood transfusion laboratories are also at risk of COVID-19 infection.

According to a previous study [12], great uncertainty concerning biohazard risks related to SARS-CoV-2 and inadequate resources such as surgical masks, protective suits and laboratory equipment pose great challenges for processes in clinical chemistry laboratories. Therefore, we sought to identify the existing problems and propose areas for improvement by investigating the current situation of laboratory biosafety protection in blood transfusion departments. Specifically, we investigated attitudes to and knowledge of COVID-19; the rate of COVID-19 infection among laboratory staff; knowledge of laboratory biosafety protection; and laboratory biosafety protection practices in pretransfusion testing during the COVID-19 pandemic in Chinese blood transfusion departments. To the best of our knowledge, this is the first report concerning the rate of COVID-19 infection and its prevention in blood transfusion departments.

Materials and methods

Study population and participants

This study was conducted online by issuing electronic questionnaires. All participants were guaranteed anonymity and provided informed consent. During the study period (1st to 6th May 2020), 653 participants from blood transfusion departments in China completed the questionnaire.

Study questionnaire

The study questionnaire consisted of four sections. The first section included five questions concerning the demographic characteristics of participants, such as their gender, age, degrees, work experience and geographical distribution. The second section included five questions concerning participants' attitude to and knowledge of COVID-19, such as anxiety levels about COVID-19 and the transmission routes, symptoms, the causative pathogen, and COVID-19 treatments. The third section included six questions concerning the rate of COVID-19 infection and participants' knowledge of laboratory biosafety protection in blood transfusion departments. The fourth section included 12 questions concerning laboratory biosafety protection practices in pretransfusion testing. Detailed questionnaire information is provided in Annex I.

Ethical approval

This cross-sectional observational study was approved by the Ethics Committee of the Third Xiangya Hospital of Central South University (2020-S351).

Statistical analysis

All responses were summarized using only descriptive statistics and expressed as frequencies and ratios [*n* (%)].

Results

Demographic characteristics of the study participants

A total of 653 participants completed the questionnaire [235 males (35.99%) and 418 females (64.01%)]. The

study participants had various degrees, including college, bachelor, master and doctorate degrees. Participants were aged from 18 years to older than 60 years. A total of 538 (82.39%) participants had more than 5 years of work experience (Fig. 1). The study participants were from 34 provinces of China (Fig. 2).

Participants' attitude to and knowledge of COVID-19

Participants' attitude to and knowledge of COVID-19 are shown in Table 1. Most participants (66.9%) were concerned about COVID-19. In terms of the common transmission routes of SARS-CoV-2, 97.5% and 99.7% of participants knew that SARS-CoV-2 can be transmitted through close contact and respiratory transmission, respectively, but 292 (44.7%) participants believed that SARS-CoV-2 can be transmitted through blood transfusion. A substantial number of participants incorrectly answered questions about the pathogen of COVID-19. For example, 187 (28.6%) and 129 (19.8%) participants thought the pathogen responsible for COVID-19 is SARS-CoV and MERS-CoV, respectively. More than 80% of participants knew the manifestations of COVID-19, including fever, cough, fatigue and no symptoms. A total of 98% participants knew that COVID-19 is treated with symptomatic and supportive treatment.

The rate of COVID-19 infection and knowledge of laboratory biosafety protection in blood transfusion departments

The rate of COVID-19 infection and knowledge of laboratory biosafety protection in blood transfusion departments are shown in Table 2. A total of two (0.3%) participants stated that there were personnel infected with COVID-19 (nosocomial infection) in their departments. Meanwhile, 31 (4.7%) participants did not receive safety and security training about COVID-19 in their departments. In terms of risk assessment of laboratory procedures, nearly all participants knew the risks of SARS-CoV-2 transmission involving contagion, droplet transmission in specimen reception and aerosol transmission caused by removing caps, centrifugation and vigorous shaking. Laboratory biosafety protection mainly involves personal biosafety protection and laboratory space and facility protection. Most participants had adequate knowledge of personal biosafety protection levels. In terms of laboratory space and facility protection, nearly all participants undertook measures such as ventilating the laboratory, partitioning items and separating clean and dirty items. However, only 60–80% of participants undertook measures such as guaranteeing sufficient experimental supplies and instrument maintenance, suggesting that laboratory personnel should pay more attention to laboratory space and facility protection.

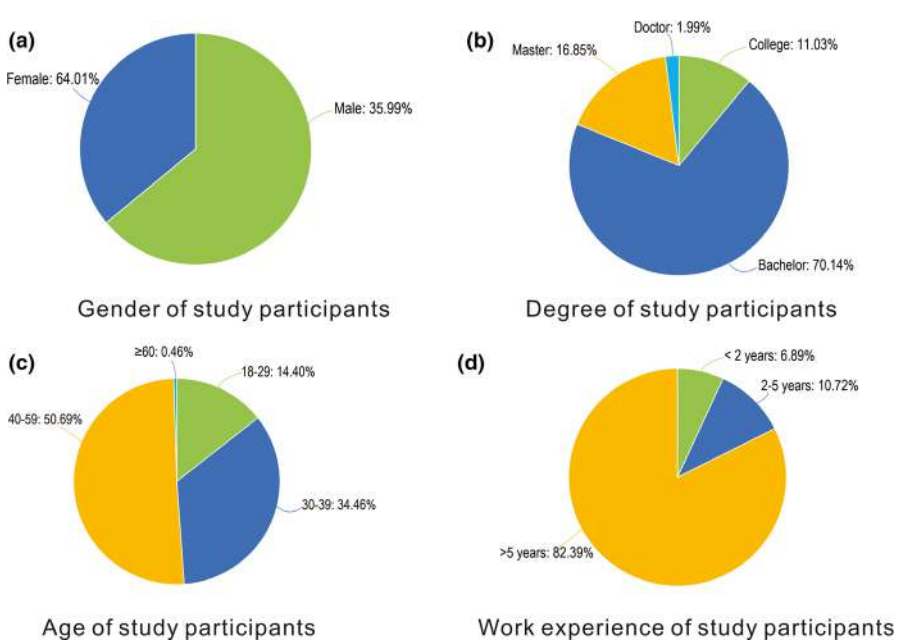

Fig. 1 Demographic characteristics of the study participants. (A) Gender, (B) degree, (C) age and (D) work experience of the study participants.

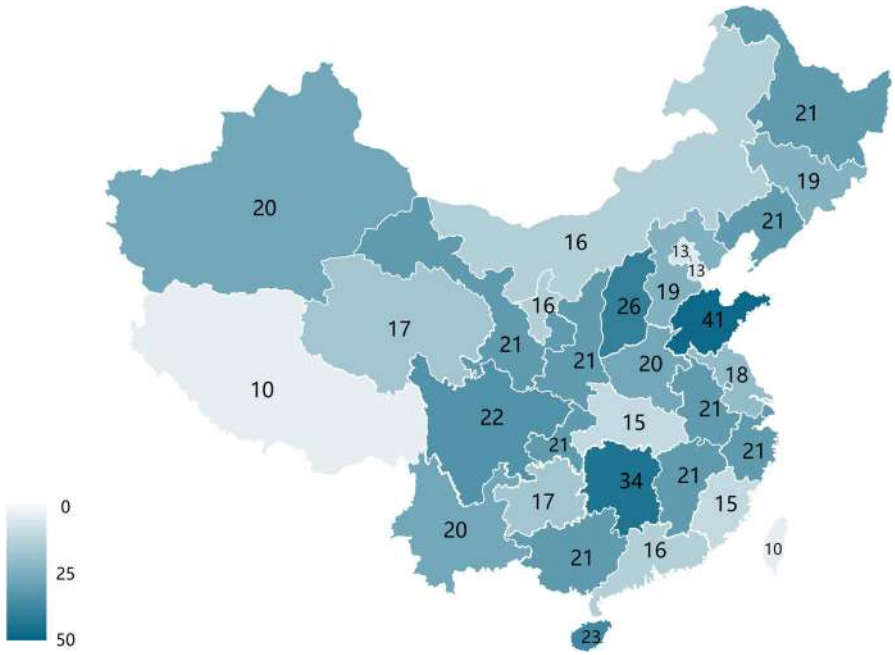

Fig. 2 The geographical distribution of the study participants.

Table 1 Participants' attitude to and knowledge of COVID-19.

Question	Response	Frequency, *n* (%)
Are you worried about COVID-19?	Very much	57 (8.7)
	A little	437 (66.9)
	Not at all	159 (24.3)
What is the transmission route of SARS-CoV-2?	Close contact	637 (97.5)
	Blood transfusion	292 (44.7)
	Respiratory transmission	651 (99.7)
What are the symptoms of COVID-19?	Fever	563 (86.2)
	Cough	553 (84.7)
	Fatigue	547 (83.8)
What is the pathogen of COVID-19?	SARS-CoV	187 (28.6)
	SARS-CoV-2	287 (44.0)
	MERS-CoV	129 (19.8)
What are the treatments for COVID-19?	Market vaccine	9 (1.4)
	Specific medicine	4 (0.6)
	Symptomatic and supportive treatment	640 (98.0)

Laboratory biosafety protection practices during pretransfusion testing

Laboratory biosafety protection practices during pretransfusion testing are shown in Table 3. A total of 523 (80.1%) participants manipulated COVID-19 patient specimens in a biosafety level 2 (BSL-2) laboratory. The pretransfusion testing procedure involves reception of the specimen, centrifugation and manipulation of the specimen, disposal of the specimen and waste and disinfection of the laboratory environment after testing. Attention must be paid to laboratory biosafety protection during each of these steps. During testing of COVID-19 patient specimens, only 225 (34.5%) participants used level 3 personal biosafety protection, and 430 (65.8%) participants stated that their departments lacked personal protective equipment (PPE). This indicates that some laboratory personnel did not use level 3 personal biosafety

Table 2 The rate of COVID-19 infection among laboratory staff and knowledge of laboratory biosafety protection in blood transfusion departments.

Question	Response	Frequency, *n* (%)
Is anyone in your department infected with COVID-19 (nosocomial infection)?	Yes	2 (0.3)
	No	651 (99.7)
Have you received safety and security training about COVID-19?	Yes	622 (95.3)
	No	31 (4.7)
What are the risks for SARS-CoV-2 transmission in laboratories?	Respiratory droplet transmission	628 (96.2)
	Close contact transmission	615 (94.2)
	Aerosol transmission	641 (98.2)
	Blood transfusion transmission	292 (44.7%)
Which situations pose a risk of COVID-19 infection?	Specimen reception	643 (98.5)
	Specimen centrifugation, removing caps or shaking	651 (99.7)
What are the levels of personal biosafety protection[a]?	Level 1 personal biosafety protection	530 (81.2)
	Level 2 personal biosafety protection	629 (96.3)
	Level 3 personal biosafety protection	562 (86.1)
What laboratory space and facility protection measures are there in your laboratory?	Laboratory ventilation	644 (98.6)
	Item partitioning	598 (91.6)
	Separating clean and dirty items	638 (97.7)
	Guaranteeing sufficient experimental supplies	509 (77.9)
	Ultraviolet radiation disinfection	638 (97.7)
	Maintenance of laboratory equipment	402 (61.6)

Level 1: a medical surgical mask, gloves, a laboratory coat, a medical protective cap and seven-step hand-washing.

Level 2: a medical surgical mask or N95 mask, gloves, an isolation gown, a medical protective cap and seven-step hand-washing.

Level 3: a medical surgical mask or N95 mask, gloves, a face shield, safety glasses, a protective suit, a medical protective cap and seven-step hand-washing.

[a]Levels of personal biosafety protection:

protection due to a lack of PPE including surgical masks, gloves and protective suits. During sample reception, most participants disinfected the surface of the plastic bag containing the specimen and application form, the inner layer of the plastic bag, the specimen tubes and the application form with 75% ethanol. Frequent hand hygiene is a personal protection measure that laboratory personnel should adhere to. A total of 608 (93.1%) participants performed seven-step hand-washing before entering and leaving the laboratory and on any occasion when contamination was known or suspected to be present on the hands; however, 45 (6.9%) participants did not strictly adhere to hand hygiene practices. Removal of the cap from the specimen tube is an aerosol-generating procedure, and automatic cap removal centrifugation can avoid aerosol generation. However, only 194 (29.7%) participants had an automatic cap removal centrifuge in their departments. Only 349 (53.4%) participants removed specimens more than 10 min after the centrifuge had stopped, and 45 (6.9%) participants removed specimens immediately. When abnormal sounds occurred during centrifugation, only 463 (71.0%) participants used personal level 3 biosafety protection and 573 (87.7%) participants removed specimens more than 30 min after the centrifuge had stopped. When faced with laboratory contamination (including broken or overturned specimen tubes) caused by blood specimens of patients with confirmed COVID-19, nearly all participants closed the laboratory and covered the contaminated area with 5500 mg/L effective chlorine disinfectant wipes for more than 30 min, but only 339 (51.9%) participants fumigated the laboratory with peroxyacetic acid following large spills. A total of 478 (73.2%) participants performed manual tests of specimens in a biosafety cabinet (BSC); however, 130 (19.9%) participants lacked a BSC in their departments. In addition, 45 (6.9%) participants did not perform manual tests of specimens in a BSC because they felt it was inconvenient. After all manipulations were completed, a few participants did not disinfect laboratory supplies, equipment, countertops or the space with appropriate disinfectants or ultraviolet radiation. Meanwhile, a few participants did not seal the specimen tube with a cap in a double yellow medical garbage bag or disinfect the surface of the garbage bag with 75% ethanol. Fortunately, all participants autoclaved the waste after the storage period.

Discussion

The COVID-19 pandemic has posed an unprecedented challenge for medical staff including laboratory personnel. According to a previous study, clinical laboratories

are facing major challenges including limited guidance and inadequate medical protection resources [12[pp1439–1440]]. Blood transfusion departments have performed pretransfusion testing during the COVID-19 pandemic. Personnel in these departments may be infected with SARS-CoV-2 due to frequent contact with potentially infected blood specimens and other personnel who are asymptomatically infected. Therefore, it is crucial to ensure that laboratory processes are safe and effective.

In the current study, 653 participants completed the questionnaire. The participants spanned a range of ages, had different degrees including college, bachelor, master and doctorate degrees, and had a variety of work experience. Additionally, the participants were from each of the 34 provinces in China, meaning that their responses to our questionnaire represent laboratory personnel in blood transfusion departments across China.

Participants' attitude to and knowledge of COVID-19 play an important role in establishing prevention awareness and promoting positive behaviours to a certain extent [13,14[pp186]]. More than half of participants were concerned about COVID-19. Nearly all participants knew the common transmission routes of SARS-CoV-2 [15,16]. However, 292 (44.7%) participants believed that SARS-CoV-2 can be transmitted through blood transfusion. Currently, there is no evidence to support this [17,18]. However, only 287 (44.0%) participants knew that the pathogen of COVID-19 is SARS-CoV-2 [19], and some thought that it is SARS-CoV or MERS-CoV. When we sought clarification from participants regarding this finding, a lack of knowledge regarding the causative pathogen of COVID-19 was confirmed. Therefore, knowledge of COVID-19 among laboratory personnel in blood transfusion departments in China must be improved via training and education.

According to interim guidance, titled 'Laboratory biosafety guidance related to the novel coronavirus (2019-nCoV)', issued by the WHO (https://www.who.int/docs/default-source/coronaviruse/laboratory-biosafety-novel-coronavirus-version-1-1.pdf?sfvrsn=912a9847_2), all personnel should receive safety and security training; all procedures should be performed based on risk assessment; and initial processing (before inactivation) of all specimens should be performed in a validated BSC. In our study, 4.7% of participants did not receive safety and security training about COVID-19 in their departments, including 23 (3.5%) individuals who replied that their hospitals did not provide training to all employees and eight (1.2%) individuals who were unable to attend training due to scheduling conflicts. This means that such training was inadequate in these departments and some laboratory personnel were unqualified to perform pretransfusion testing of COVID-19 patients, which is an obstacle to ensuring laboratory processes are safe. Nearly all participants knew the risks of SARS-CoV-2 transmission involving contagion, droplet transmission and aerosol transmission due to specimen reception, centrifugation, vigorous shaking and removal of caps [20,21]. Personal biosafety protection plays an important role in decreasing infection of HCWs with COVID-19 [22–24]. Most participants had adequate knowledge of laboratory biosafety protection levels. A safe laboratory environment is essential for laboratory personnel [20[pp581]]. However, some participants did not perform measures such as ventilating the laboratory, partitioning items, separating clean and dirty items, guaranteeing sufficient experimental supplies, disinfecting with ultraviolet radiation and maintaining laboratory equipment. This may be related to the safety and security training of participants and some participants' inadequate knowledge of COVID-19.

Patients can have suspected or confirmed COVID-19 or even be infected with SARS-CoV-2 but not display symptoms. Laboratory personnel may be infected with SARS-CoV-2 during laboratory processes involving specimen reception before testing, centrifugation and manipulation of specimens and disposal of specimens and waste. Therefore, laboratory biosafety protection measures are vital to avoid infection of laboratory personnel. According to recommendations of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Taskforce, COVID-19 specimens should be handled in a BSL-2 laboratory [21]. However, 130 (19.9%) participants did not perform pretransfusion testing in a BSL-2 laboratory, meaning that the laboratory conditions in some blood transfusion departments in China did not meet the requirements for COVID-19 testing. Due to the presence of asymptomatic or undiagnosed COVID-19 patients and the highly contagious nature of COVID-19, use of appropriate personal biosafety protection is universally recommended to avoid COVID-19 infection [25]. However, some participants did not perform proper disinfection during specimen reception, and only 225 (34.5%) participants used level 3 personal biosafety protection when testing specimens of COVID-19 patients. Additionally, 430 (65.8%) participants stated that their departments lacked PPE, suggesting that the reason why most participants did not use level 3 personal biosafety protection was a lack of PPE. The characteristics of SARS-CoV-2 transmission, especially aerosol transmission, play an important role in infection of laboratory personnel. Therefore, personnel should be cautious about generating aerosols during all laboratory manipulations such as centrifugation, testing, vigorous shaking and removal of caps. An automatic cap removal centrifuge avoids aerosol generation during

Table 3 Laboratory biosafety protection practices during pretransfusion testing.

Question	Response	Frequency, *n* (%)
Do you perform all manipulations of COVID-19 patient specimens in a BSL-2 or higher-level laboratory?	Yes	523 (80.1)
	No	130 (19.9)
Which of the following personal biosafety protection levels do you use when testing blood specimens of COVID-19 patients?	Level 1 personal biosafety protection	89 (13.6)
	Level 2 personal biosafety protection	339 (51.9)
	Level 3 personal biosafety protection	225 (34.5)
Does your department lack PPE?	Yes	430 (65.8)
	No	223 (34.2)
Which of the following do you perform during specimen reception?	Disinfect the surface of the plastic bag containing the specimens and application form with 75% ethanol	603 (92.3)
	Disinfect the inner layer of the plastic bag, specimen tubes and the application form	568 (87.0)
When do you perform seven-step hand-washing?	Before entering and leaving the laboratory, after handling any specimen and on any occasion when contamination is known or suspected to be present on the hands	608 (93.1)
	Only after handling any specimen	9 (1.4)
	I do not perform seven-step hand-washing	36 (5.5)
Is there an automatic cap removal centrifuge in your department?	Yes	194 (29.7)
	No	459 (70.3)
How long do you wait to remove the specimen after the centrifuge stops?	0 min	45 (6.9)
	5–10 min	259 (39.7)
	More than 10 min	349 (53.4)
How do you respond when abnormal sounds occur during centrifugation?	Use personal level 3 biosafety protection	463 (71.0)
	Remove the specimen more than 30 min after the centrifuge has stopped	573 (87.7)
How do you handle laboratory contamination (including broken or overturned specimen tubes) caused by blood specimens of patients with confirmed COVID-19?	Close the laboratory and cover the contaminated area with 5500 mg/L effective chlorine disinfectant wipes for more than 30 min	638 (97.7)
	Fumigate the laboratory with peroxyacetic acid following large spills	339 (51.9)
Do you perform manual tests in a BSC?	Yes	478 (73.2)
	No, the department lacks a BSC	130 (19.9)
	No, the manipulation is inconvenient in a BSC	45 (6.9)
What disinfection do you perform after testing?	Disinfect laboratory instruments and supplies with 75% ethanol	553 (84.7)
	Wipe laboratory countertops, the floor and equipment with 75% ethanol or 500 mg/L effective chlorine disinfectant	618 (94.6)
	Disinfect the laboratory space with ultraviolet lamps for more than 30 min	613 (93.9)
	Seal specimens inside double yellow medical garbage bags	588 (90.0)
How do you dispose specimens after testing?	Disinfect the surface of the above bags with 75% ethanol	533 (81.6)
	Autoclave waste after storage	578 (88.5)

BSC, biosafety cabinet; PPE, personal protective equipment (a surgical mask, gloves, a protective suit, safety glasses, a face shield, etc.).

removal of specimen caps. However, only 194 (29.7%) participants had an automatic cap removal centrifuge in their departments, meaning that insufficient laboratory equipment is a challenge for ensuring laboratory procedures are safe. According to the recommendation of the Chinese Society of Laboratory Medicine (http://www.cslm.org.cn/cn/news.asp?id=73.html), laboratory personnel should remove specimens more than 10 min after the centrifuge has stopped, and, if abnormal sounds occur during centrifugation, laboratory personnel should use level 3 personal biosafety protection and remove specimens more than 30 min after the centrifuge has stopped. Nevertheless, only 349 (53.4%) participants removed specimens more than 10 min after the centrifuge had stopped, and 463 (71.0%) participants used level 3 personal biosafety protection, and 573 (87.7%) participants removed specimens more than 30 min after the centrifuge had stopped when abnormal sounds occurred during centrifugation. According to the recommendation of the WHO, COVID-19 specimens must be handled in a BSC using standard precautions, especially during manual specimen tests. However, 130 (19.9%) participants lacked a BSC in their departments, and 45 (6.9%) participants did not perform manual specimen tests in a BSC because it was inconvenient, indicating that laboratory facilities are insufficient and laboratory personnel do not have adequate laboratory biosafety knowledge. After all manipulations were completed, a few participants did not disinfect the laboratory, indicating that they did not pay attention to the specimen disposal procedure after testing. Hand hygiene is vital in laboratory biosafety protection. Furthermore, strict adherence of laboratory staff to hand hygiene measures is required to prevent inadvertent transmission of pathogens to themselves or others [20,23]. However, 6.9% of participants did not strictly adhere to seven-step handwashing. In conclusion, laboratory biosafety protection practices of some laboratory personnel in Chinese blood transfusion departments were poor, which may have been due to their inadequate laboratory biosafety knowledge and insufficient laboratory facilities such as a BSC, an automatic cap removal centrifuge and PPE. Moreover, two (0.3%) participants stated that there were personnel infected with COVID-19 in their departments. We contacted these two participants to investigate the reasons why these personnel were infected. One participant stated that the infected personnel did not use level 3 personal protection during testing of COVID-19 specimens due to a lack of PPE and did not receive security and safety training about COVID-19. The other participant stated that the infected personnel did not perform manual tests of specimens in a BSC.

Limitations

This is the first study to focus on the rate of COVID-19 infection and its control in blood transfusion departments. There are several limitations of our study. First, the limited number of questions does not reflect the imprecise current situation of laboratory biosafety protection. Second, the responses provided likely reflect a snapshot of national practices at the time of survey and may change over time. Hospital blood transfusion departments in China order blood components from blood centres and distribute them to recipients when necessary. Blood processing, including preparation and modification of components, is performed in blood centres and is prohibited in hospital blood transfusion departments. Therefore, the risks associated with blood products are beyond the scope of this study.

Conclusion

In this study, some participants thought that SARS-CoV-2 can be transmitted through blood transfusion and did not know the pathogen of COVID-19, meaning that knowledge of COVID-19 among laboratory personnel must be improved via training and education. Some personnel did not have adequate knowledge of laboratory biosafety protection and were unqualified to work in a laboratory during the COVID-19 pandemic because they did not receive security and safety training. During pretransfusion testing of COVID-19 patients, some participants did not strictly perform laboratory biosafety protection. This was because some laboratory personnel were unqualified and laboratory facilities such as a BSC, an automatic cap removal centrifuge and PPE were lacking. Meanwhile, two participants stated that there were personnel infected with COVID-19 in their departments. This may have been due to insufficient laboratory biosafety protection. Consequently, on the one hand, we should enhance security and safety training of laboratory personnel to improve their laboratory biosafety protection knowledge and practices. On the other hand, we should ensure that there is sufficient equipment such as a BSC, an automatic cap removal centrifuge and PPE in blood transfusion departments to guarantee that laboratory procedures are safe.

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Conflict of interest

The authors declare that they have no conflicts of interest relevant to the manuscript.

Author contributions

Haiting Liu, Leping Liu, Rong Gui and Rong Huang were involved in questionnaire design. Leping Liu, Xueling Shang, Sai Chen, Xisheng Li and Junhua Zhang were involved in sample processing, data acquisition and analysis and manuscript writing. Haiting Liu wrote the manuscript. Rong Gui and Rong Huang edited the manuscript.

References

1 Zhang YZ, Chen N, Zhou M, *et al.*: Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Nature* 2020; **395**:507–13

2 Lai CC, Shih TP, Ko WC, *et al.*: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and coronavirus disease-2019 (COVID-19): The epidemic and the challenges. *Radiology* 2020; **55**:105924

3 Muniyappa R, Gubbi S: COVID-19 pandemic, coronaviruses, and diabetes mellitus. *Am J Physiol Endocrinol Metab* 2020; **318**:E736-e41

4 Jin YH, Huang Q, Wang YY, *et al.*: Perceived infection transmission routes, infection control practices, psychosocial changes, and management of COVID-19 infected healthcare workers in a tertiary acute care hospital in Wuhan: a cross-sectional survey. *Mil Med Res* 2020; **7**:24

5 Huang L, Lin G, Tang L, *et al.*: Special attention to nurses' protection during the COVID-19 epidemic. *Crit Care* 2020; **24**:120

6 Chu J, Yang N, Wei Y, *et al.*: Clinical characteristics of 54 medical staff with COVID-19: A retrospective study in a single center in Wuhan, China. *J Med Virol* 2020; **92**:807–813

7 van Doremalen N, Bushmaker T, Morris DH, *et al.*: Aerosol and surface stability of HCoV-19 (SARS-CoV-2) compared to SARS-CoV-1. *N Engl J Med* 2020; **382**:1564–1567

8 Asadi S, Bouvier N, Wexler AS, *et al.* The coronavirus pandemic and aerosols: Does COVID-19 transmit via expiratory particles? *Aerosol Sci Technol* 2020; 1–4

9 https://www.who.int/zh/emergencies/diseases/novel-coronavirus-2019/question-and-answers-hub/q-a-detail/q-a-how-is-covid-19-transmitted.

10 Kiely P, Hoad VC, Seed CR, *et al.*: Severe acute respiratory syndrome coronavirus-2: implications for blood safety and sufficiency. *Vox Sang* 2020. https://doi.org/10.1111/vox.13009.

11 **Peng** L, **Liu** J, **Xu** W, *et al.*: SARS-CoV-2 can be detected in urine, blood, anal swabs, and oropharyngeal swabs specimens. *J Med Virol* 2020; **92**(9):1676–1680. https://doi.org/10.1002/jmv.25936

12 Loh TP, Horvath AR, Wang CB, *et al.*: Laboratory practices to mitigate biohazard risks during the COVID-19 outbreak: an IFCC global survey. *Clin Chem Lab Med* 2020; **58**(9):1433–1440. https://doi.org/10.1515/cclm-2020-0711

13 Pambuccian SE: The COVID-19 pandemic: implications for the cytology laboratory. *J Am Soc Cytopathol* 2020; **9**:202–11

14 Zhang M, Zhou M, Tang F, *et al.*: Knowledge, attitude, and practice regarding COVID-19 among healthcare workers in Henan. *China. J Hosp Infect* 2020; **105**:183–7

15 Guo YR, Cao QD, Hong ZS, *et al.*: The origin, transmission and clinical therapies on coronavirus disease 2019 (COVID-19) outbreak - an update on the status. *Mil Med Res* 2020; **7**:11

16 Shereen MA, Khan S, Kazmi A, *et al.*: COVID-19 infection: Origin, transmission, and characteristics of human coronaviruses. *J Adv Res* 2020; **24**:91–8

17 Chang L, Yan Y, Wang L: Coronavirus disease 2019: coronaviruses and blood safety. *Transfus Med Rev* 2020; **34**:75–80

18 Cho HJ, Koo JW, Roh SK, *et al.*: COVID-19 transmission and blood transfusion: A case report. *J Infect Public Health* 2020; **13**(11):1678–1679. https://doi.org/10.1016/j.jiph.2020.05.001

19 Gorbalenya AE, Baker SC, Baric RS, *et al.*: The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat Microbiol* 2020; **5**:536–544

20 Ağalar C, Öztürk Engin D: Protective measures for COVID-19 for healthcare providers and laboratory personnel. *Turk J Med Sci* 2020; **50**:578–84

21 Lippi G, Adeli K, Ferrari M, *et al.*: Biosafety measures for preventing infection from COVID-19 in clinical laboratories: IFCC Taskforce Recommendations. *Clin Chem Lab Med* 2020; **58**:1053–62

22 Zhang Z, Liu S, Xiang M, *et al.*: Protecting healthcare personnel from 2019-nCoV infection risks: lessons and suggestions. *Front Med* 2020; **14**:229–31

23 Ferioli M, Cisternino C, Leo V, *et al.*: Protecting healthcare workers from SARS-CoV-2 infection: practical indications. *Eur Respir Rev* 2020; **29**:200068

24 Mourya DT, Sapkal G, Yadav PD, *et al.*: Biorisk assessment for infrastructure & biosafety requirements for the laboratories providing coronavirus SARS-CoV-2/(COVID-19) diagnosis. *Indian J Med Res* 2020; **151**:172–6

25 Hellewell J, Abbott S, Gimma A, *et al.*: Feasibility of controlling COVID-19 outbreaks by isolation of cases and contacts. *Lancet Glob Health* 2020; **8**: e488–e496

Supporting Information

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

Annex I The detailed questionnaire information
Annex II Recommendations of COVID-19 laboratory biosafety

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Establishment of transfusion-relevant bacteria rzeference strains for red blood cells

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Abstract

Background and objectives Red blood cell concentrates (RBCC) are susceptible to bacterial contamination despite cold storage. A reliable evaluation of strategies to minimize the risk of RBCC-associated bacterial transmission requires the use of suitable reference bacteria. Already existing Transfusion-Relevant Bacteria Reference Strains (TRBRS) for platelet concentrates fail to grow in RBCC. Consequently, the ISBT TTID, Working Party, Bacterial Subgroup, conducted an international study on TRBRS for RBCC.

Materials and methods Six bacterial strains (*Listeria monocytogenes* PEI-A-199, *Serratia liquefaciens* PEI-A-184, *Serratia marcescens* PEI-B-P-56, *Pseudomonas fluorescens* PEI-B-P-77, *Yersinia enterocolitica* PEI-A-105, *Yersinia enterocolitica* PEI-A-176) were distributed to 15 laboratories worldwide for enumeration, identification, and determination of growth kinetics in RBCC at days 7, 14, 21, 28, 35 and 42 of storage after low-count spiking (10–25 CFU/RBCC).

Results Bacterial proliferation in RBCC was obtained for most strains, except for *S. marcescens*, which grew only at 4 of 15 laboratories. *S. liquefaciens*, *S. marcescens*, *P. fluorescens* and the two *Y. enterocolitica* strains reached the stationary phase between days 14 and 21 of RBCC storage with a bacterial

concentration of approximately 10^9 CFU/ml. *L. monocytogenes* displayed slower growth kinetics reaching 10^6–10^7 CFU/ml after 42 days.

Conclusion The results illustrate the importance of conducting comprehensive studies to establish well-characterized reference strains, which can be a tool to assess strategies and methods used to ameliorate blood safety. The WHO Expert Committee on Biological Standardization adopted the five successful strains as official RBCC reference strains. Our study also highlights the relevance of visual inspection to interdict contaminated RBC units.

Key words: bacteria, contamination, red blood cells, sepsis, blood safety, reference material, validation.

Introduction

The risk of septic reactions from transfusion of blood components has greatly diminished over the last decade due to the enhancement in the donor deferral criteria, improved donor skin disinfection, diversion of the initial aliquot of donated blood and enhanced blood component processing [1,2]. These measures were primarily introduced for platelet concentrates (PC) as they are stored at 20–24°C, which is optimal for platelet viability and function, but also allows many bacterial species to grow. Compared to PC, the rate of bacterially-contaminated red blood cell concentrates (RBCC) is significantly lower with approximately 1 per 2 million transfused RBCC [3]. This is mainly attributable to the refrigerated storage of RBCCs impeding the growth of the majority of transfusion-associated bacterial species.

The reduced risk of bacterial transmission from RBCC units is partly offset by a more frequent transfusion of these type of units to treat patients. Consequently, several cases of sepsis following RBCC transfusion have been reported in the past few years. Funk et al. reported four fatalities between 1997 and 2010 caused by transfusion of bacterially-contaminated RBCC in Germany [4]. The causative bacteria were *Staphylococcus aureus*, *Serratia marcescens,* and *Yersinia enterocolitica*. From 2014 to 2018, the FDA reported 4 fatalities caused by RBCC contaminated with *Pseudomonas fluorescens, Pseudomonas veronii, Enterococcus faecium* and *Anaplasma phagocytophilum* [5]. Similarly, there was one case of a fatal transfusion reaction involving RBCC contaminated with *Pseudomonas koreensis* documented in the SHOT report of 2009 [6]. *Klebsiella pneumoniae* has also been implicated in septic transfusion events involving contaminated RBCC [7–9]. More recently, Frati and colleagues published a case report of a transfusion-transmitted septic reaction with a fatal outcome in Italy caused by a RBC unit contaminated with *Yersinia enterocolitica* [10].

Identification and categorisation of the most prevalent RBC associated contaminants reveal the presence of mainly psychrotrophic bacteria that are able to proliferate at low temperatures (1°C–6°C). Hence, compared to PC, the spectrum of transfusion-relevant bacteria for RBCC is more limited and covers different species. As a consequence, the established transfusion-relevant bacterial reference strains (TRBRS) panel for PC [11,12] cannot be applied arbitrarily to cold-stored RBCC.

In this study, we present the establishment of a panel of defined, quantified, and functionally characterized bacterial strains that have proven their growth ability in RBCC under cold storage temperatures.

Materials and methods

Study partners

The study was coordinated by the Paul-Ehrlich-Institut (PEI) in collaboration with 15 centres in 10 different countries under the auspices of the International Society for Blood Transfusion, Transfusion-Transmitted Infectious Diseases Working Party (ISBT TTID WP) Bacterial Subgroup (Table S1). Frozen bacterial stocks were sent to the study partners on dry ice. The following information was provided by study participants: (a) the number of viable cells of the bacterial stock solutions, (b) the inoculum per RBC unit, (c) the number of colony forming units (CFUs) over time for each RBC unit, (d) identification of bacteria from each RBC unit at the end of the study, and (e) a completed questionnaire with additional information including details of RBCC manufacturing. Sites used RBC units according to local practice. Ethical Committee approval for the research use of clinical RBC units was obtained at those centres where approval was required.

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Bacterial strains

In the course of the selection of suitable bacterial strains, 32 isolates previously related to RBC contamination were collected from laboratories worldwide. All strains were pre-tested for their growth properties in cold-stored RBCC via low-count inoculation [13]. Strains that consistently grew in RBCC were designated as candidate strains and included following: *Listeria monocytogenes* PEI-A-199, *Serratia liquefaciens* PEI-A-184, *Yersinia enterocolitica* PEI-A-105 and *Yersinia enterocolitica* PEI-A-176. In addition, the candidate panel contained *Serratia marcescens* PEI-B-P-56 and *Pseudomonas fluorescens* PEI-B-P-77 from the PC WHO repository [11]. For the preparation of the frozen batches, bacteria were cultivated in CASO medium (Merck, Darmstadt, Germany) and harvested in the logarithmic growth phase to ensure optimal viability. Aseptically, cells were mixed 1:2 with 20% albumin (Biotest Pharma GmbH, Dreieich, Germany) as a cryoprotectant, dispensed into cryotube vials and then frozen immediately at −80°C. Colony counts of samples were determined before and after the freezing stage to determine the impact of freezing on bacterial viability. In addition, the within-batch variation of the CFU/ml was analysed by CFU counting of vials filled at the beginning, middle and end of production. Stability testing was performed after the production of the frozen bacterial suspensions and within the following period in an interval of several months. For this purpose, six vials of each strain stored at −80°C were defrosted and two independent dilution series of each vial were prepared. Samples of a predefined dilution of each dilution series were plated and the mean colony count/ml was determined the following day after incubation under appropriate conditions for each bacterial strain.

Study protocol

In order to prove the suitability of the selected strains, a protocol similar to one used to characterize TRBRS for PC [11,12] was used with a few modifications (Fig. 1). Three RBC units, not older than day seven after blood collection, were artificially contaminated with approximately 10–25 CFU/unit. Prior to RBCC spiking, the baseline sterility of the units was tested according to the routine standard operating procedures used in each participating laboratory. Bacterial stocks were thawed and serially diluted with sterile saline to achieve the desired concentration. Inocula were confirmed by plating of 100 µl of the last three dilutions onto agar plates in triplicate, and colonies were counted the following day after incubation under appropriate conditions for each bacterial strain. After inoculation, RBCC were stored under standard RBC storage conditions (no agitation at 2–6°C in Europe or 1–6°C in North America). Aseptic sampling was performed on days 7, 14, 21, 28, 35, and 42 of RBC storage from the 3 RBC units inoculated with each bacterial strain. The RBC samples were serially diluted with sterile 0·85% saline solution and samples from each dilution were plated onto agar plates in triplicate. The colonies were counted after overnight incubation and species identity was confirmed at day 42 by the standard identification methods routinely used in each participant laboratory. This included classical biochemical reactions (7 labs), MALDI-TOF mass spectrometry (7 labs), 16S rRNA gene sequencing (3 labs) and fatty acid methyl ester analysis (1 lab).

Statistical methods

Statistical analysis was performed on the data submitted by the participants transformed to $\log_{10}$ CFU/ml; zero CFU/ml were set to 0·01 before log transformation. Based on the data submitted with an inoculation of 1–215 CFU/RBCC and RBC volumes of 187–419 ml, the starting inoculum per millilitre of RBCC ranged from 0·002 to 1·15 CFU/ml (i.e. - 2·7 to 0·06 $\log_{10}$ CFU/ml). Growth data were analysed per strain on each sampling day. Overall mean for each strain was estimated per strain by means of a mixed linear model with $\log_{10}$ CFU as the dependent variable and including participant as random factor. The statistical analysis was performed with SAS®/STAT software, version 9.4, SAS System for Windows.

Results

Verification of bacterial stock concentration

Prior to the shipment of the bacteria to the study partners, batches of the respective strains were manufactured and submitted to QC testing. The impact of the freezing procedure on the viability of the strains was generally low. When comparing the CFU before and after freezing, the most significant difference was observed for *S. marcescens* with a reduction of approximately $0{\cdot}4 \times 10^7$ CFU/ml. Based on the post-freeze concentrations, dilution schemes were prepared for the study protocol to achieve an inoculum of 10–25 CFU/RBC unit. With respect to the within-batch CFU variation, the bacterial distribution in the vials remained homogenous during the filling procedure. Following the batch production, long-term viability of the bacteria was assessed during the ongoing study. The viability of frozen stocks of the different bacteria remained highly stable, with a maximum variation of $\pm 0{\cdot}2 \times 10^6$ CFU/ml over a two year period.

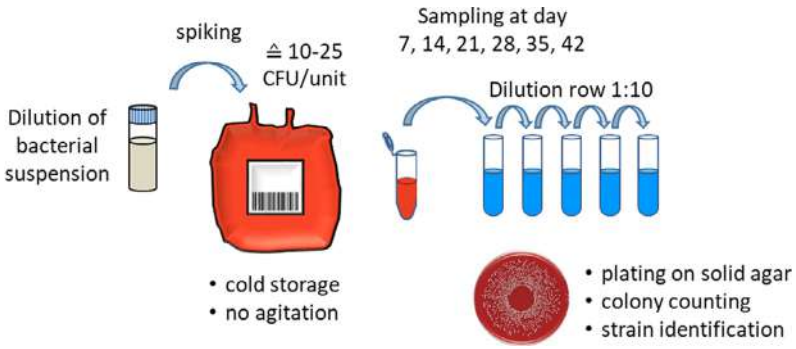

Fig. 1 Study overview. Bacterial stock suspension were diluted and each RBC unit was inoculated with 10–25 bacteria per bag. Sampling was performed at a weekly interval and the number of bacteria was determined by colony counting.

Results received from the participant laboratories showed that shipment and storage of the strains had no significant impact on the bacterial count, which were predominately consistent with the PEI results (Fig. 2). The most significant variations were observed for the two *Y. enterocolitica* strains PEI-A-176 and PEI-A-105, where three and five of the participants reported inocula outside of the specified min./max.-limits of ±0·5 log CFU/ml, respectively. For PEI-A-105, all three outliers were more than 1 log CFU/ml below the expected range. The variation was less significant for PEI-A-176, where all outliers were at or within a range of ±1 log CFU/ml of the mean concentration. The inoculum of the other strains met the specifications with only very few outliers (see Fig. 2). Each participant performed the low-count spiking of RBC units in order to mimic a low-level contamination scenario aiming at an initial inoculum of approximately 10–25 CFU per RBC unit. The average reported inoculum, as well as the final concentration, were 20 CFU/unit or 0·07 CFU/ml for *S. liquefaciens*, 19 CFU/unit or 0·06 CFU/ml for *S. marcescens*, 15 CFU/unit or 0·05 CFU/ml for *P. fluorescens*, 31 CFU/unit or 0·1 CFU/ml for *L. monocytogenes*, and 15 CFU/unit or 0·05 CFU/ml for both *Y. enterocolitica* strains.

Bacterial growth in cold-stored RBCC

The graphical analysis of the individual growth behaviour is summarized in Fig. 3. A similar growth pattern was demonstrated for *S. liquefaciens*, *P. fluorescens* and the two *Y. enterocolitica* strains, which all grew rapidly under RBC storage conditions. After 21 days, all four strains had reached concentrations that were greater than 10^8 CFU/ml. Growing much slower than the previously mentioned organisms, *L. monocytogenes* only reached a concentration of approximately 10^2 CFU/ml after 21 days of RBCC storage. This isolate generally demonstrated a slow and steady growth pattern throughout storage and did eventually reach a concentration of 10^7 CFU/ml on day 42. In summary, robust and reliable growth was observed in 100% of the units for *Y. enterocolitica* PEI-A-176, 98% for both *P. fluorescens* and *S. liquefaciens*, 96% for *Y. enterocolitica* PEI-A-105, and 90% for *L. monocytogenes*. A total of 48 inoculated RBC units were tested per strain.

S. marcescens showed the greatest amounts of growth variability among the different laboratories. At four of the participating laboratories, including at the PEI, this organism grew in all three inoculated RBC units. Three other sites reported an occasional failure of this organism to grow in 1–2 of the three inoculated units after 42 days of incubation. However, at nine participating sites, no colonies were ever observed on any of the plates throughout the testing period. Laboratories who performed final sterility testing by inoculation of samples into BacT/Alert media bottles reported negative results, indicating that *S. marcescens* did not persist but died over time.

Based on the individual growth kinetics, the generation time calculated for each strain for the linear range on the semi-log plots. A comprehensive growth analysis of the strains showing an exponential growth behaviour of the bacteria in RBC is the basis for the respective calculations [13]. Exponential growth was observed between day 7 and day 14 for strains *P. fluorescens*, *S. liquefaciens*, *Y. enterocolitica* 105, *Y. enterocolitica* 176 and between day 7 and day 28 for *L. monocytogenes*. The fastest growth rate was determined for the two *Y. enterocolitica* strains with a generation time of 9·24 h for strain PEI-A-176, 9·38 h for PEI-A-195 followed by *S. liquefaciens* with 10·24 h, and *P. fluorescens* with 12·27 h per generation. The slowest growth parameters were calculated for *L. monocytogenes* with a generation time of 26·91 h.

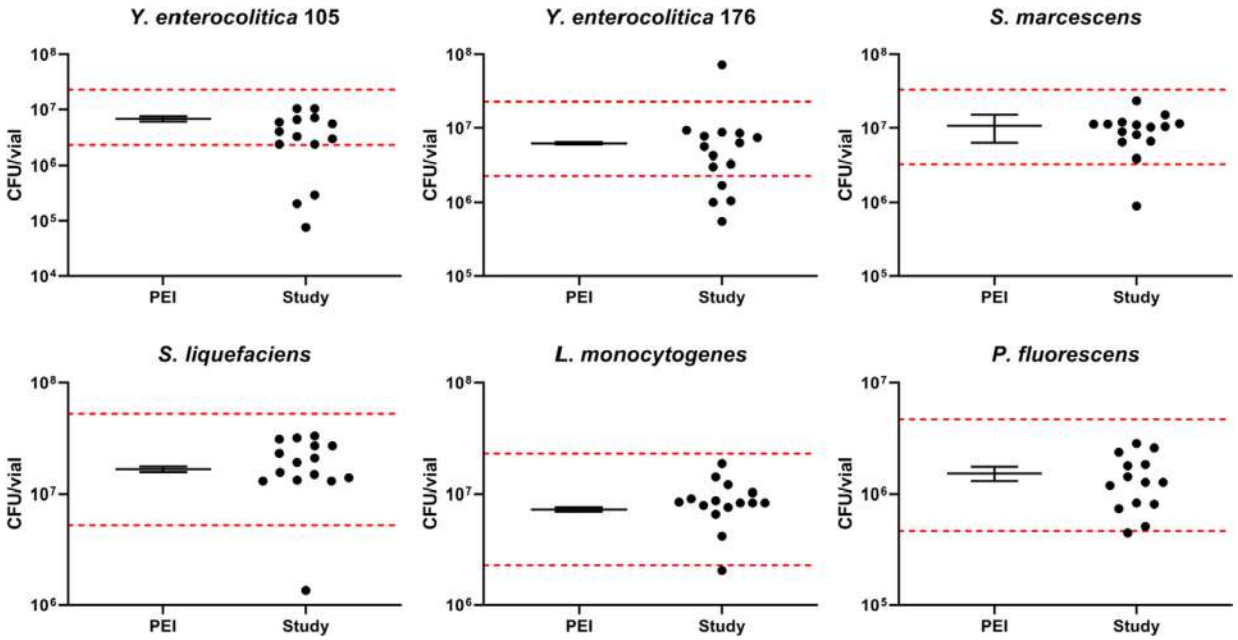

Fig. 2 Match of inoculum. Recalculated values of the bacterial stock suspensions are compared with the pre-determined PEI values. PEI results are presented as mean values with 95% CI. Red dashed lines show ±0·5 log of the mean value. [Colour figure can be viewed at wileyonlinelibrary.com]

Bacteria-dependent colour change of RBCC and species identification

During the storage period of the contaminated RBC units, the colour of the red cells changed considerably upon bacterial growth (Fig. 4). For *S. liquefaciens*, bacterial loads of more than 10^7 CFU/ml led to a colour change from red to black by day 21. Similar findings were seen for *P. fluorescens* and the two *Y. enterocolitica* strains, although the latter showed a delayed colour change between days 21 and 28. RBC units contaminated with *L. monocytogenes* showed no discoloration even after storage for 42 days and a bacterial concentration of more than 10^6 CFU/ml.

The strain identification after 42 days following routine local procedures generally corresponded with the results provided by PEI. Minor discrepancies in the species identification were reported for *P. fluorescens*, which was also identified as *P. synxantha, P. veronii, P. cedrina*. One site identified *S. liquefaciens* as *S. proteamaculans*. The two *Y. enterocolitica* strains were two times identified as member of the *Y. enterocolitica* group and one site reported an inconclusive identification as *Y. enterocolitica/frederiksenii*.

Discussion

Despite the cold storage of RBCC, bacterial contamination caused by mainly psychrotrophic organisms can lead to severe and even fatal transfusion reactions [10,14]. In contrast to PC, RBCC are not routinely screened for bacteria, thus, leaving potential contaminations undetected. In order to reduce this residual risk, either bacterial culture screening or pathogen inactivation of RBCC, which is currently under development [15], can be considered. However, prior to a widespread implementation the efficiency of these systems must be properly validated. A prerequisite for this is the use of qualified bacterial strains with known and defined characteristics. Here we established and characterized a novel bacterial reference panel specifically developed and validated for growth in RBCC.

Due to different storage conditions of RBCC, the already existing TRBRS for PC are not suitable for studies in RBCC. The selected bacterial strains tested in this study have proven, with one exception, their suitability for robust growth in RBCC, independent of external factors. The growth success in 90–100% of RBC units is comparable with results of the two previous studies for establishment of PC-relevant reference strains with rates between 70 and 100% [11]. Even though *S. marcescens* is part of the WHO PC TRBRS panel, it failed to grow in RBC units in nine out of 13 laboratories. In contrast, four sites reported successful growth in the respective RBCC.

In order to mimic a low-level RBC contamination scenario, all participants performed spiking of RBC units with circa 25 CFU per bag. A prerequisite for the low-count inoculation was the correct CFU count of the vials provided by PEI and the proper dilution of the stock

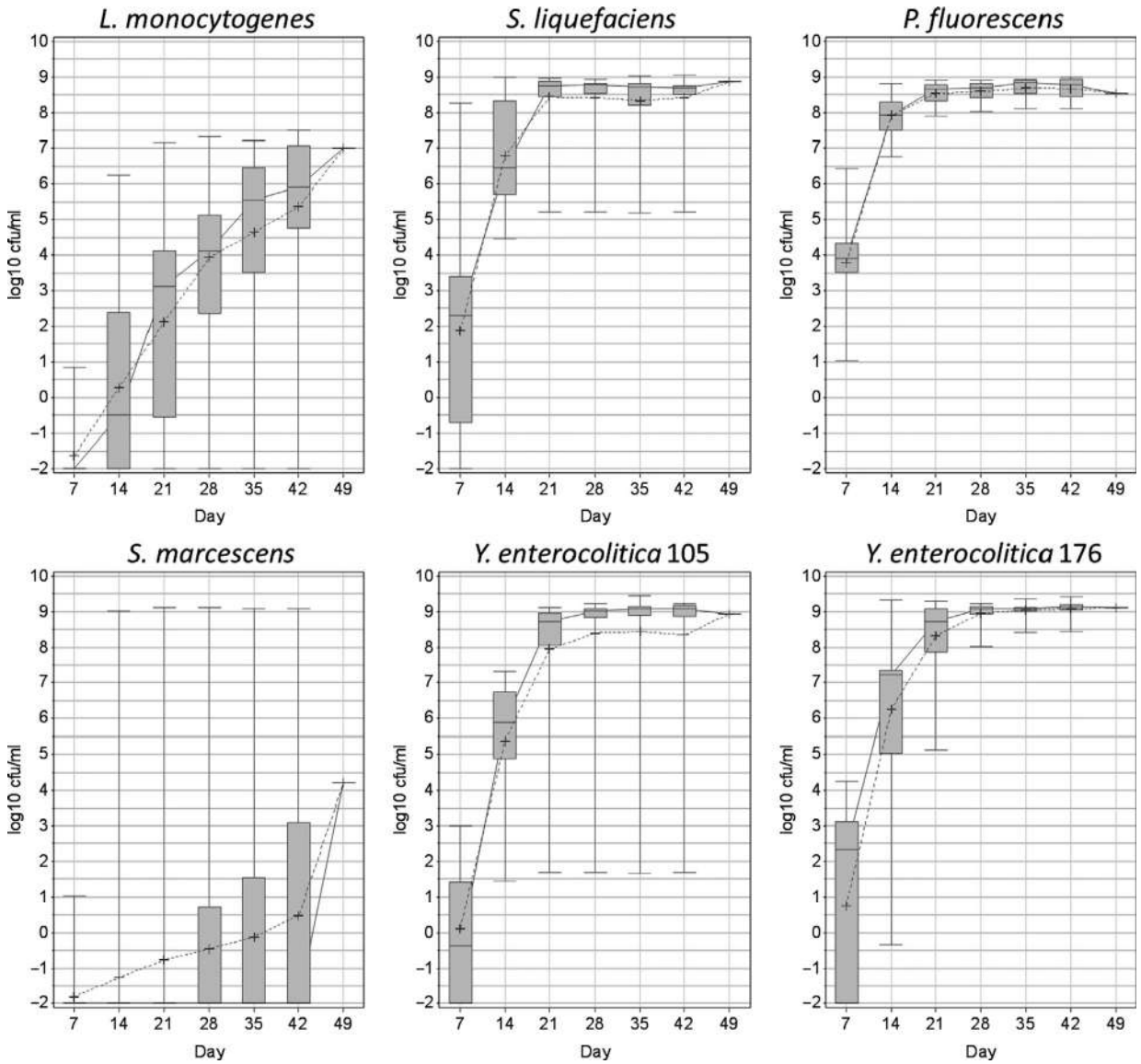

Fig. 3 Box- and Whisker plots of averaged growth curves. Continuous line connecting the median values per day, denoted by horizontal line within each box; dotted line connecting mean values, denoted by '+'). Additional data for day 49 was reported by one study partner.

suspension by the participants. Based on the control inoculum, 12·5% of the reported inocula did not match the specifications with a deviation of more than ±0·5 log CFU/ml of mean. This might be attributed to dilution errors that can occur during the preparation of the final inoculum. In addition, the nutrient agar itself can have an impact on the plate count result. Factors such as the quality of the nutrient components, the humidity level of the agar or the level of chemo-oxidized molecules due to physical factors like heat can affect the media quality as well as the number of colonies [16]. Despite the occasional failure of matching the respective inoculum, the varying inocula particularly for the two *Y. enterocolitica* strains did not result in growth failure. The growth performance of both *Y. enterocolitica* strains was 100% and 96% after several days. This corroborates the robust and reliable growth of the strains even at starting inocula below 25 CFU/bag. A statistical calculation yielded a potential starting concentration between 0·02 and 0·74 CFU/ml for PC [17]. There is probable great variation between initial contamination levels between incidents with the bacteremic donor and a skin donor derived contamination. Recent spiking studies of whole blood demonstrated an uneven distribution of bacteria in the subsequent fractioning process [18]. Therefore, the initial bacterial load in a RBC unit might be underestimated. Nonetheless, the

Fig. 4 Time dependent discoloration of RBC units upon bacterial growth. Colour change from red to black depends on the bacterial counts and took place upon an average concentration of approximately 10e7 CFU/ml.

average inocula of 0·002–1·15 CFU/ml reached within the study clearly fulfil the low-count criteria.

The growth kinetics of the candidate strains in refrigerated RBCs can be divided into three categories comprised of 'fast growers' such as *S. liquefaciens*, *P. fluorescens*, both *Y. enterocolitica* strains, 'slow growers' like *L. monocytogenes* and 'inconsistent growers' like *S. marcescens*. All of these organisms are well-known for their growth ability under low-temperature conditions, hence their association with food poisoning and subsequent infections like yersiniosis or listeriosis [19]. Interestingly, the minimum and maximum growth rate differed by a factor of three with 0·075/h for *Y. enterocolitica* 176 and 0·026/h for *L. monocytogenes*. Compared to values between 0·8 to 2·8 originating from bacteria grown in PC, the growth rate in RBC is reduced by a factor of 10–100 depending on the respective strains [20]. Nevertheless, due to the duration of the RBC shelf life, which in some countries is up to 49 days [21], even bacteria with slow growth rates can reach a critical concentration over time. The current results are consistent with previous growth experiments in RBC units using *Y. enterocolitica* as a model organism. Bacterial loads in this work reached concentrations between 8^{10} to 10^{10} CFU/ml after 21–35 days post-inoculation [22].

A comparison of the intra-laboratory growth kinetics showed some variability, particularly within the first 2 weeks of the incubation, when displayed in larger boxplots (Fig. 3). Several factors might contribute to this heterogeneity. Different starting inocula, with a min-/max-range of 1–215 CFU/bag observed in this study, and a subsequent exponential growth of the cells inevitably generates varying growth patterns in the single RBC bags, especially at an early time. Additionally, the composition of each RBCC, with respect to the remaining number of leucocytes and the volume of plasma, can vary among the units. Finally, the donor specific variation of the RBC

matrix itself can lead to increased or attenuated antimicrobial activity of the RBC suspension. A recent study analysed the impact of different additive solution and/or RBCC manufacturing variables on the growth capacity of different bacteria [23]. A negligible influence was observed for different additive solutions, whereas the manufacturing process itself might have a stronger impact. The underlying cause is still not clear and further work is needed to identify critical parameters. Of note, several additional strains, most of them originating from transfusion incidents, did not show any growth in cold-stored RBC when tested at PEI. This is corroborated by a former study in which strains from the American Type and Culture Collections (ATCC), with known transfusion-associated background, were used for growth experiments in RBCC [24]. Only five out of 12 tested organisms, four of them (*S. liquefaciens, P. fluorescens, Y. enterocolitica, L. monocytogenes*) covering the successful species demonstrating growth in this study, showed growth in cold-stored RBCC. Of note, in this study the slowest growth was observed for an isolate of the species *L. monocytogenes* as well. Similar results of growth failure of bacteria in RBCs was shown in another study, where ATCC strains all involved in adverse transfusion reactions exhibited no growth under cold storage conditions except for *S. liquefaciens* [25].

All laboratories provided additional information on their test setup and consumables that might influence the growth behaviour of bacteria in RBCs retrospectively. However, none of the examined parameters such as bag material, type of additive solution or the content of remaining plasma and leucocytes gave a clear indication to the different growth results. Particularly for *S. marcescens*, it would be valuable to identify factors or conditions that led to the observed heterogeneous growth. Interestingly, other studies using different *S. marcescens* isolates as model bacteria also revealed heterogeneous growth behaviour in RBCC [25–27]. This indicates that isolates of the *S. marcescens* species generally comprise a rather broad geno-/phenotype variation.

Regarding the identification of the candidate strains to species level, results for *P. fluorescens* varied between the laboratories. However, based on taxonomic classifications, all identified species.

P. synxantha, P. veronii, P. cedrina are part of an intrageneric cluster called *P. fluorescens* species complex [28]. Based on the identification method used, the species accuracy might reach its limit. Similarly, the incorrect identification of *S. liquefaciens* as *S. proteamaculans* is probably based on their close evolutionary relationship [29]. For *Y. enterocolitica*, the correct determination of the genetic relatedness of *Yersinia* species within the *Y. enterocolitica* group is challenging and, dependent on the method applied, may lead to a misidentification of certain isolates [30]. Therefore, the identification results received from the participating sites are all within an acceptable range.

A visual inspection of blood components is highly recommended in order to prevent the administration of contaminated blood components. With respect to RBCC, bacterial growth is accompanied with oxygen consumption and subsequent discoloration of red cells. Based on the current results, the main drawback of the visual quality control is based on a delayed colour change in RBC units emerging only with bacterial loads exceeding 10^7 CFU/ml. In the worst case, highly contaminated RBC units maybe potentially transfused without any apparent anomalies in their appearance. Nevertheless, as long as no other safety measures are in place, a visual control of each single blood unit is important to withdraw highly contaminated RBC units.

The establishment of new bacteria reference strains for RBC is the result of a successful collaboration of experienced and strongly dedicated laboratories worldwide. Due to their commitment, the WHO Expert Committee on Biological Standardization (ECBS) approved five bacterial strains as the 1st WHO International Repository of Red Blood Cell-Transfusion Relevant Bacteria Reference Strains (WHO/BS/2019.2377). The strains can be acquired through the PEI. The new reference panel can serve as a valuable tool for the further development of new techniques in the field of transfusion safety. The heterogeneous growth of *S. marcescens* in the study is a prime example for the relevance of TRBRS and confirms once again their raison d'être.

Acknowledgements

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Author contributions

E. Spindler-Raffel initiated the study and designed the study protocol. M. Prax continued and led the planning and execution of the study with international partners. M. Prax, O. Krut, S. Ramirez-Arcos and C.P. McDonald wrote the manuscript. All co-authors performed testing enumeration, identification and growth measurement of the bacterial strains and contributed to the final version of the manuscript.

References

1 Busch MP, Bloch EM, Kleinman S: Prevention of transfusion-transmitted infections. *Blood* 2019; **133**:1854–1864

2 McDonald CP, Roy A, Mahajan P, *et al.*: Relative values of the interventions of diversion and improved donor-arm disinfection to reduce the bacterial risk from blood transfusion. *Vox Sang* 2004; **86**:178–182

3 Kleinman S, Stassinopoulos A: Risks associated with red blood cell transfusions: potential benefits from application of pathogen inactivation. *Transfusion* 2015; **55**:2983–3000

4 Funk MB, Lohmann A, Guenay S, *et al.*: Transfusion-transmitted bacterial infections - haemovigilance data of German blood establishments (1997–2010). *Transfus Med Hemother* 2011; **38**:266–271

5 FDA: Fatalities reported to FDA following blood collection and transfusion. Annual Summary for Fiscal Year 2018 2020.

6 Cohen H, Taylor C, Mold D, *et al.*: Serious Hazards of Transfusion - Annual Report 2009, 2010.

7 Niu MT, Knippen M, Simmons L, *et al.*: Transfusion-transmitted Klebsiella pneumoniae fatalities, 1995 to 2004. *Transfus Med Rev* 2006; **20**:149–157

8 Perez P, Salmi LR, Folléa G, *et al.*: Determinants of transfusion-associated bacterial contamination. Results of the French BACTHEM Case-Control Study. *Transfusion* 2001; **41**:862–872

9 Warnke P, Kiefel V, Schäffler H, *et al.*: Transfusion reaction due to Klebsiella pneumoniae-contaminated red blood cells. A case report. *Transfus Med* 2013; **23**:445–446

10 Frati P, Busardò FP, Di Stefano MA, *et al.*: A fatal case of post-transfusion sepsis caused by Yersinia enterocolitica after delivery. *Blood Transfus* 2015; **13**:528–531

11 Spindler-Raffel E, Benjamin RJ, McDonald CP, *et al.*: Enlargement of the WHO international repository for platelet transfusion-relevant bacteria reference strains. *Vox Sang* 2017; **112** (8), 713–722

12 Störmer M, Arroyo A, Brachert J, *et al.*: Establishment of the first international repository for transfusion-relevant bacteria reference strains. ISBT working party transfusion-transmitted infectious diseases (WP-TTID), subgroup on bacteria. *Vox Sang* 2012; **102**:22–31

13 Ramirez-Arcos S, McDonald C, Deol P, *et al.*: Bacterial safety of blood components–a congress review of the ISBT transfusion-transmitted infectious diseases working party, bacterial subgroup. *ISBT Science Series* 2019; **14**:239–247

14 Woodring TS, Farrell JJ: Pseudomonas poae-Associated Fatal Septic Transfusion Reaction, Peoria, Illinois, USA, 2017. *Emerging Infect Dis* 2019; **25**:1445–1451

15 Winter KM, Johnson L, Kwok M, *et al.*: Red blood cell in vitro quality and function is maintained after S-303 pathogen inactivation treatment. *Transfusion* 2014; **54**:1798–1807

16 Helou C, Marier D, Jacolot P, *et al.*: Microorganisms and Maillard reaction products: a review of the literature and recent findings. *Amino Acids* 2014; **46**:267–277

17 Benjamin RJ, Wagner SJ: The residual risk of sepsis. Modeling the effect of concentration on bacterial detection in two-bottle culture systems and an estimation of false-negative culture rates. *Transfusion* 2007; **47**:1381–1389

18 Taha M, Kalab M, Yi Q-L, *et al.*: Bacterial survival and distribution during buffy coat platelet production. *Vox Sang* 2016; **111**:333–340

19 Chlebicz A, Śliżewska K: Campylobacteriosis, salmonellosis, yersiniosis, and listeriosis as zoonotic foodborne diseases: a review. *Int J Environ Res Public Health* 2018; **15**:863–891

20 Dumont LJ, Wood TA, Housman M, *et al.*: Bacterial growth kinetics in ACD-A apheresis platelets: comparison of plasma and PAS III storage. *Transfusion* 2011; **51**:1079–1085

21 Bundesärztekammer: Richtlinie zur Gewinnung von Blut und Blutbestandteilen und zurAnwendung von Blutprodukten. Richtlinie Hämotherapie, 2017

22 Guinet F, Carniel E, Leclercq A: Transfusion-transmitted Yersinia enterocolitica sepsis. *Clin Infect Dis* 2011; **53**:583–591

23 Ramirez-Arcos S, Kou Y, Cayer M-P, *et al.*: The impact of red blood cell manufacturing variables on bacterial growth dynamics: a pilot study. *Vox Sang* 2019; **114**:478–486

24 Chen CL, Yu J-C, Holme S, *et al.*: Detection of bacteria in stored red cell products using a culture-based bacterial detection system. *Transfusion* 2008; **48**:1550–1557

25 de Grandmont MJ, Ducas E, Girard M, *et al.*: Quality and safety of red blood cells stored in two additive solutions subjected to multiple room temperature exposures. *Vox Sang* 2014; **107**:239–246

26 Ramirez-Arcos S, Mastronardi C, Perkins H, *et al.*: Evaluating the 4-hour and 30-minute rules. Effects of room temperature exposure on red blood cell quality and bacterial growth. *Transfusion* 2013; **53**:851–859

27 Ramirez-Arcos S, Perkins H, Kou Y, *et al.*: Bacterial growth in red blood cell units exposed to uncontrolled temperatures. Challenging the 30-minute rule. *Vox Sang* 2013; **105**:100–107

28 Scales BS, Dickson RP, LiPuma JJ, *et al.*: Microbiology, genomics, and clinical significance of the Pseudomonas fluorescens species complex, an unappreciated colonizer of humans. *Clin Microbiol Rev* 2014; **27**:927–948

29 Grimont PAD, Irino K, Grimont F: TheSerratia liquefaciens-S. proteamaculans-S. grimesii complex: DNA relatedness. *Curr Microbiol* 1982; **7**:63–67

30 Kuhm AE, Suter D, Felleisen R, *et al.*: Identification of Yersinia enterocolitica at the species and subspecies levels by Fourier transform infrared spectroscopy. *Appl Environ Microbiol* 2009; **75**:5809–5813

Supporting Information

Additional Supporting Information may be found in the online version of this article:
Table S1

VoxSanguinis
The International Journal of Transfusion Medicine
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Vox Sanguinis (2020)

ORIGINAL PAPER

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Assessing inpatient platelet ordering practice: evaluation of computer provider order entry overrides

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Vox Sanguinis

Background and Objectives Judicious utilization of platelet products protects a limited resource and mitigates risks of transfusion. At many institutions, computer physician order entry systems provide prompts to guide transfusion decisions; many capture the indication for transfusion, and generate metadata when orders are dissonant with guidelines. We conducted a retrospective review to examine adherence to and overrides of hospital guidelines for platelet transfusion to identify opportunities for improved transfusion practice.

Materials and methods Physician override reports (1/1/2018–3/31/2019) were examined and physician-entered justification comments accompanying override orders were extracted, in addition to patient-specific data (clinical service, age, sex, and pretransfusion platelet count). Two transfusion medicine physicians independently assessed comments in context of patient data and institutional guidelines and categorized as: indicated, protocol driven, or not indicated. Following adjudication, consensus was reached between the two reviewers. Override keyword frequencies were also determined.

Results Over 15-months, 1373 override orders were placed for 558 unique patients (25% of all adult inpatient platelet transfusions). haematology/oncology providers placed 573 (42%) override orders (261 unique patients), 46% of which were categorized as "not indicated", based on consensus review. Overall, 470 (34%) override orders were categorized as "not indicated". Examples of recurring key words included "bleeding/risk of bleeding", "falling platelet count", "platelet goal of XX".

Conclusions A large percentage of override orders for platelet transfusions were determined to be "not indicated" and out of compliance with institutional guidelines. The metadata captured identified concerns regarding clinical transfusion practice and opportunities for revised indications (e.g. threshold for retinal haemorrhage).

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Key words: haemovigilance, patient blood management, platelet transfusion, transfusion medicine.

Introduction

Approximately 2 million platelets are transfused annually in the United States [1]. Given their comparatively short shelf life, which often results in limited product availability, as well as the recognized risk for transfusion-associated septic reactions secondary to their room temperature storage, the benefits and risks of platelet transfusion must be carefully considered [2,3].

In clinical practice, a high proportion of platelet transfusions are given prophylactically in response to a threshold platelet count; the remainder are administered to treat active bleeding [4]. However, audits of platelet utilization reveal wide variability in the appropriate use of platelets

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across many clinical settings [5,6]. A recent study by Etchells *et al.* suggested that one in five platelet transfusions may be unnecessary [7]. These reports emphasize the need for continued reinforcement of safe and evidence-based decision-making when considering platelet transfusions.

Increasingly, computer physician order entry systems (CPOE) combine institutional recommendations and patient-specific information to guide appropriate transfusion decisions at the time of product order. Some systems have the ability to provide immediate feedback through a computer generated best practice alert (BPA), giving the physician an opportunity to cancel the transfusion order based on this information. Alternatively, clinicians may proceed with placing the transfusion order after documenting the reason for "overriding" the recommended indications. CPOE systems that have the capacity to collect information on "override" orders present an opportunity for quality review, particularly when a hard stop countermanding the transfusion order is not in place.

Our medical centre implemented an institution-developed CPOE for blood components in 2002 [8]. The CPOE ordering screens for blood products display institutional guidelines for adult inpatient transfusion as well as the patient's most recent, relevant, laboratory value, if available in an effort to guide appropriate blood product ordering practice. As an example, when a platelet product is requested, the CPOE ordering screen displays institutional guidelines (Fig. 1) for platelet transfusion and the patient's most recent platelet count. In cases where laboratory data (e.g. platelet count) conflict with the selected indication for transfusion, the provider is prompted to justify the order by entering a free-text comment. These comments are recorded within the blood bank module and are provided monthly to the transfusion service for retrospective review.

The purpose of this study was to examine platelet transfusion order practice at a single academic medical centre that utilizes an institutional developed CPOE and to investigate the reasons why clinicians order outside of recommended indications for platelet transfusion as presented in the CPOE at time of blood product order.

Methods

BIDMC is a 673-bed tertiary care academic institution with highly complex services including adult and neonatal critical care, trauma surgery, cardiothoracic surgery, neurosurgery, obstetrics and gynaecology, and solid-organ transplant and haematopoietic stem cell transplant services.

The study was limited to adult inpatients for the following reasons: (1) we do not have separate institutional paediatric guidelines for platelet transfusion, and (2) we do not currently have CPOE for outpatient blood component ordering. At the time of the study, institutional guidelines for platelet transfusion to adult inpatients were in place (Table 1). These guidelines were originally developed through consensus of the hospital's multidisciplinary transfusion committee. Institutional pre-procedural guidelines were developed with input from the relevant clinical services, including interventional radiology and pulmonology, neurosurgery, and anaesthesiology. Due to occasional differences between national transfusion guidelines and specialty-specific guidelines, consensus decisions were achieved following discussion between the transfusion service and the clinical services performing the procedures. All guidelines and changes in guidelines are presented to the institutional transfusion committee for approval.

In addition, at the time of the study, service-specific blood product transfusion protocols applicable to specific patient populations (e.g. liver transplant recipients and patients on extracorporeal membrane oxygenation (ECMO)) were in place (Table 1). Such protocols were developed by the clinical groups and approved by the transfusion service and the institutional transfusion committee, but were not incorporated into the CPOE system for blood product ordering due to limitations with the current information system.

Regardless of the situation or indication provided, there are no automatic hard stops in the blood ordering system when one platelet product is requested; all single product requests are filled provided inventory is not limited. If more than one platelet product is requested, laboratory policy dictates that a single product is released, and then the technologist immediately pages the transfusion medicine resident who is charged with reviewing the request with the ordering provider to determine appropriateness of multiple platelet products. Additionally, manual restrictions may be placed on individual patients based on clinical diagnosis. For instance, when a diagnosis of immune thrombocytopenic purpura (ITP) or thrombotic thrombocytopenic purpura (TTP) is reported to the Blood Bank, the technologist enters a blood bank restriction indicating the need to page the transfusion medicine resident prior to release of any platelet products.

Monthly reports on platelet product override orders were extracted and collated. It is worth noting that due to limitations of the CPOE system, it was not possible to capture altered or cancelled orders. Data elements examined included: the medical record number (MRN), order date/time, the override comment, name of ordering physician, the hospital service of the ordering physician, laboratory values for most recent platelet count, and patient's attending physician name.

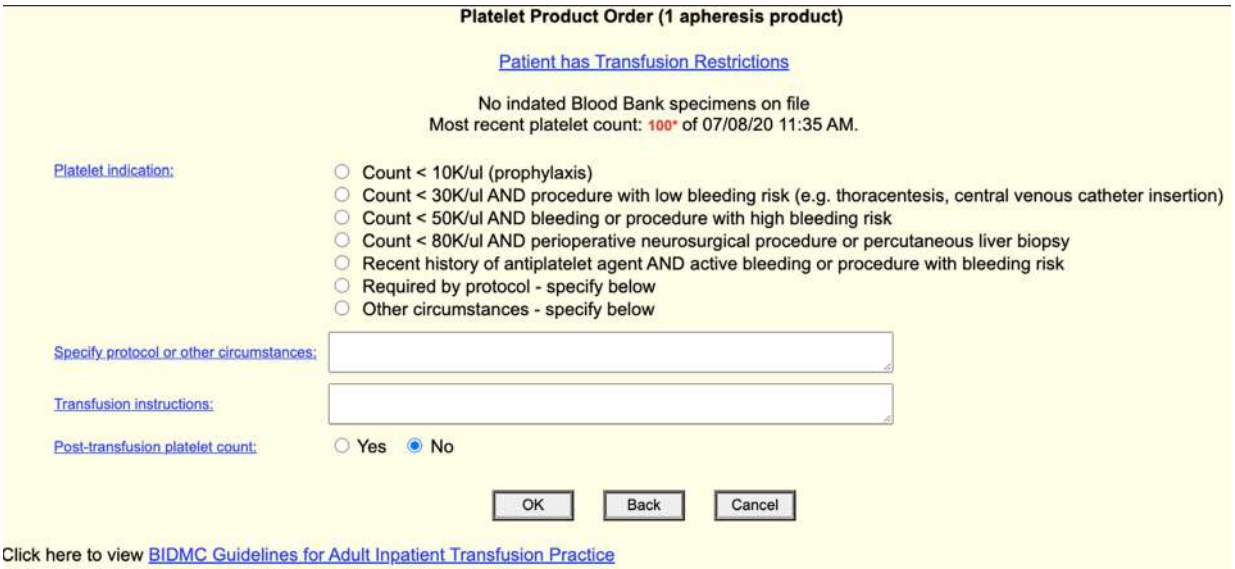

Fig. 1 Provider order entry screen for platelet orders.

Table 1 Approved institutional indications for platelet transfusion

	Indication	Transfuse when platelet count
Institutional guideline	Prophylaxis in a stable, non-bleeding patient	<10 000/μl
	Bleeding patient	<50 000/μl
	Pre-procedure (Low risk)	<30 000/μl
	Pre-procedure (Moderate/High risk)	<50 000/μl
	Pre-procedure (Neurosurgical)	<80 000/μl
Service-specific guideline	ECMO (bleeding pathway)	≤75 000/μl
	ECMO (non-bleeding pathway)	≤50 000/μl
	Liver TXP (bleeding pathway)	≤100 000/μl for day 0 and POD1
	Liver TXP (non-bleeding pathway)	≤50 000/μl for day 0 and POD1

Low risk procedures include (but are not limited to) arthrocentesis, joint and soft tissue injections, central line placement, paracentesis, thoracentesis, and bronchoscopy with lavage; Moderate/High risk procedures include (but are not limited to) percutaneous biopsies, transbronchial biopsies, lumbar puncture, and subcutaneous port placement; Neurosurgical procedures include (but are not limited to) epidural catheter placement or removal and epidural injections.

ECMO, extracorporeal membrane oxygenation; POD, post-operative day; TXP, transplant.

Each platelet override order was assigned to one of six categories:

(1) Indicated: aligned with institutional transfusion guidelines in the context of patient lab results and comments made by the ordering provider.
(2) Indicated by approved protocol: aligned with one of several approved, institution-specific protocols for platelet transfusion that contradicted institutional guidelines and were only applicable to particular patient groups, as described previously.
(3) Not indicated: lacked clear indications for transfusion and/or represented potential harm to recipients which outweighed any obvious benefit.
(4) Not indicated by approved protocol: not indicated based on institutional guidelines or one of the several approved, institution-specific protocols for platelet transfusion described previously.
(5) Need more information: provided relevant but incomplete information and, as such, these orders could not be assessed for the appropriateness of transfusion.
(6) Instruction: orders for which free-text comments included information that, while useful for the care of the particular patient (e.g., "give on route to operating room"), did not provide data pertinent for the purposes of assessing the appropriateness of transfusion.

Two transfusion medicine physicians (KO and LU) independently reviewed the override comments in the context of institutional guidelines and approved clinical service-specific protocols. Each physician independently categorized each order. Following independent assignment to one of the six categories, data were manually assessed for agreement between the two independent reviewers. Disagreements between categorization were discussed at an in-person consensus meeting between the two subject matter experts; consensus for categorization was achieved at this time.

To investigate patterns in ordering practice and reasons for overrides, the comments were manually reviewed for recurring "key words" and ranked by frequency of occurrence. Similarly, common combinations of "key words" were ranked by frequency of occurrence.

Descriptive statistics were used to derive percentages and frequencies for categorical variables. Results were displayed as bar charts using Microsoft Excel and Microsoft PowerPoint, version 2010 (Microsoft Corporation, Redmond, VA, USA). Median pre-platelet count was further analysed by ordering service group using Kruskal–Wallis test. A P-value of <0·05 was considered statistically significant. Statistical analyses were performed in SAS, version 9.3 (SAS Institute Inc., Cary, NC, USA). Boxplots were created to visualize pre-platelet count results in PowerBI (Microsoft Corporation, Redmond, VA, USA).

Because this study was designed as a quality improvement activity, the Institutional Review Board of the Beth Israel Deaconess Medical Center (BIDMC), Boston, MA waived the requirement for review.

Results

Between January 1, 2018 and March 31, 2019, a total of 5456 platelets were transfused to 962 adult inpatients. Overall, 1373 (25%) platelet orders placed for 558 unique patients triggered a CPOE system override. Demographic characteristics of patients, by category, for whom override orders were placed, are shown in Table 2. Orders placed by surgical services (Surgery), including general surgery, orthopaedic surgery, gynaecological surgery, and vascular surgery were combined into one group for the purposes of this analysis. The male:female ratio ranged from 0·15 (Neurosurgery) to 1·38 (Surgery) across all groups (Table 3). It was notable that women were the predominant gender type only in the Surgery group, which included all general surgery subspecialties as well as vascular surgery. The median age of patients ranged from 56 to 71 years.

Orders placed for patients on the haematology/oncology service most frequently triggered a CPOE system override (42%), followed by Transplant Surgery (14%), and Surgery (8%) and Internal Medicine (8%) (Fig. 2). Statistically significant differences in median pre-platelet counts were observed between service groups for patients for whom a physician order triggered an override ($P < 0·001$, Kruskal–Wallis test, Fig. 3).

Based on consensus between the two reviewers, 470 of 1373 overrides (34%) were categorized as "Not Indicated"; 430 (31%) were categorized as "Need more information"; 246 (18%) were categorized "Indicated"; 180 (13%) were categorized as "Indicated per Protocol"; 11 (1%) were categorized as "Not Indicated per Protocol", and 36 (3%) were "Instruction" orders that did not

Table 2 Patient demographics for whom platelet transfusion orders triggered an override

N = 558 patients		
Age	65 (median)	54–73(IQR)
Sex		
Male	341	61·1%
Female	217	38·9%
N = 1373 order overrides		
Platelet counts	44 (median)	16–75 (IQR)
Ordering services		
Haematology/Oncology	573	41·7%
Transplant Surgery	190	13·8%
Surgery[a]	109	7·9%
Internal Medicine	104	7·6%
Cardiac Surgery	98	7·1%
Intensive Care	89	6·5%
Gastroenterology	81	5·9%
Cardiology	54	3.9%
Emergency Department	26	1·9%
Neurosurgery	25	1·8%
Neurology	24	1·7%
Categorization of Overrides[b]		
Not Indicated	470	34·2%
Need More Information	430	31·3%
Indicated	246	17·9%
Protocol-Indicated	180	13·1%
Instruction	36	2·6%
Protocol-Not Indicated	11	0·8%

[a]Surgery service includes all general surgical services and vascular surgery. Cardiac surgery and neurosurgery are separately represented.

[b]Indicated: aligned with institutional transfusion guidelines in the context of lab results and comments made by the ordering provider; Not Indicated: lacked clear indications for transfusion and/or represented potential harm to recipients which outweighed any obvious benefit; Need More Information: provided relevant but incomplete information, and as such, these orders could not be assessed for appropriateness of transfusion; Protocol-Indicated and Protocol-Not indicated: transfusions ordered under institutionally approved protocols for specific patient populations, as described above; Instruction: free-text comments included information that, while useful for the care of the patient, did not provide data pertinent for the purposes of assessing the appropriateness of transfusion.

Table 3 Distribution of patient gender and age by ordering service groups

Ordering Service	Number of patients (F:M, ratio)	Age, Median (IQR)
Haematology/Oncology	151 (55:96, 0.57)	67 (58–74)
Transplant Surgery	38 (17:21, 0.81)	59 (50–66)
Surgery[a]	69 (40:29, 1.38)	61 (43–69)
Internal Medicine	76 (36:40, 0.90)	67 (53–74)
Cardiac Surgery	64 (20:44, 0.46)	71 (62–79)
Intensive Care	56 (25:31, 0.81)	65 (52–76)
Gastroenterology	38 (7:31, 0.23)	56 (47–62)
Cardiology	16 (3:13, 0.23)	60 (48–72)
Emergency Department	25 (9:16, 0.56)	65 (50–73)
Neurosurgery	15 (2:14, 0.15)	63 (44–76)
Neurology	10 (3:7, 0.43)	56 (41–70)

[a]Surgery service includes all general surgical services and vascular surgery. Cardiac surgery and neurosurgery are separately represented.

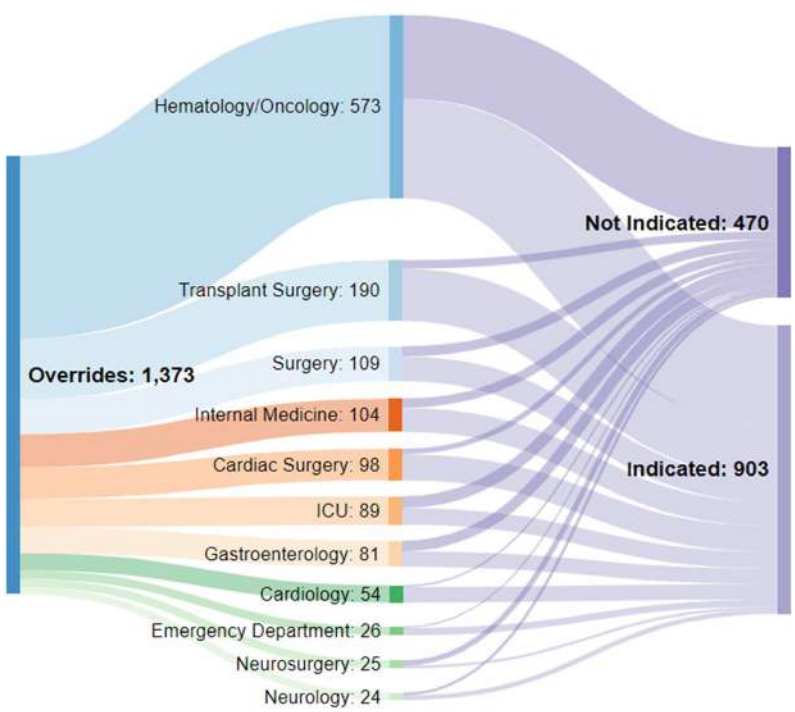

Fig. 2 Distribution of triggered overrides by ordering service.

provide any information as to appropriateness of platelet order (Table 2). Notable was the observation that 46% of the override orders placed by haematology/oncology were categorized as "not indicated".

Keyword frequencies are shown in Fig. 4. The top ten most common keywords were "bleeding/concern for bleeding" (335), "platelet goal >XX" (147) (with XX representing a variety of platelet counts not defined by any current institutional guidelines), "per liver transplant protocol" (120), "CNS (or concern for) bleed" (111), "prior to/peri procedure" (87), "down trending" (76), "massive transfusion protocol" (69), "per ECMO protocol" (64), "platelet count is 10" (55), and "in need of anticoagulation" (54). Pretransfusion platelet counts associated with these keywords varied widely (Fig. 5); notably, median pre-platelet counts for orders in the setting of massive transfusion was 123 x 10^9/l (IQR, 74–206) as compared to pretransfusion platelet counts where "down trending" was listed in the override comment, 12 x 10^9/l (IQR, 11–16).

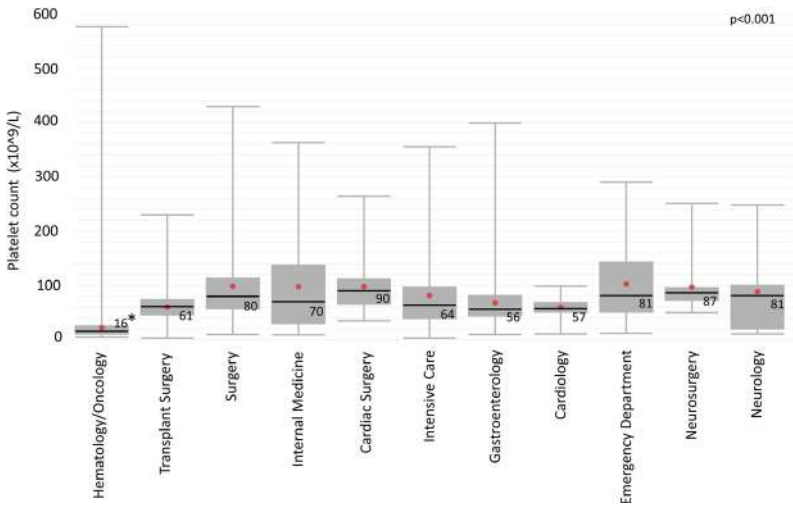

Fig. 3 Pretransfusion platelet counts Boxplots of median and interquartile range, mean, maximum and minimum pretransfusion platelet counts of patients for whom a physician order entry triggered an override order, by ordering service. Asterisk (*) indicates statistical significance ($P < 0{\cdot}001$) in median platelet count relative to other ordering service groups.

The frequencies of keyword combinations are shown in Fig. 6. The top ten most common keyword combinations were "status post-surgery" or "post-procedure and bleeding" (146), "central nervous system (CNS) bleed and platelet count <100 x 10^9/l (80)", "ECMO and bleeding" (63), "count <XX and bleeding" (60) (with XX representing a variety of platelet counts not currently defined by any institutional guidelines), "bone marrow transplant (BMT) patient with goal >XX (58)", "need anticoagulation, so goal >XX" (57), "need to give a balanced resuscitation" (49), "count <XX and high risk of bleeding" (31), "coagulopathy and count <XX/bleeding" (31), and "liver failure needing intracranial pressure monitoring/bleeding" (24). Pretransfusion platelet counts associated with keyword combinations varied widely (Fig. 7). The median pretransfusion platelet count for orders associated with the keyword combination "status post-surgery or post-procedure and bleeding" was 81 x 10^9/l (IQR, 56–101) vs 19 x 10^9/l (IQR, 14–24) for orders associated with the keyword combination "BMT patient with goal >XX".

Discussion

Our retrospective study demonstrated that a considerable proportion of CPOE platelet orders were not in immediate agreement with established, evidence-based institutional guidelines. Despite BPAs, 25% (1373/5456) of platelet orders triggered an override comment, 42% (573/1373) of which were placed by the haematology/oncology service. Furthermore, 34% of the all overrides were categorized as "not indicated" since they fell outside of institutional guidelines (Table 2) and the clinical information provided in the override comment did not support the need for transfusion. Fifty-six per cent of all of the "not indicated" orders were placed by the haematology/oncology service.

Our observations are similar to those published by others examining the usefulness of BPAs in guiding transfusion practice [5–7,9,10]. In a retrospective review of a year of platelet transfusions in New South Wales, Australia, Schofield and colleagues found 33% of all orders to be inappropriate [6]. Qureshi *et al.* [5] found 43% of platelet transfusions were non-compliant with institutional guidelines. The Canadian study published by Etchells and colleagues showed 22% of platelet transfusions at four large academic medical centres to be out of compliance with institutional guidelines [7].

Our examination of the override comments revealed recurring key words and phrases which show opportunities for educational interventions as well as a need for modification of current transfusion guidelines. In terms of existing educational initiatives directed at transfusion practice, the Transfusion Service presently provides monthly didactic lectures to trainees in the haematology/oncology department throughout the academic year. Additionally, informal education takes place in the form of transfusion medicine clinical consultations. However, these interactions, by their nature, are sporadic, thus, an inconsistent method of education on even the most commonly encountered transfusion-related topics. A more formalized approach may improve clinician understanding on best practices related to patient transfusion [11]. For example, development of an online recorded lecture on appropriate indications for platelet transfusion that is available through our institution's intranet and available

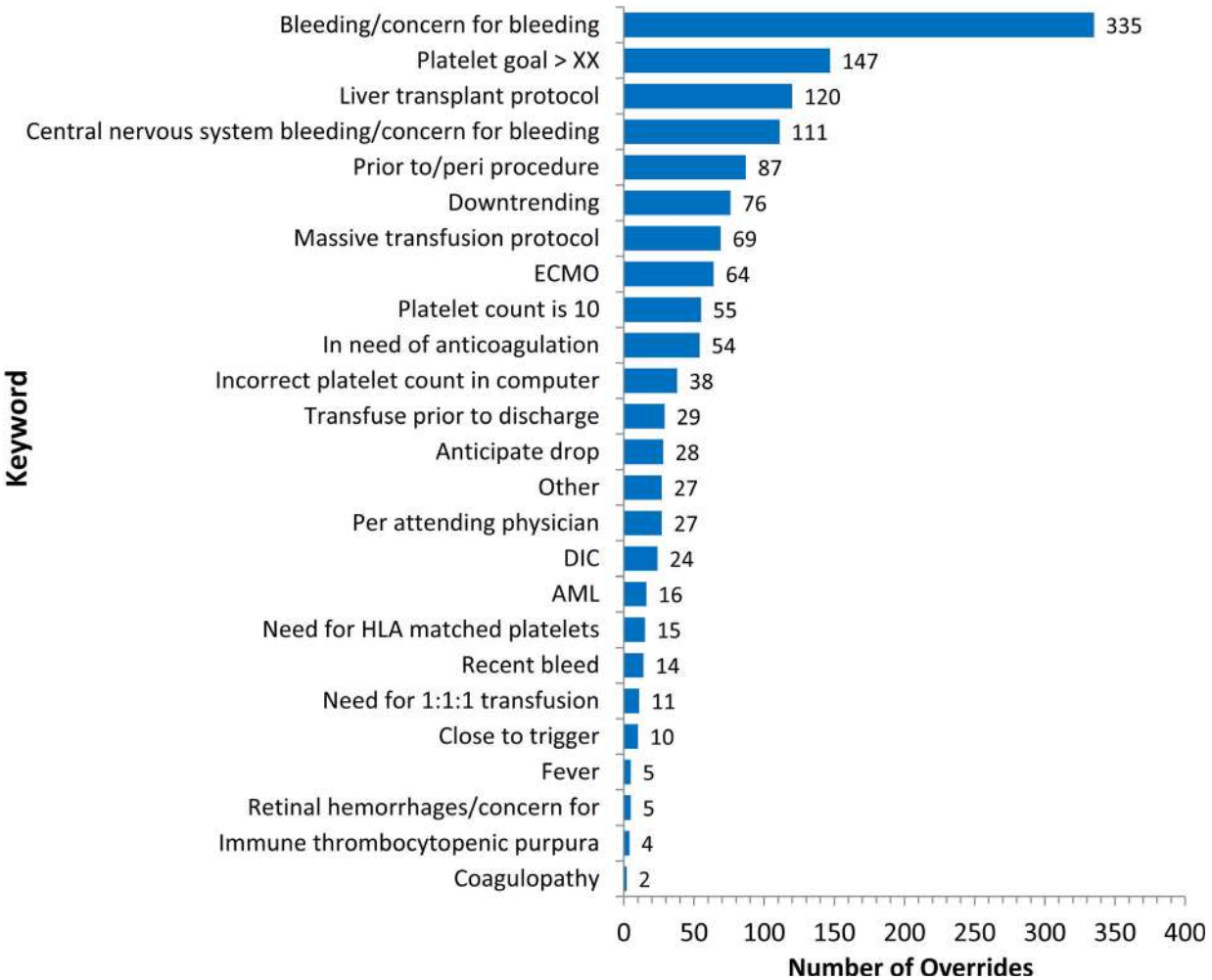

Fig. 4 Most common keywords entered in the comment field by ordering providers.

at any time for review may be beneficial. Also, a brief, targeted yearly required online training module for all staff and trainee physicians may provide reinforcement of institutional guidelines.

The observation of free-text comments such as "per attending" and "per service" reflect the tendencies of trainees to order on behalf of an attending physician or consult service, possibly in conflict with their own medical judgment. The concept of authority gradients in medicine is not new. First described in the aviation industry where ineffective communication between pilots and copilots in stressful situations was observed when there was a significant difference in their experience or perceived authority, the concept was first applied to medicine in 2000 [12,13]. The practice of medicine is historically hierarchical, where the more experienced attending physicians educate and train students and junior housestaff through lectures, simulations and modelling. There may be instances when less experienced trainees believe that guidance/recommendations for medical intervention are incorrect and potentially harmful. When the less experienced physician fails to heed their inner beliefs of what is appropriate and, instead puts their trust in their more experienced teammember despite internal dissonance, medical errors may result [12]. Institutional endorsement of an environment where all team members and their ideas are considered in medical management decisions helps to avoid the detrimental pitfalls of authority gradient.

Specific ways to address platelet orders where trainees are ordering a product "per attending physician", include the incorporation of vignettes in didactic sessions with solutions to the scenario or offering trainees access to transfusion medicine attendings who may facilitate a discussion with the patient's attending about the rationale for a platelet order that is not in accordance with institutional guidelines. Additionally, specifically monitoring

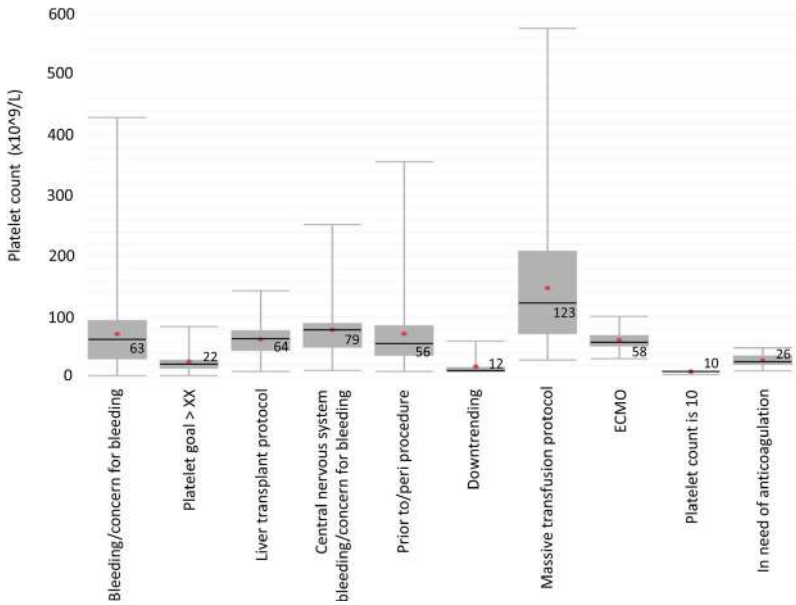

Fig. 5 Pretransfusion platelet counts of patients for whom physician order entry triggered an override order for the top ten most common keyword frequencies Boxplots show median and interquartile range, mean, maximum and minimum pretransfusion platelet counts of these patients.

these overrides separately for ordering service/physician offers opportunities to identify concerning practices which warrant involvement of departmental leadership to assist in correcting poor practice.

Individualized feedback directed at attending level physicians may further complement department-wide education initiatives [14]. Transfusion Guideline Compliance reports, which show individual providers' transfusion guideline compliance rates and allow comparison to peers within their own specialty, have been shown in some cases to be impactful [15]. Staples and colleagues describe a multi-pronged approach involving monthly review with junior haematology clinicians, a hospital-wide dashboard allowing visualization of ordering data, quarterly reports sent to senior members of the clinical staff by email, and daily multidisciplinary team review of all override orders [16]. As previously noted, override reports are generated monthly by our institution's decision support department, thus providing timely feedback to providers is difficult. More timely reporting would likely be fruitful. Additionally, review of override reports at the hospital quarterly transfusion committee meetings would enhance visibility of clinician transfusion practice.

Many if not most haematology/oncology providers are aware of institutional guidelines for inpatient platelet transfusions, however, many of their reasons for transfusing fall outside of the limited indications listed on the CPOE ordering screen. For example, platelet transfusions were frequently ordered for haematology/oncology inpatients on the day of planned discharge from the hospital, despite the fact that the patients were stable. There are no published guidelines for platelet thresholds for outpatients (or for patients transitioning from the inpatient to the outpatient setting) who are otherwise stable. The American Society for Clinical Oncology (ASCO) advises a threshold of >10 000/μl in haematology/oncology patients, especially if transfusion may not be readily available in cases of emergencies [17]. The AABB similarly endorses a more liberal platelet threshold, indicating that it affords more practical management of outpatients, but a specific threshold is not provided [4]. The British Committee for Standards in Haematology (BCSH) goes a little further in declaring that consideration should be given for increasing the threshold from 10 to 20 000/μl in patients judged to have additional risk factors for bleeding; if outpatient status is thought to be an additional risk factor for bleeding, 20 000/ μl would appear to be an acceptable threshold [18]. The absence of harmonized guidelines across professional organizations regarding outpatient transfusion practice contributes to the wide variation in practice that is not necessarily evidence-based. Joint efforts among organizations with respect to guideline development would be welcomed by the transfusion medicine and haematology communities.

Our review also showed that the haematology/oncology providers ordered platelets for thrombocytopenic patients with retinal haemorrhages and those patients with thrombocytopenia and receiving anticoagulation therapy for documented thromboses; there are currently no

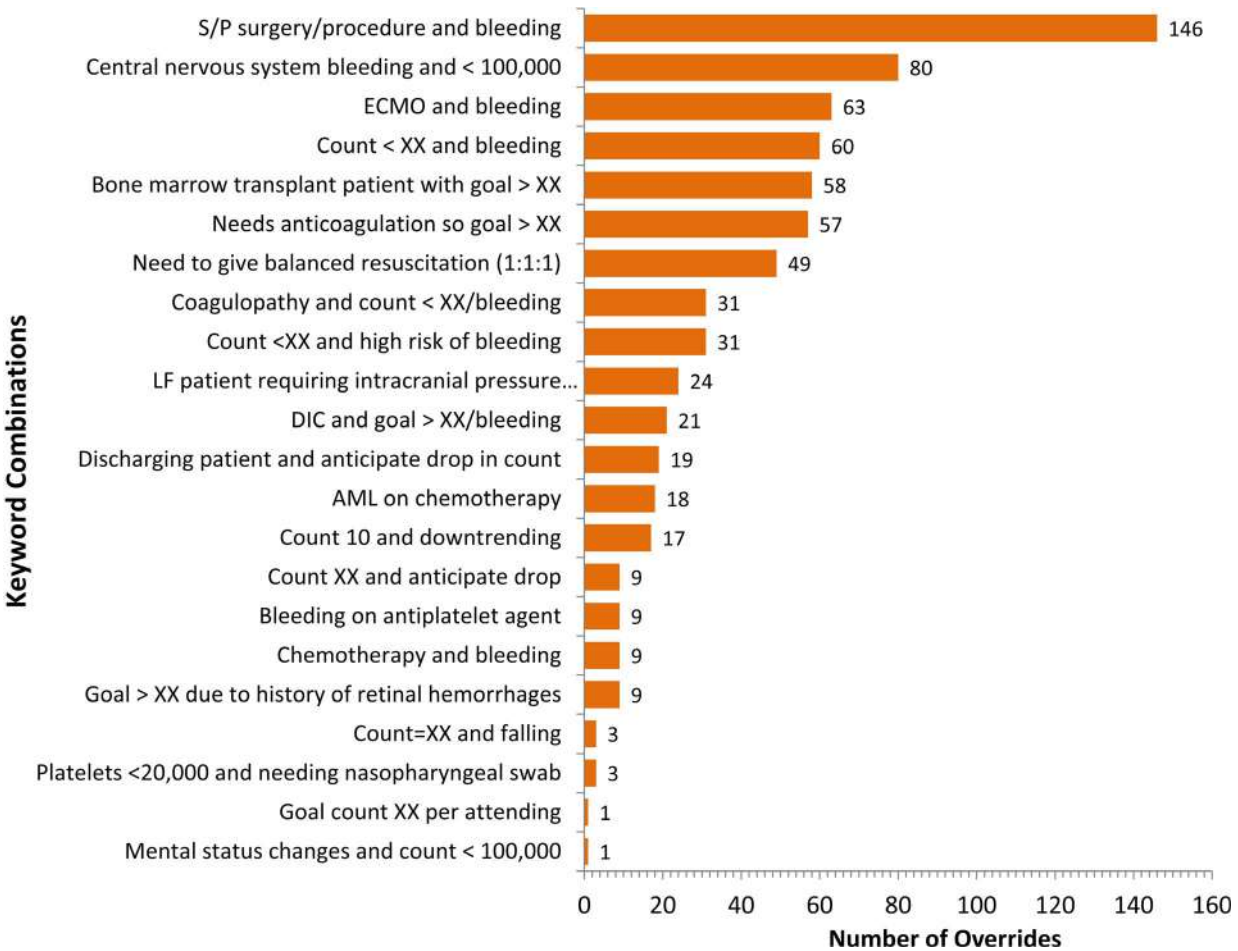

Fig. 6 Most common keyword combinations entered in the comment field by ordering providers.

institutional guidelines or published guidelines from AABB, ASCO or BCSH for either indication.

While rare, the risk for ocular/retinal bleeding at low platelet counts is reported to be multifactorial in nature, and includes retinal damage in the setting of CMV retinitis or pre-transplant conditioning and immunosuppressive regimens [19,20]. In light of this review, we plan to update current platelet transfusion guidelines allowing for this indication (transfusion goals in patients with retinal haemorrhages with thrombocytopenia) by working in concert with our colleagues in haematology/oncology to develop an evidence-based threshold.

Our review also revealed 57 overrides for transfusion of platelets to thrombocytopenic patients in need of chemical anticoagulation, including unfractionated heparin, low molecular weight heparin, and the novel oral anticoagulants. Thrombosis is a well-recognized complication of malignancy, but the approach to anticoagulation in thrombocytopenic patients with a malignancy is challenging [21–23]. Recommendations in the literature offer approaches to anticoagulation based on both the type of anticoagulant and depth of thrombocytopenia [21,23]. Our review highlighted the need for an institutional-wide approach to anticoagulation management of thrombocytopenic patients, particularly in our haematology/oncology patient population where triggered overrides were associated with very low median pretransfusion platelet count (26 x 10^9/l). Once established, the guidelines can be incorporated into the CPOE system with a goal of improving platelet utilization and reducing the number of triggered platelet overrides. Again, working with colleagues in haematology/oncology to establish institutional evidence-based guidelines for this indication would be a first step (threshold for thrombocytopenic patients on chemical anticoagulation). Once a threshold is agreed upon, data would be presented to the institution transfusion committee for approval prior to incorporation into CPOE.

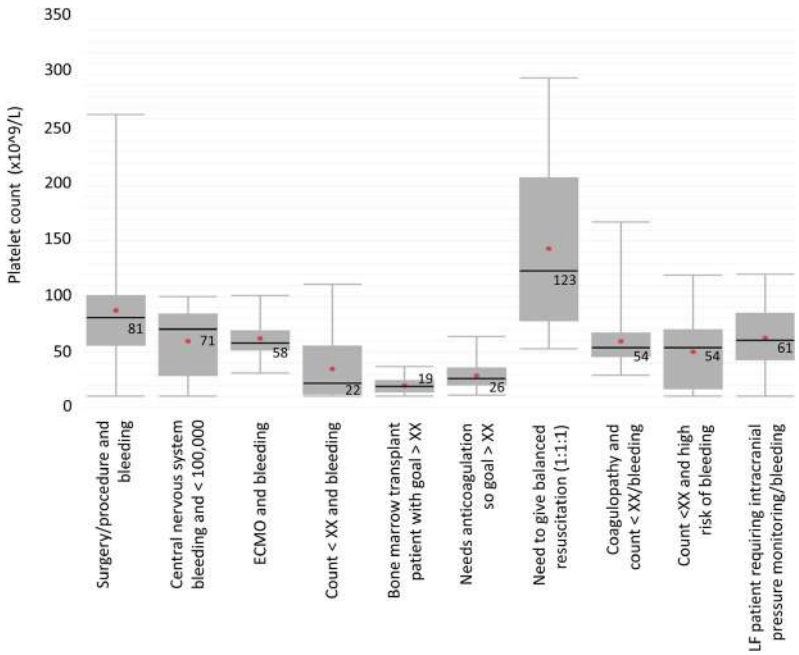

Fig. 7 Pretransfusion platelet counts of patients for whom physician order entry triggered an override order for the top ten most common keyword combination frequencies Boxplots show median and interquartile range, mean, maximum and minimum pretransfusion platelet counts of these patients.

Another opportunity to reduce overrides, and therefore override fatigue, would be to add our institution's massive haemorrhage transfusion protocol (MHTP) activation to the CPOE, which is not available presently. Outside of a massive or rapid bleed, we advise providers to monitor laboratory data obtained either by centralized or point of care testing, along with patient symptoms, to direct transfusion of blood products, including platelets. Educational efforts to dispel the notion that 1:1:1 blood product ratio should be used in non-rapidly or non-massively bleeding patients should be aimed at physicians at all levels, but especially physicians in the intensive care, emergency department, and critical care settings [24–26]. Not only does this offer an opportunity to decrease the number of override orders and unnecessary transfusions, but these efforts would also assist in reducing blood component wastage.

Our study has several limitations, mostly related to the means by which the data were captured. While our review of CPOE system orders clearly highlighted problematic platelet transfusion practices, a deeper analysis through individual chart reviews could have possibly clarified the more ambiguous categorizations such as the "need more information" category. Additionally, for the sake of categorization, the analysis necessarily assumed that all free-text justification comments were reflective of the ordering provider's knowledge of transfusion best practices, indications, and clinical judgment. As previously mentioned, external factors may have contributed to questionable orders, such as trainees ordering on behalf of the responsible attending physician. Another limitation to our study is the absence of inter-rater comparability assessment (kappa statistic) of the override categorization secondary to our methodological approach; however, we are confident that our consensus assignment provides reliable data on the characteristics of the override comments. Finally, we were unable to capture data through the CPOE system on platelet transfusion orders that were initiated, but cancelled after acknowledging a best practice alert.

In conclusion, the examination of comments provided by ordering physicians in a CPOE system which incorporates best practice alerts is a valuable tool for retrospective review of platelet transfusion order practices. Beyond the appraisal of transfusion appropriateness, this system provides meaningful insight into the knowledge, motivations, and ordering habits of its users – which in turn can serve as a starting point for targeted educational initiatives or consideration for modification of indications. Our review demonstrated that almost half of the override orders originated from the haematology/oncology service; one-third of platelet orders triggering an override were not indicated based on the existing guidelines. These observations are likely not unique to our institution and suggest an ongoing need for improved educational initiatives, as well as periodic review of institutional policies to adequately reflect society guidelines [27].

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None.

Conflict of interests

The authors report no conflict of interest.

References

1 Ellingson KD, Sapiano MRP, Haass KA, *et al.*: Continued decline in blood collection and transfusion in the United States-2015. *Transfusion* 2017; **57** (Suppl 2):1588–1598
2 Bloch EM, Marshall CE, Boyd JS, *et al.*: Implementation of secondary bacterial culture testing of platelets to mitigate residual risk of septic transfusion reactions. *Transfusion* 2018; **58**:1647–1653
3 FDA, CBER: Bacterial risk control strategies for blood collection establishments and transfusion services to enhance the safety and availability of platelets for transfusion, 2016
4 Kaufman RM, Djulbegovic B, Gernsheimer T, *et al.*: Platelet transfusion: a clinical practice guideline from the AABB. *Ann Intern Med* 2015; **162**:205–213
5 Qureshi H, Lowe D, Dobson P, *et al.*: National comparative audit of the use of platelet transfusions in the UK. *Transfus Clin Biol* 2007; **14**:509–513
6 Schofield WN, Rubin GL, Dean MG: Appropriateness of platelet, fresh frozen plasma and cryoprecipitate transfusion in New South Wales public hospitals. *Med J Aust* 2003; **178**:117–121
7 Etchells M, Spradbrow J, Cohen R, *et al.*: Audit of appropriate use of platelet transfusions: validation of adjudication criteria. *Vox Sang* 2018; **113**:40–50
8 Haspel RL, Uhl L: How do I audit hospital blood product utilization? *Transfusion* 2012; **52**:227–230
9 McWilliams B, Triulzi DJ, Waters JH, *et al.*: Trends in RBC ordering and use after implementing adaptive alerts in the electronic computerized physician order entry system. *Am J Clin Pathol* 2014; **141**:534–541
10 Collins RA, Triulzi DJ, Waters JH, *et al.*: Evaluation of real-time clinical decision support systems for platelet and cryoprecipitate orders. *Am J Clin Pathol* 2014; **141**:78–84
11 Lin Y, Tinmouth A, Mallick R, *et al.*: BEST-TEST2: assessment of hematology trainee knowledge of transfusion medicine. *Transfusion* 2016; **56**:304–310
12 Cosby KS, Croskerry P: Profiles in patient safety: authority gradients in medical error. *Acad Emerg Med* 2004; **11**:1341–1345
13 Kohn LT, Corrigan JM, Donaldson MS (eds): *To Err is Human: Building a Safer Health System*. Washington, DC: The National Academies Press. 2000
14 Beaty CA, Haggerty KA, Moser MG, *et al.*: Disclosure of physician-specific behavior improves blood utilization protocol adherence in cardiac surgery. *Ann Thorac Surg* 2013; **96**:2168–2174
15 Frank SM, Thakkar RN, Podlasek SJ, *et al.*: Implementing a health system-wide patient blood management program with a clinical community approach. *Anesthesiology* 2017; **127**:754–764
16 Staples S, Salisbury RA, King AJ, *et al.*: How do we use electronic clinical decision support and feedback to promote good transfusion practice. *Transfusion* 2020; **60**:1658–1665
17 Schiffer CA, Bohlke K, Delaney M, *et al.*: Platelet transfusion for patients with cancer: American Society of Clinical Oncology clinical practice guideline update. *J Clin Oncol* 2018; **36**:283–299
18 Estcourt LJ, Birchall J, Allard S, *et al.*: Guidelines for the use of platelet transfusions. *Br J Haematol* 2017; **176**:365–394
19 Inamoto Y, Petricek I, Burns L, *et al.*: Non-GVHD ocular complications after hematopoietic cell transplantation: expert review from the Late Effects and Quality of Life Working Committee of the CIBMTR and Transplant Complications Working Party of the EBMT. *Bone Marrow Transplant.* 2019; **54**:648–661
20 Yoo YS, Na KS, Shin JA, *et al.*: Posterior eye segment complications related to allogeneic hematopoietic stem cell transplantation. *Retina*. 2017; **37**:135–143
21 Al-Samkari H, Connors JM: Managing the competing risks of thrombosis, bleeding, and anticoagulation in patients with malignancy. *Blood Adv* 2019; **3**:3770–3779
22 Houghton DE, Key NS, Zakai NA, *et al.*: Analysis of anticoagulation strategies for venous thromboembolism during severe thrombocytopenia in patients with hematologic malignancies: a retrospective cohort. *Leuk Lymphoma* 2017; **58**:2573–2581
23 Khanal N, Bociek RG, Chen B, *et al.*: Venous thromboembolism in patients with hematologic malignancy and thrombocytopenia. *Am J Hematol* 2016; **91**:E468–E472
24 Sommer N, Schnuriger B, Candinas D, *et al.*: Massive transfusion protocols in nontrauma patients: a systematic review and meta-analysis. *J Trauma Acute Care Surg* 2019; **86**:493–504
25 Dunbar NM, Olson NJ, Szczepiorkowski ZM, *et al.*: Blood component transfusion and wastage rates in the setting of massive transfusion in three regional trauma centers. *Transfusion* 2017; **57**:45–52
26 Motameni AT, Hodge RA, McKinley WI, *et al.*: The use of ABC score in activation of massive transfusion: the Yin and the Yang. *J Trauma Acute Care Surg* 2018; **85**:298–302
27 Lin Y, Tilokee E, Charge S, *et al.*: Transfusion Camp: a prospective evaluation of a transfusion education program for multispecialty postgraduate trainees. *Transfusion* 2019; **59**:2141–2149

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An international effort and use of social media to save a young girl

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Vox Sanguinis

Abstract

Background and objectives A 2-year-old female with neuroblastoma needed In(b–), E– red blood cells (RBCs). No units were available at the blood centre (BC) nor in the rare donor programme member's inventories. BC's Immunohematology Reference Laboratory (IRL) and its marketing department concentrated on recruiting and testing those donors more likely to be antigen negative based on ethnicity.

Materials and methods The BC's communication plan utilized social and traditional media to assist in the search for In(b–) blood. Media strategies directed donors in the United States (US) and Canada to go to their nearest BC for collection, tagging and testing of units. Two segments from each donation were shipped overnight to the BC's IRL (associated with the patient) for testing. Diluted anti-Inb sera was tested by microtechniques to conserve resources. Additionally, the American Rare Donor Program (ARDP) facilitated the international searches and acted as a liaison to the Food and Drug Administration (FDA).

Results More than 25 000 people responded to the appeal. Seventy-seven BCs submitted segments from 4197 units. Two donors were In(b–) but E+ and thus not compatible with the patient but were submitted to ARDP for future needs. The prevalence of In(b–) units identified in the search was 0·048%. In total, five known In(b–) donors, two from the US and three from international sources, provided units for this patient.

Conclusion Social media sparked a viral response to the rare blood need. While a match was not found among the units tested, domestic and international searches were able to meet the patient's blood needs.

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Key words: immunohematology, blood components, transfusion-paediatrics, blood groups, donor motivation, RBC antigens and antibodies.

Introduction

Hospitals encounter patients with alloantibodies to very high prevalence antigens for whom provision of blood products is difficult due to the paucity of units and identified donors in a country's rare donor database. At this point, there are three choices for transfusion: (1) transfusion of incompatible blood matched for the most number of antigens for which blood is available – suitable if the patient needs blood urgently; (2) request that ARDP contact the International Society of Blood Transfusion's (ISBT) International Rare Donor Panel (IRDP) to search

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outside of the country (in this case outside of the US) – this process may take several weeks and involves working with the government agency (FDA in the US) to allow importation of units; and (3) ask BCs to screen members of the community most likely to possess blood lacking the antigen-the time frame would be a minimum of several days for the BC to engage its marketing and recruitment teams and the laboratory to be ready to test samples provided. The interaction between the BC, the patient's physicians, the hospital's transfusion service and the patient's parents caring for a 2-year-old female who had a rare blood type and needed an autologous hematopoietic progenitor cell transplant will be described. Both an international search and recruitment of samples from the US and Canada required a coordinated effort among all parties including ARDP, FDA and BCs outside the US. The use of social media sparked a viral response that was unprecedented resulting in both media and public interest in the vital role of BCs. The donor response was tremendous, and unique strategies were created to optimize testing and manage collaborations between BCs.

Materials and methods

Antibody detection was performed by the admitting hospital The patient had no history of previous transfusion. Antibody identification was performed by the local BC IRL, and additional testing was performed by another IRL for molecular genotyping. The monocyte monolayer assay (MMA) testing was performed by a third IRL to determine clinical significance.

The BC's Media department created a communication plan that utilized several social and traditional media platforms to assist in the search for In(b–), E– blood worldwide. The communication plans included a prepared Facebook Live show with video of the patient, interviews with her parents, as well as the IRL team and gave insight into the behind-the-scenes efforts taking place to find blood for transfusion. The media message detailed the ethnically diverse donor criteria needed to be a possible match for the patient and featured a special website page with detailed information on where individuals matching the specific donor criteria could go to donate.

Media messages directed donors in the US and Canada to go to their nearest BC for collection, tagging and testing of units. Two segments from each donation were shipped overnight to the BC's IRL (associated locally with the patient) for testing. Diluted anti-In^b human source plasma was tested by microtechniques to conserve resources. Additionally, ARDP facilitated the international searches through the ISBT's IRDP and acted as a liaison to the Food and Drug Administration (FDA).

Results

A 2-year-old female of Pakistani origin presented to a children's hospital complaining of abdominal pain, constipation and fevers. Radiologic studies revealed a large mass involving the left kidney, aorta, coeliac and mesenteric arteries. She was diagnosed with stage IV neuroblastoma with metastasis to the bone marrow The patient was anaemic (haemoglobin-8·2 g/dl) on admission, and two units of RBCs were ordered. Initial testing performed at the admitting hospital included a blood type and antibody detection. An antibody panel using Gel technique (Ortho Clinical Diagnostics, Raritan NJ) detected nonspecific weak reactivity. The autocontrol was non-reactive. All commonly encountered clinically significant alloantibodies were excluded. Additional testing was performed using tube technique. Testing performed with low ionic strength saline (LISS) (Alba Bioscience, Peniduik, UK) and with polyethylene glycol (PEG) (Immucor, Norcross, GA) techniques were negative.. On hospital day two, two crossmatch compatible RBCs were transfused, and the post-transfusion haemoglobin was 9·1 g/dl. As the patient's haemoglobin had decreased to 7·5 g/dl by hospital day five, another unit of RBCs was ordered. The antibody detection test was negative. One unit of crossmatch compatible RBCs was transfused to the patient. During the transfusion, the patient developed an increase in temperature and a transfusion reaction investigation was initiated. There was no evidence of clerical error, haemolysis or icterus detected in the post-transfusion sample. Pre- and post-transfusion direct antiglobulin tests (DAT) were microscopically positive with polyspecific antihuman globulin (AHG) and anti-IgG, negative with anti-C3. The eluate panel was non-reactive. The post-transfusion haemoglobin on hospital day six was 8·2 g/dl. The transfusion reaction interpretation attributed the fever to the patient's underlying medical condition. On hospital day six, after the patient was transfused with 60 ml of RBCs she developed an increase in temperature. A second transfusion reaction investigation was initiated. Findings were the same as the previous transfusion reaction and the fever was again attributed to the patient's underlying condition. The patient's haemoglobin decreased to 7·2 g/dl by hospital day 10. This time the antibody detection test was positive (3+) in the Gel technique with a positive autocontrol (2+).

The patient's sample was sent to the BC's IRL to determine if there were any underlying alloantibodies and for antibody identification. Initial pretransfusion testing consisted of ABO/Rh, RBC phenotyping, DAT, elution, tube testing with PEG, LISS, ficin and DTT methods. Based on the test results and reported patient Middle-Eastern

ethnicity, selected (rare) red cells were chosen for testing with an In(b–) red cell being non-reactive. An anti-E, reactive only in ficin-IAT technique was also identified. Puzzling was the patient's RBCs were reactive with two examples of anti-In^b . The sera was a frozen human source. It is still unclear as to why this sera was reactive with the patient's cells. One possibility is that the sera may have contained an additional undetermined antigen specificity. A decision was made to send the sample for *IN* genotyping, and antibody identification confirmation to ensure a variant antigen was not present.

The patient's genotype was determined to be *IN*01/01* by sequencing of *IN* exons 2, 3, 5 and 15. The antibody was confirmed by the second IRL to be anti-In^b. The patient's red cells were predicted to be In(b–).

The MMA results showed 68% reactive monocytes (negative control range 0–3%) indicating the anti-In^b was clinically significant, a finding that was unsurprising based on the results of a decrease in haemoglobin with the initial two transfusions [1].

Once the anti-In^b was identified, the clinicians were informed of the risk of transfusion and no blood was provided until compatible units could be identified. The case was discussed extensively with both the clinical team and the patient's parents. The usual haemoglobin threshold for transfusion of paediatric oncology patients at the children's hospital was 7·0 g/dl. Due to the anticipated difficulty of obtaining units and the projected course of treatment, the clinical team agreed to decrease the transfusion threshold in this case to 6·0 g/dl with close monitoring.

The BC began working with other BCs in the US and Canada in an attempt to obtain compatible units. The efforts by the local BC's IRL to contact BCs in the US and Canada with large Middle-Eastern demographics resulted in discovery of the first compatible donor, which also became a vital source of screening reagent as the donor also had anti-Inb. The ARDP database search of over 65 000 rare donors yielded no registered donors matching the type needed and the request from the ARDP to members did not yield units or suitable donors. As there were no US donors or units initially identified, no possibility of autologous donation, no eligible siblings, and with the patient's haemolytic reaction to In(b+) units, it was decided that an international search was needed. In(b–) units were ordered. An emergency investigational new drug (eIND) application was initiated by the patient's physician at the children's hospital, approved by the parents and submitted to the FDA along with a letter stating the blood would be tracked, transfused only to this patient and destroyed if not used by her. FDA approval allowed ARDP to request In(b–) RBC units from the ISBT's IRDP managed by the International Blood Group Reference Laboratory (IBGRL) at the National Health Service (NHS) in Bristol, United Kingdom (UK), and since the need was urgent, members of the ISBT Working Party on Rare Donors were also contacted.

The BC media strategies directed prospective US and Canadian donors to go to their nearest BC for collection and tagging of donated blood. The unit was held at the local BC and two segments and a specialized form for this particular patient were sent via overnight shipping to the BC's IRL (local to the patient) for testing. Referring BC's were emailed test results within 18 h of segment receipt. Donor source anti-In^b reagent was diluted 1:64 with 3% albumin and tested using microtechniques(either microplate or microcapillary) to maximize the reagent availability [2].

The rarity of the blood necessitated creation of 100 ml doses based on the weight of the patient. This was to provide the optimal transfusion dose required and conserve the remainder of the unit's RBCs. The patient received a total of ten aliquots and one unit was used to prime the apheresis device for the autologous peripheral blood stem cell collection.

The patient began chemotherapy with topotecan and cytoxan on 13 September 2018 her haemoglobin decreased from 7·2 g/dl to 5·8 g/dl 2 days later. A California IRL had an unconfirmed (In^b type with a single unlicensed antisera) but probable In(b–), E– unit with the donor's plasma with presumed anti-In^b. The unit was compatible, shipped, thawed and aliquoted for two transfusions within 24 h. Her haemoglobin improved to 8·5 g/dl post-transfusion. (Fig. 1).

In the US and Canada, 77 BC's submitted segments from 2262 units combined with the IRL's BC donors (1935) totalled 4197 units tested. Only two donors were In(b–), but unfortunately, both were E+ and not suitable the patient. They were entered into the ARDP database for future needs of other patients requiring In(b–) units.

After the Facebook Live show, more than 25 000 people meeting ethnicity donation criteria responded by email. Once a comprehensive show regarding the patient was aired and a press release was issued to local and national news media, the story went viral. By the next morning, national news in the US followed by international media were reporting the story. The BC's communications team provided the media with easy to understand information along with a video tool kit that included interviews from the Facebook Live show resulting in a consistent message resonating around the world. The communications team documented every development of the patient's journey and produced eight Facebook Live shows between December 2018 and April 2019. Each development resulted in media coverage and additional donor response.

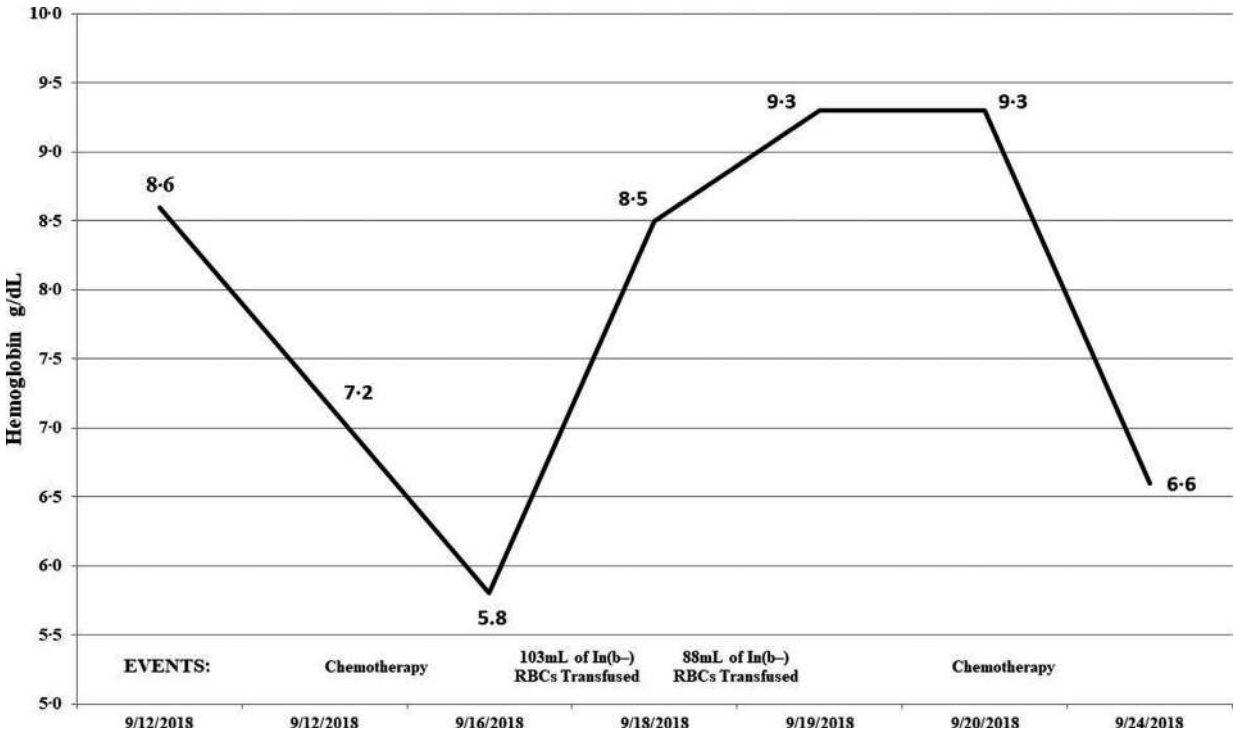

Fig. 1 Patient's haemoglobin shows changes that occurred due to hospital events. Note improvement with transfusion of In(b–) red blood cells.

Outside of the US, donors and units were identified first by the ISBT's IRDP with two donors from the UK and subsequently one donor in Australia. One unit was freshly collected in the UK by the NHS Blood and Transplant and ARDP facilitated the transportation arrangements into the US (Fig. 2). The second donor from the UK and the donor from Australia were contacted, both presented and were collected, and the units sent to the US. FDA was notified of importation. These three units and subsequent donations by two US donors completed the required five In (b–), E– units needed for the patient to start aggressive chemotherapy.

Discussion

Neuroblastoma (NB) is a paediatric tumour presenting at diagnosis either as localized or metastatic disease, which mainly involves the bone marrow (BM).The physical occupancy of BM space by metastatic NB cells has been held responsible for impairment of BM function resulting in anaemia. A recent study showed that the impairment was selective and occurred only in the erythrocyte lineage, regardless of the presence of metastatic NB cells. Both myeloid and lymphoid-lineages were normally represented in the BM, as well as the proportion of pro-erythroblasts, basoerythroblasts and polychromatophilic erythroblasts. The authors of this study were unable to identify a potential cause for such a specific impairment of late stage erythrocyte maturation [3].

The In^a antigen was discovered in 1973 with the detection of an antibody directed against a low-prevalence antigen in the serum of hyperimmunized Indian blood donors [4]. Subsequently, Giles showed that cells that did not react with the Salis antibody (the historical name for anti-In^b) were positive with two anti-In^a sera. Two families were available for study and Salis became In^b, the high frequency antithetical antigen to In^a [5]. The gene encoding the In^a and In^b antigens is inherited in an autosomal dominant fashion independent of other blood group systems. This antigen polymorphism is encoded by the single nucleotide polymorphism 252G > C in CD44 [6]. The In^a and In^b antigens are efficient immunogens and the anti-In^a and anti-In^b antibodies are considered clinically significant [7]. The calculated prevalence of the In(a+ b–) phenotype, based on the estimated frequency of the In^a antigen, ranges from 0·01 to 0·09 per cent in the Indian subcontinent to 0·3 per cent in Iranians and 0·4 per cent in Saudi Arabians visiting hospitals in Bombay [7]. The extremely low number of In (b–) donors identified by the BC's IRL two out of 4197 donors tested (0·048%), was unexpected.

The patient's story sparked a global call to action to donate blood and re-emphasized the need for an ethnically diverse donor base Social media sparked a viral response that was unprecedented resulting in both media and public

Fig. 2 BC Immunohematology Reference Laboratory personnel receive first internationally shipped unit from the United Kingdom.

interest in the vital role of BCs and the ARDP in supplying rare blood.[8] The donor response was tremendous, and unique strategies were created to optimize testing and managing collaborations between centres.

The organized logistical collaboration from participating BC allowed for responsive donors nationwide to be tested expeditiously, and the use of microtechniques exponentially increased the testing capacity. While a match was not found among the units tested, domestic donors and international searches by ARDP ultimately resulted in identifying five compatible donors and the patient's blood needs were met. She subsequently underwent tumour resection, tandem autologous bone marrow transplants and radiation therapy. Treatment was completed approximately seven months after her hospital admission. She was discharged to home and to date has been in remission.

Constant communication between BCs, hospital transfusion services, ARDP, IRDP, FDA as well as grass roots efforts to recruit donors for compatible blood negative for high prevalence antigen In^b ensured RBCs were available and contributed to a young girl's survival.

The carefully executed communications strategy resulted in additional compatible donors being found for the patient, brought the importance of rare blood donors to the forefront and gave the world an awareness on why diversity of the blood supply is important.

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Conflict of interest

The authors have no conflicts of interest to report.

References

1 Nance S, Arndt P, Garratty G: Predicting the clinical significance of red cell alloantibodies using a monocyte monolayer assay. *Transfusion* 1987; **27**:449–452

2 Judd JW, Johnson ST, Storry JR: *Judd's Methods in Immunohematology*, 3rd edn. Bethesda, MD, AABB Press, 2008:216–225

3 Morandi F, Barco S, Stigliani S, *et al.*: Altered erythropoiesis and decreased number of erythrocytes in children with neuroblastoma. *Oncotarget* 2017; **8**:53194–53209

4 Xu Q: The Indian blood group system. *Immunohematology* 2011; **27**:89–93

5 Giles CM: Antithetical relationship of anti-In^a with the Salis antibody. *Vox Sang* 1975; **29**:73–6

6 Lopez GH, McBean RS, Wilson B: Molecular typing for the Indian blood group associated 252G>C single nucleotide polymorphism in a selected cohort of Australian blood donors. *Blood Transfus* 2015; **13**:78–85

7 Joshi SR: Immediate haemolytic transfusion reaction due to anti-In^b. *Vox Sang* 1992; **63**:232–233

8 Nance S, Scharberg EA, Thornton N, *et al.*: International rare donor panels: a review. *Vox Sang* 2016; **110**:209–218

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First annual report of Chinese haemovigilance network

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Abstract

Background and objectives Haemovigilance involves surveillance of the whole chain of blood transfusion with the aim of identifying adverse events and errors and improving outcomes for patients. The Chinese Haemovigilance Network, founded in August 2017, has witnessed a rapid development in the last three years.

Materials and methods Based on the 1,022 cases in 2019, we analysed the adverse reactions (ARs) by blood component, clinical outcome severity and demography of recipients in an effort to publish the first annual Chinese haemovigilance report.

Results The AR rate associated with blood transfusion in 2019 was 0·2% in China. Allergic reactions and FNHTR were the two most common adverse symptoms, accounting for 97·7% of the reports. Two-thirds of the TAD, AHTR and TACO and all of the HTR and DHTR resulted in hospitalization or prolongation of hospitalization. Plasma and AP were usually associated with allergic reaction (81·1%), whereas red cells more commonly cause FNHTR (68·8%) and all the AHTR, HTR, DSTR and DHTR. 84·1% of patients were aged 16 years or over, and the majority of the TAD, AHTR, TACO and HTR involved patients aged 60 and above. The ratio of serious adverse reactions (SARs) was 8·2%. Allergic reaction and FNHTR were top two (85·7%) SARs. The first case related to anti-D immunoglobulin was detected in a DHTR report.

Conclusion This report provides the world's first overview of transfusion-related adverse reactions in China. This report is useful for better understanding transfusion risks in China.

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Key words: haemovigilance, blood transfusion, adverse reaction, annual report, Chinese Haemovigilance Network, 2019.

Introduction

Haemovigilance is 'a set of surveillance procedures covering the whole transfusion chain (from the collection of blood and its components to the follow-up of recipients), intended to collect and assess information on unexpected or undesirable effects resulting from the therapeutic use of labile blood products, and to prevent their occurrence or recurrence' [1]. China has witnessed a consistent growth in the number of voluntary blood donations since the Law of Blood Donation took effect in 1998. In 2018, around 25 million units of whole blood were collected for clinical use by 353 blood banks all over the country (compared to < 5 million units in 1998). When blood products became more available in China, patient safety was given higher priority [2]. All blood services are administered by the National Health Commission (NHC), while transfusion medicine is promoted by the Chinese Society of Blood Transfusion (CSBT). To guide clinical transfusion and the preparation of blood components, NHC promulgated the 'Standard Operating Procedure of Blood Transfusion' in 2000, updated the 'Measures for the Administration of the Clinical Use of Blood in Medical Institutions' in 2012 and has revised the 'Standard Operating Procedures for Blood Banks' once every three years. Although China has made impressive achievements in blood safety over the past two decades, universal leucocyte reduction, FFP (fresh-frozen plasma) from male-only donors and a national surveillance system for transfusion-related adverse events, among other arrangements, are still absent.

In response to the call for the Healthy China 2030 strategy proposed by the Chinese government [3], and the Global Effort launched by the World Health Organization (WHO) to halve medication-related errors in 5 Years [4], five institutions of the Chinese Academy of Medical Sciences (CAMS), Institute of Blood Transfusion, Peking Union Medical College Hospital, Cancer Hospital (National Cancel Center), Fuwai Hospital (National Center for Cardiovascular Diseases), Institute of Basic Medicine, and Beijing Red Cross Blood Center, jointly applied for and were granted the CAMS Innovation Fund for Medical Sciences in November 2016. This project, entitled *Mechanisms and Intervention of Adverse Transfusion Reactions*, aimed at promoting quality, safety and efficacy of blood products, and in doing so, improving recipient outcome.

Supported by the grant and learned from other countries (e.g. UK, United States of America, Australia), project participants defined a framework of a haemovigilance system in line with international recommendations [5,6], published standard definitions of adverse reactions, severity and imputability, designed the clinical diagnostic criteria and treatment guidance; developed web-based reporting application, provided webinar content and in-person education and training to doctors, nurses, and laboratory scientists all over the country in the last three years.

Chinese Haemovigilance Network (CHN) was founded and became a member of the International Haemovigilance Network (IHN) in August 2017. Of the 188 participating hospitals from 29 of 31 provinces (only covering the Chinese mainland in this programme, as Hong Kong, Macao and Taiwan have independent systems), all of them are Grade III and Class A healthcare institutions (the highest quality in China, for which there are a total of 1,442 in the country), and they all consumed the most of blood products in their respective administrative areas. CHN Online Reporting System was officially launched to collect cases in May 2018. Up to 1 April 2020, there were 2,158 AR reports submitted from 62 hospitals in 28 provinces.

In line with the spirit of 'sharing and analysing data for benchmarking and practice improvement', as called for by the IHN [7], here we published the first annual report of CHN. We hope that the international community would find this work useful for better understanding of the Chinese efforts to improve patient outcome. Feedback is always welcome.

Materials and methods

Adverse reaction case classification criteria

There were totally 13 transfusion-related adverse reactions be defined: transfusion-associated circulatory overload (TACO), transfusion-related acute lung injury (TRALI), transfusion-associated dyspnoea (TAD), allergic reaction, hypotensive transfusion reaction (HTR), febrile non-haemolytic transfusion reaction (FNHTR), acute haemolytic transfusion reaction (AHTR), delayed haemolytic transfusion reaction (DHTR), delayed serologic transfusion reaction (DSTR), transfusion-associated graft *vs*. host disease (TAGVHD), post-transfusion purpura (PTP),

transfusion-transmitted infection (TTI) and Other/Unknown. For each reaction, three diagnostic criteria (definitive, probable and possible), five severity levels (non-severe, severe, life-threatening, death and not determined) and six imputability categories (definite, probable, possible, doubtful, ruled out and not determined) were established for clinical investigation (Fig. S1). These criteria were translated from the U.S. CDC recommendations [8].

Data collection

CHN Online Reporting System (https://www.ihn-cn.com:8000) is a web-based application for haemovigilance data reporting. In this system, 8 modules (Patient Details, Component Details, Vital Signs, Pre-transfusion Testing, Post-transfusion Testing, Reaction Details, Treatment/Support, Investigation Results) are operated by healthcare facilities to create new reports, and 2 modules (Procedure Review, Confirmation and Finalization of a notification for an adverse reaction) are used by CHN Steering Group to review (Table S1). Currently, the system only captures adverse reactions in patients, as process- and procedure-related errors and outcomes are not yet captured. All participating hospitals gave their reports freely and voluntarily. All submitted cases were reviewed and were given feedback regardless of whether they met the classification criteria or not.

Statistical analysis

Reports made to CHN Online Reporting System were collected from 1 January 2019 31 to December 2019. In statistical analysis, cases with imputability being possible, doubtful, ruled out or not determined were excluded. Serious adverse reactions (SARs) were defined as the severity of the cases being severe, life-threatening and death-causing. Given the monthly bulletin of patients transfused (Fig. S2 and Table S2), some hospitals apparently did not submit all ARs observed in their recipients. Therefore, only the data of top ten hospitals in Table S2 were selected for calculation of the annual rate of ARs in 2019.

Results

In 2019, there were totally 1,022 transfusion-related adverse cases reported to CHN (Fig. S2 and Table S2), with 8 cases proved irrelevant to blood transfusion, and 108 cases with imputability being possible, doubtful, ruled out or not determined. The 56 reporting hospitals were from 38 cities in 27 provinces.

Rate of ARs

Based on data from the top ten reporting hospitals, the rate of ARs in 2019 was 0·2% (543/270,990) in China (Table S3). Each of these hospitals had more than 2·6 million outpatient encounters this year. One in three (270,990/703,707) surgeries needed to be handled with blood transfusion. For each transfused case, patient received on average 0·9 U red cells, 108·4 mL plasma, 2·5 doses AP and 0·3 U cryoprecipitate. Whole blood was not so common in use (3/10), while pooled platelet was chosen by only one hospital. Eight out ten institutions applied cell salvage (autologous) transfusion methods; however, the average consumption of red cells was only a handful of 1·9 mL.

Reactions in patient

After the CHN Steering Group review, 906 cases were confirmed as definite or probable adverse reactions associated with blood transfusion and further analysed in the annual report (Fig. 1, Table 1). These cases involved 9 reaction types in patient, 9 blood products for clinical use and 9 age groups of recipients. For ARs, allergic reaction was the biggest risk of transfusion with 668/906 (73·7%), followed by FNHTR 217/906 (24%). These 2 reactions contributed to 97·7% ARs of this year, while the remaining 7 were 2·3% (TAD 0·7%, Others 0·4%, AHTR 0·3%, TACO 0·3%, HTR 0·2%, DSTR, 0·2%, DHTR 0·1%).

813/885 (91·9%) of allergic reactions and FNHTR, however, were classified as non-severe, which means the patient may have required symptomatic treatment, but lack of such would not result in immediate risk to the life. In contrast, the development of two-thirds of the TAD, AHTR, TACO and all of HTR and DHTR might bring about an inpatient hospitalization or prolongation of hospitalization. There were no severe DSTR cases this year.

For age distribution of recipients, the majority of ARs occurred during 40-70 years of age and among children, 575/906 (63·5%), with the next groups being 16-40 and 70-80, 281/906 (31%). The majority of severe reactions (i.e. TAD, AHTR, TACO, HTR) involved patients aged 60 and above. The frequency of the AR cases followed a normal distribution across different age groups ($P = 0·8$, one-sample K-S test). Overall, there were 53·5% male and 46·5% female cases in 2019. It is important to note that more cases are needed to statistically support the gender ratio.

Reactions by product

Further investigation was launched into blood components as a possible source of the reactions (Fig. 1,

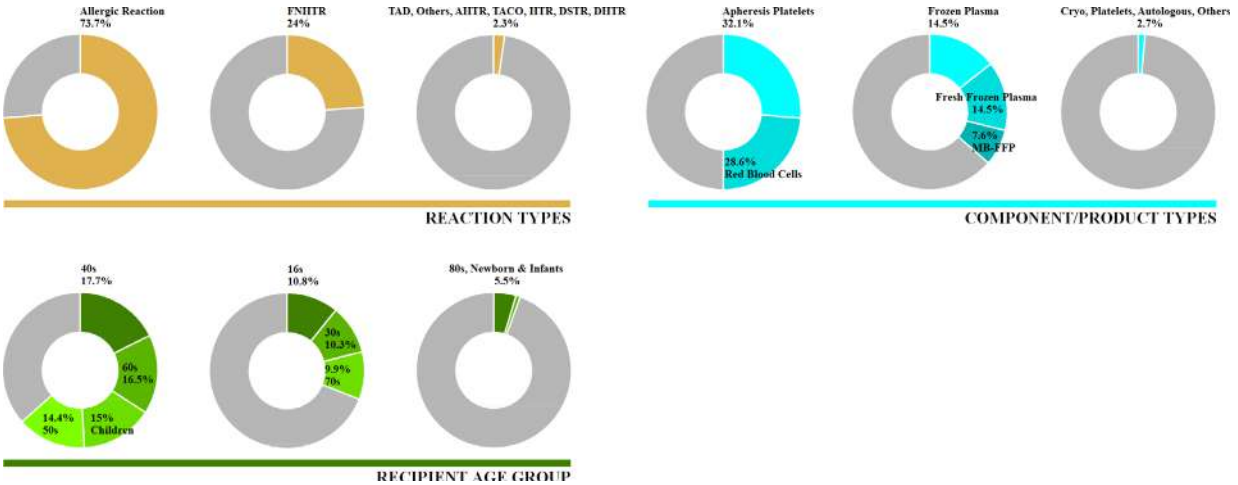

Fig. 1 Percentages of 906 reactions in each category. MB-FFP, methylene blue-treated FFP (fresh-frozen plasma); Cypo, cryoprecipitate, derived from the centrifugation of 200mL of FFP (1U); Platelets, derived from the centrifugation of 200mL of whole blood (1U); Autologous, autologous red cells, for cell salvage transfusion; Children, >=1 year to < 16 years; Newborn, >=0 days and < 28 days; Infants, >28 days and < 1 year

Table 1). AP and RBC were the 2 major causes and accounted for 60·7% of the ARs. The use of plasma components (e.g. FP, FFP, MB-FFP) reached more than one-third (36·6%). Cryoprecipitate, pooled platelets, autologous blood and others together were 2·7%.

As the only reaction involving all blood components, allergic cases were most often implicated in usage of plasma and AP, accounting for 81·1% of the reports. Red cells caused 68·8% of FNHTR, and all haemolytic transfusion reactions (e.g. AHTR, DHTR; 4/4), HTR (2/2) and DSTR (2/2). 66·7% of the incidence of TACO were linked to FFP. It is worth noting that only a very small proportion of reactions were related to the transfusion of cryoprecipitate (allergic reactions 1·6%, TACO 33·3%).

3 AHTR cases occurred between 127 and 175 minutes after starting red cell transfusion. Three patients developed symptoms consistent with TACO during 50 to 199 minutes of the transfusion. Two patients exhibited profound hypotension during transfusion over 5-20 minutes. For 96·7% of the times, reaction occurrence was within standard hours. Overtime cases implied that overnight/out-of-hours transfusion should be given with the same attention to patient observation [9].

Serious adverse reactions

SAR reports made to CHN during 2019 were 84/1022 (8·2%) (Table S4). Allergic reaction (42/84, 50%) and FNHTR (30/84, 35·7%) were the top two SARs. Despite gender ratio of SARs being similar, male patients with allergic reaction and AHTR were apparently more than females (26 *vs.* 16, 2 *vs.* 0, respectively). In contrast, women outnumbered men for FNHTR and TAD in the ratio of three to one. 38/42 (90·5%) of serious allergic symptoms were found within 2 hours of transfusion, while more than half of the reactions (24/42, 57·1%) occurred during an AP transfusion.

Red cells (16/30, 53·3%) and AP (11/30, 36·7%) were the two main components that induced FNHTR between 1 and 4 hours of transfusion. 2/2 TACO cases involved the use of FFP. 2/2 HTR occurred within 20 minutes of transfusion, and 2/2 AHTR between 2 and 3 hours of transfusion. The only DHTR case developed on the 11th day after cessation of transfusion was related to anti-D immunoglobulin, where a 53-year-old lady (D-negative) with multiple myeloma was issued with 5·5 units of D-positive leucocytes reduced red cells. TAD was characterized by respiratory distress within 24 hours of transfusion [10]; however, all 4 cases in the report were observed with 90 minutes of transfusion. A likely explanation was that 3/4 of the TAD patients were children.

There was no definite or probable life-threatening case or death. The only 'Death' record in 2019 was an 87-year-old woman with coronary atherosclerotic heart disease. She developed shortness of breath and a rise in blood pressure from 140/80 to 210/110 mmHg during the second unit RBC. Despite aggressive treatment, the patient died of an acute left heart failure. For lack of laboratory evidence of brain natriuretic peptide, central venous pressure levels, chest X-ray or positive fluid balance, this case was confirmed as '**possible** TACO'.

Errors related to reaction investigation

264 reports failed to meet the classification criteria in 2019, which accounted for 25·8% of ARs cases (Table S5).

Table 1 Reactions by product, clinical outcome severity, gender, age group and transfusion time, 2019-2020

	Allergic Reaction	FNHTR	TAD	AHTR	TACO	HTR	DSTR	DHTR	Others	*sum*
Cases	668	217	6	3	3	2	2	1	4	906
AP	259 (38·3%)	29 (13·3%)	2 (33·3%)						3 (75%)	293 (32·1%)
RBC	105 (15·5%)	150 (68·8%)	1 (16·7%)	3 (100%)		2 (100%)	2 (100%)	1 (100%)		261 (28·6%)
FP	121 (17·9%)	11 (5·1%)								132 (14·5%)
FFP	111 (16·4%)	16 (7·3%)	2 (33·3%)		2 (66·7%)				1 (25%)	132 (14·5%)
MB-FFP	57 (8·4%)	11 (5·1%)	1 (16·7%)							69 (7·6%)
Cryo	11 (1·6%)				1 (33·3%)					12 (1·3%)
Platelets	7 (1·0%)									7 (0·8%)
Autologous	1 (0·2%)									1 (0·1%)
Others	4 (0·6%)	1 (0·5%)								5 (0·5%)
Non-severe	626 (93·7%)	187 (86·2%)	2 (33·3%)	1 (33·3%)	1 (33·3%)		2 (100%)		3 (75%)	822 (90·7%)
Severe	42 (6·3%)	30 (13·8%)	4 (66·7%)	2 (66·7%)	2 (66·7%)	2 (100%)		1 (100%)	1 (25%)	84 (9·3%)
Male	372 (55·7%)	103 (47·5%)	2 (33·3%)	2 (66·7%)	2 (66·7%)	1 (50%)	1 (50%)		2 (50%)	485 (53·5%)
Female	296 (44·3%)	114 (52·5%)	4 (66·7%)	1 (33·3%)	1 (33·3%)	1 (50%)	1 (50%)	1 (100%)	2 (50%)	421 (46·5%)
Newborn	2 (0·3%)									2 (0·2%)
Infants	6 (0·9%)									6 (0·7%)
Children	129 (19·3%)	4 (1·9%)	3 (50%)							136 (15·0%)
16s	80 (12·0%)	17 (7·9%)							1 (25%)	98 (10·8%)
30s	77 (11·5%)	16 (7·4%)								93 (10·3%)
40s	125 (18·7%)	33 (15·2%)							2 (50%)	160 (17·7%)
50s	87 (13·0%)	39 (18·0%)				1 (50%)	1 (50%)	1 (100%)	1 (25%)	130 (14·4%)
60s	87 (13·0%)	56 (25·8%)	2 (33·3%)	1 (33·3%)	2 (66·7%)	1 (50%)				149 (16·5%)
70s	48 (7·2%)	38 (17·5%)	1 (16·7%)	1 (33·3%)	1 (33·3%)		1 (50%)			90 (9·9%)
80s	27 (4·0%)	14 (6·5%)		1 (33·3%)						42 (4·6%)
Time of reaction occurrence (hour, during transfusion)										
0·5	119 (17·8%)	34 (15·7%)	2 (33·3%)			2 (100%)				157 (17·3%)
1	236 (35·3%)	40 (18·4%)	1 (16·7%)		1 (33·3%)					278 (30·7%)
2	189 (28·3%)	78 (35·9%)	3 (50%)				1 (50%)		4 (100%)	275 (30·4%)
4	101 (15·1%)	58 (26·7%)		3 (100%)	2 (66·7%)					164 (18·1%)
24	23 (3·4%)	7 (3·2%)								30 (3·3%)
>24							1(D28) (50%)	1(D11) (100%)		2 (0·2%)

AP, apheresis platelets; RBC, red blood cells in additive solution (1 U, 200 mL); FP, frozen plasma; FFP, fresh-frozen plasma; MB-FFP, methylene blue-treated FFP; Cypo, cryoprecipitate, derived from the centrifugation of 200 mL. of FFP (1 U); Platelets, derived from the centrifugation of 200 mL. of whole blood (1 U.); Autologous, autologous red cells, for cell salvage transfusion; Newborn, >=0 days and < 28 days; Infants, >28 days and < 1 year; Children, >=1 year to < 16 years; D28/D11, the 28^{th}/11^{th} day after cessation of transfusion.

In these errors, wrong imputability contributed to 51·4%, wrong severity to 28·9%, and wrong reaction to 19·7%. The number of cases that none of case definition, severity or imputability fit the criteria was 44/264 (16·7%). For case definition, primary errors were allergic reactions and FNHTR. The presence/absence of fever and chills, meanwhile, made allergic reactions and FNHTR two most confusing ARs to each other (14/20 allergic reactions mistaken for FNHTR, 16/19 FNHTR mistaken for allergic reactions).

Combining the monthly horizon of reporting (Fig. S2), a strong uphill linear relationship between the error rates and the number of cases submitted was shown ($r = 0{\cdot}8$, $P < 0{\cdot}01$, Pearson's correlation). Linear regression analysis revealed that one case would be wrong for every five cases reported ($\hat{Y} = 5{\cdot}1 + 0{\cdot}2X$, $R^2 = 0{\cdot}6$). Unfortunately, the error rate did not change as time went on. From this perspective, clinical staffs have not learned from haemovigilance yet.

Discussion

On behalf of CHN, we conducted the first annual Chinese haemovigilance report. This report provides an overview of transfusion-related adverse reactions in 2019. It represents historical progress in understanding the risks associated with transfusion in China. In this report, 9 categories of reactions related to transfusion were reviewed. No cases of TRALI, TTI, PTP and TAGVHD were reported. First, the incidences of TAGVHD and PTP were rare in most countries [9,10]. Then, for lack of TRALI cases, the reason might be due to fewer female donors, and the majority of plasma collected in China is sent for further manufacture into plasma derivatives [2]. For lack of TTI cases, it was partly because nucleic acid test (NAT) was adopted for all types of donation since 2016 and partly because only three viruses (HIV, hepatitis B and hepatitis C) were required for NAT screening.

There were no definite life-threatening cases or deaths reported in 2019. Considering that, on average, more than 27,000 patients received transfusion in each of the hundreds of hospitals, this result clearly does not tell the whole story. It might be associated with poor recognition of medication-related errors and the 'blame' culture, where managers feared to take responsibility for the incidents. The health administrative departments were under huge pressure in recent years, given the huge media interest in the tensions between patients and medical providers. Another barrier that must be mentioned is that the Department of Blood Transfusion (DBT) holds a very weak position in Chinese hospitals. Big part of DBT's job is laboratory tests and administration of blood components, with almost no voice in clinical decision-making. This has greatly limited the development of transfusion medicine.

The establishment of CHN provides a national focus on haemovigilance with the aim of improving transfusion practice in China. From adverse reaction to adverse incident, from blood transfusion to blood donation, a quality assurance system will be built from vessel to vessel, promoting safety and quality in the Chinese blood sector.

Conflict of interest

The authors have no conflict of interest.

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References

1 IHN: About: Haemovigilance [Internet]. Schuttersveld, 2020. [cited 2020 Oct 9]. Available from: https://www.ihn-org.com/about/haemovigilance.
2 Yin YH, Li CQ, Liu Z: Blood donation in China: sustaining efforts and challenges in achieving safety and availability. *Transfusion* 2015; **55**:2523–2530
3 Tan X, Liu X, Shao H: Healthy China 2030: a vision for health care. *Value Health Reg Issues* 2017; **12**:112–114
4 WHO: WHO launches global effort to halve medication-related errors in 5 years. Geneva, 2017 [cited 2020 Feb 29]. Available from: http://www.who.int/mediacentre/news/releases/2017/medication-related-errors/en/
5 Wood EM, Stevenson L, Bielby L, *et al.*: Haemovigilance: concepts and frameworks. *ISBT Sci Series* 2014; **9**:86–90
6 WHO: A guide to establishing a national haemovigilance system [Internet]. Geneva, 2016 [Cited 2020 Mar 1]. Available from: https://www.who.int/bloodsafety/haemovigilance/haemovigilance-guide/en/
7 IHN: What we do [Internet]. Schuttersveld, 2020. [cited 2020 Mar 2]. Available from: https://www.ihn-org.com/
8 U.S.CDC: National Healthcare Safety Network Biovigilance Component Hemovigilance Module Surveillance Protocol [Internet]. Atlanta, 2018. [cited 2020 Mar 2]. Available from: http://www.cdc.gov/nhsn/PDFs/Biovigilance/BV-HV-protocol-current.pdf
9 National Blood Authority: Australian Haemovigilance Report, Data for 2013-14 [Internet]. Canberra, 2016.

[cited 2020 Apr 2]. Available from: https://www.blood.gov.au/haemovigilance-reporting.

10 Narayan S, Poles D, *et al.* on behalf of the Serious Hazards of Transfusion (SHOT) Steering Group: The 2018 Annual SHOT Report, ed 1. Manchester (UK), SHOT Office, 2019

Supporting Information

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

Figs S1 and S2
Tables S1–S5

VoxSanguinis
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A newly devised flow cytometric antibody binding assay helps evaluation of dithiothreitol treatment for the inactivation of CD38 on red blood cells

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Vox Sanguinis

Abstract

Background and objectives Anti-CD38 monoclonal antibodies, including daratumumab and isatuximab, often interfere with pretransfusion testing. Dithiothreitol (DTT) treatment of red blood cells (RBCs) negates this interference. However, the optimum DTT concentration and treatment time have not been well defined. Here, we quantified CD38 on RBCs before and after DTT treatment using a flow cytometric antibody binding assay (FABA) to specify the optimum conditions for CD38 inactivation.

Materials and methods For FABA, untreated or DTT-treated RBCs were incubated with fluorescein isothiocyanate-labelled anti-CD38 antibody, in the presence or absence of 100-fold or more excess of unlabelled anti-CD38 antibody, and then analysed by flow cytometry (FCM). Dissociation of CD38-positive and control histograms was determined from the D-value using the Kolmogorov–Smirnov test. The results from FABA were compared with those from conventional FCM, indirect antiglobulin test (IAT) and Western blotting.

Results The results from FABA were more consistent than those from conventional FCM. The D-value was found to be reliable in the analysis of difference between CD38 before and after DTT treatment. Our data showed that 0·0075 mol/l DTT for 30 min is sufficient to inactivate CD38 on RBCs. These results were stable and consistent with the findings from IAT.

Conclusion Flow cytometric antibody binding assay is an objective way of evaluating the efficacy of DTT treatment for CD38 on RBCs. This approach allows the detection of a small number of cell surface antigens and will be useful for assessing the various chemical treatments to denature RBC antigens.

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Key words: multiple myeloma, daratumumab, CD38, dithiothreitol treatment, flow cytometry.

Introduction

Despite improvements in the response and survival rates resulting from novel therapies, most patients with multiple myeloma (MM) experience recurrence and must receive several lines of treatment [1]. In recent years, new proteasome inhibitors have been used for the treatment of MM patients [2,3]. In addition, histone deacetylase inhibitors and monoclonal antibodies (MoAbs) targeting SLAMF7 or CD38 play an important role in the treatment of MM [4,5]. Anti-CD38 MoAbs, including daratumumab (DARA) and isatuximab (ISA), have been shown to display a high level of efficacy for relapsed and refractory MM cases [6–9].

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CD38 is a 45 kDa single-chain transmembrane glycoprotein and contains disulphide bonding to maintain secondary structure [10,11]. The high-level expression of CD38 on MM cells makes it an important therapeutic target [12]. Anti-CD38 MoAbs induces cell death through complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), induction of apoptosis and modulation of CD38 enzyme activity [13]. However, CD38 is also present on the surface of red blood cells (RBCs) [10] to which residual free anti-CD38 MoAbs in the plasma can bind. Thus, panreactivity often arises in the indirect antiglobulin test (IAT) used for antibody detection, antibody identification and crossmatch. This panreactivity can conceal the presence of clinically significant RBC antibodies [14–16], leading to time-consuming pretransfusion testing and complicating the management of patients. Appropriate mitigation strategies are needed to minimize delays and allow the safe provision of RBCs to patients.

Several approaches have recently emerged to overcome problems resulting from panreactivity. Such strategies include inactivation of CD38 on RBCs by dithiothreitol (DTT) or trypsin, introduction of CD38-negative reagent RBCs, neutralization of free anti-CD38 MoAbs in plasma with soluble CD38 or anti-CD38 MoAbs idiotype-matched antibody, and antigen-matched RBC transfusions [15,17–19]. Of these, pretreatment of RBCs with DTT, a sulfhydryl agent that cleaves disulphide bonds and disrupts the protein secondary structure, has been widely introduced because this technique is inexpensive and easy to apply [14,20]. Appropriate DTT treatment disrupts the antigenic binding sites of CD38 on RBCs, and prevents anti-CD38 MoAbs from binding to reagent RBCs. The DTT treatment disrupts and weakens all antigens of the Kell blood group system, with anti-K being the most clinically important antibodies against this group [14]. Additionally, other minor antigens, such as Dombrock, Indian, John Milton Hagen, Landsteiner-Wiener, Lutheran, Raph, Cartwright and Cromor, are also DTT-sensitive, although antibodies to them are rarely found [21]. This unwanted side-effect results in impaired sensitivity for the detection of antibodies against these blood group antigens, which can lead to delayed transfusion haemolytic reactions (DHTR) after antigen-positive RBC transfusion [22]. Therefore, it is important to perform DTT treatment of reagent RBCs under more appropriate conditions [23]. Reducing the concentration of DTT might be sufficient to prevent the destruction of K and other minor antigens [24,25]. However, the optimum concentration and treatment time for DTT are not well defined, especially in the treatment of anti-CD38 MoAbs [15,26]. For example, the evaluation of RBC agglutination during the IAT relies on the visual judgement of medical staff. Moreover, the protocol used for the DTT treatment of reagent RBCs varies between facilities, and reagent RBCs supplied from different sources vary in their characteristics.

We previously developed a newly devised flow cytometric antibody binding assay (FABA, Pat. No. 2585973) for receptor analysis in our laboratory [27–32]. This assay enables the quantitative detection of low levels of cell surface receptors by employing the D-value in the Kolmogorov–Smirnov (K-S) test [33]. Here, using this highly sensitive method, we quantified CD38 on RBCs before and after DTT treatment and then assessed the optimum DTT concentration and treatment time for CD38 inactivation on RBCs. We also demonstrate that FABA is a valuable method for evaluating low levels of RBC antigens.

Materials and methods

DTT treatment of RBCs

Dithiothreitol treatment of RBCs was performed according to the AABB technical manual (Fig. 1) [34]. A 0·2 mol/l DTT solution was prepared by dissolving 1 g of DTT (Bio-Rad Laboratories Inc., Tokyo, Japan) in 32 mL of phosphate-buffered saline, pH 8·0 (PBS; Fujifilm-Wako Pure Chemical Co., Ltd., Tokyo, Japan). RBCs from healthy donors were suspended to give a 3% RBC suspension in PBS, pH 7·3 (Sysmex Co., Tokyo, Japan). The suspensions and reagent cells (Surgiscreen; Ortho-Clinical Diagnostics, Tokyo, Japan) (100 μl; $n = 3$, respectively) were washed four times with PBS, pH 7·3 (Sysmex Co., Tokyo, Japan) using an automatic cell washer (MC450; Koki Holdings Co., Ltd., Tokyo, Japan). One volume of washed RBCs (3 μl) was treated with four volumes of the prepared DTT solution (12 μl; 0·2, 0·1, 0·05, 0·01, 0·0075, 0·0050, 0·0025, 0·001 and 0·0001 mol/l). The final concentrations of DTT were 0·16, 0·08 0·04, 0·008, 0·006, 0·004, 0·002, 0·0008 and 0·00008 mol/l, respectively. The mixtures were incubated at 37°C for 5-min intervals from 0 to 30 min with periodic mixing. The treated RBCs were subsequently washed four times and resuspended to a 3% suspension in PBS.

Detection of CD38 on RBCs by flow cytometry

For conventional flow cytometry (FCM) analysis (Fig. 2A), the 3% suspensions of untreated or DTT-treated RBCs ($6{\cdot}0 \times 10^5$/tube) were stained with fluorescein isothiocyanate (FITC) mouse anti-human CD38 MoAb (Becton Dickinson, Tokyo, Japan) or, as controls, FITC mouse IgG1κ isotype control (Becton Dickinson) in a total volume of 100 μl PBS. The optimum concentration of each antibody was determined according to the manufacturer's

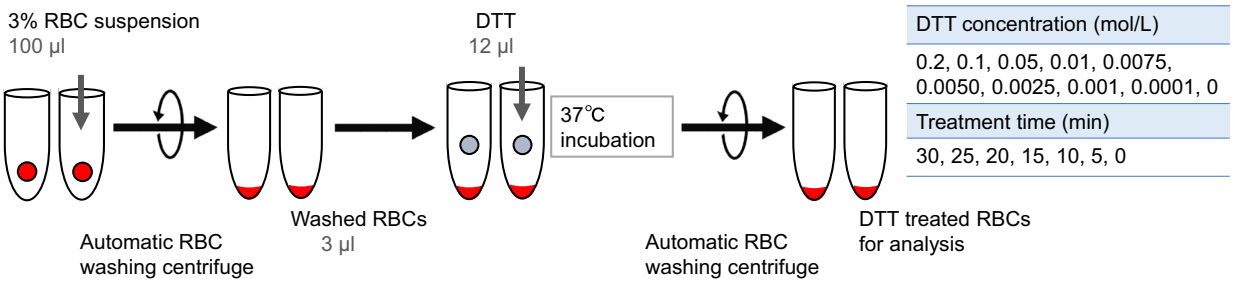

Fig. 1 DTT treatment of RBCs. One volume of RBCs from healthy donors or reagent cells ($n = 3$, respectively) was treated with four volumes of the prepared DTT solution according to the AABB technical manual. The mixtures were incubated at 37°C for 5-min intervals from 0 to 30 min with periodic mixing by inversion.

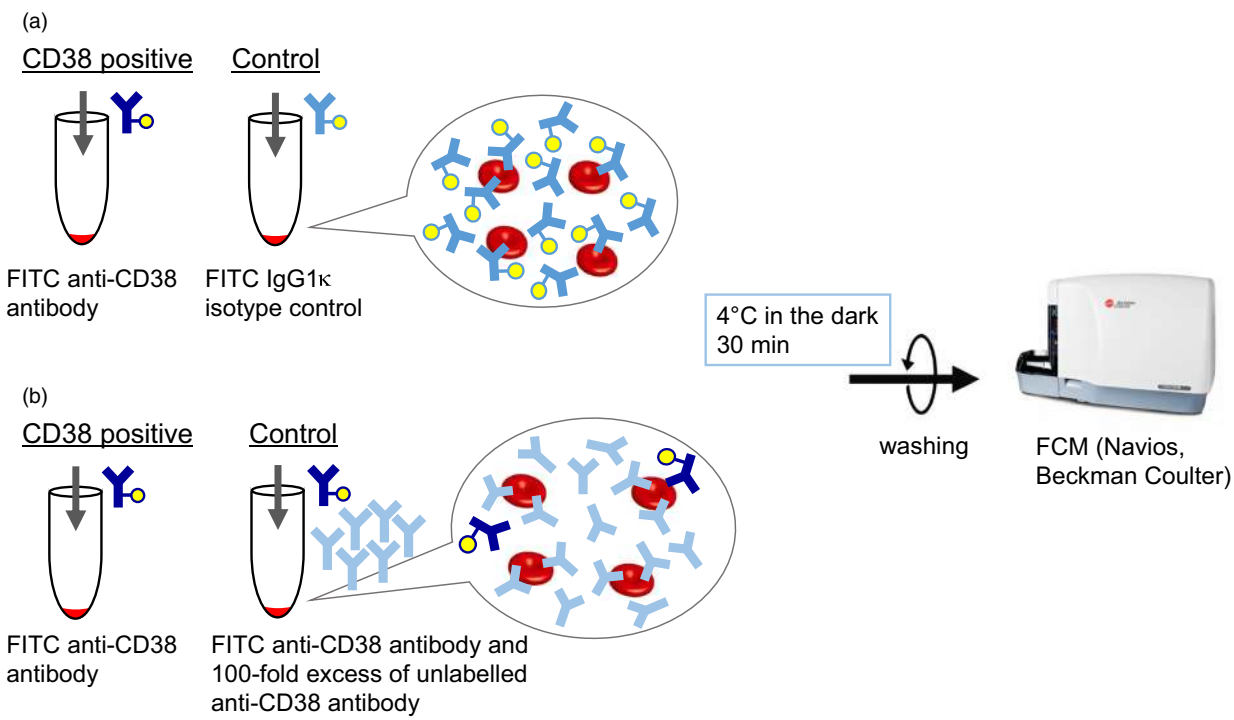

Fig. 2 Evaluation of DTT-treated RBCs using FCM. (A) In conventional FCM, untreated or DTT-treated RBCs were stained with FITC anti-CD38 antibody or, as controls, FITC $IgG1_{\kappa}$ isotype control. (B) In flow cytometric antibody binding assay (FABA), untreated or DTT-treated RBCs were stained with FITC anti-CD38 antibody in the presence or absence of a 100-fold excess of unlabelled anti-CD38 antibody. In both methods, RBCs were incubated at 4°C for 30 min in the dark, washed and analysed by flow cytometry.

recommendations. Each antibody was incubated with the RBCs at 4°C for 30 min in the dark. After washing three times with PBS, RBCs were resuspended in 250 µl of PBS and then analysed by FCM (Navios; Beckman Coulter, Inc., Tokyo, Japan).

For FABA (Fig. 2B), the 3% suspensions of untreated or DTT-treated RBCs ($6{\cdot}0 \times 10^5$/tube) were stained with FITC mouse anti-human CD38 MoAb (Becton Dickinson) in the presence or absence of a 100-fold excess of unlabelled anti-human CD38 MoAb (Janssen R&D, Spring House, PA) in a total volume of 100 µl PBS. The cells were incubated at 4°C for 30 min in the dark, washed and then analysed by flow cytometry in the same way as conventional FCM.

List mode data recorded 10 000 events per sample. Data analysis and graphics generation were performed using Kaluza software 1.2 (Beckman Coulter, Inc.).

Quantification of CD38 on RBCs after DTT treatment

In conventional FCM, dissociation of CD38-positive and control histograms is usually based on mean fluorescence intensity (MFI). In FABA, CD38 is quantified from the D-

value in a K-S test. Details of the calculation method have been described previously [29–32]. Briefly, two histograms are generated, A and B, composed of n_1 and n_2 cells, respectively (Fig. 3). From the histograms, $Fn_1(r)$ and $Fn_2(r)$ are calculated as shown below:

$$Fn_1(r) = \sum_{x=0}^{r} fn_1(x),\ Fn_2(r) = \sum_{x=0}^{r} fn_2(x)$$

$$Fn_1(0) = Fn_2(0) = 0,\ Fn_1(\infty) = Fn_2(\infty) = 1.$$

where the fluorescence intensity, $fn_1(r)$ and $fn_2(r)$, is the relative frequency at r in histograms A and B, and $Fn_1(r)$ and $Fn_2(r)$ are the accumulative relative frequency at r. The D-value is calculated over the total value or r:

$$D - \text{value} = \text{maximum value}|Fn_1(r) - Fn_2(r)|$$

As shown in Figure 3C, the D-value is the maximum vertical difference between two cumulative frequency distributions as a ratio of the total number of cells analysed [33]. The cumulative frequency distribution of FITC fluorescence intensity in the absence of unlabelled anti-human CD38 MoAb (total binding) was compared with that in the presence of an excess of unlabelled anti-human CD38 MoAb (non-specific binding). This comparison allowed the detection of an increase in fluorescence intensity resulting from specific binding of FITC anti-human CD38 MoAb to CD38. If two histograms are identical, the D-value is 0, which means that tested RBCs have no CD38.

IAT tube testing

CD38 on untreated or DTT-treated RBCs was analysed by polyethylene glycol (PEG; Immucor Inc., Tokyo, Japan)-IAT tube testing with plasma (healthy donors without RBC antibodies; $n = 3$) added in DARA (final concentration 10 μg/ml; Janssen R&D). The method for PEG-IAT followed the steps outlined in the AABB technical manual [34]. In addition, K antigens on untreated or DTT-treated RBCs of reagent cells were also analysed by IAT tube testing with the anti-K sera (Ortho-Clinical Diagnostics). In both cases, agglutination reactions were assessed based on the AABB technical manual as follows: 0, w+, 1+, 2+, 3+ and 4+.

Determination of anti-K titration

Anti-K titration was performed using a tube test technique following the standard procedure described in the AABB technical manual [34]. Briefly, serial twofold dilutions of anti-K sera in PBS were prepared. Each diluted anti-K sera (100 μl) was placed into a test tube, and then, the 3% suspensions of untreated or DTT-treated RBCs (50 μl) added. The mixtures were incubated at 37°C for 15 min according to the manufacturer's protocol. This step was performed based on the IAT tube testing described previously. The titre was determined from the highest dilution of anti-K sera that gives a reaction 1+.

Western blotting (WB)

Red blood cell membranes were obtained by single-step haemolysis [35]. Briefly, untreated or DTT-treated RBCs were resuspended in PBS (20 mOsm, pH 7·4). After 10 min, RBC membranes were sedimented at 30 000× g for 25 min, washed four times and then lysed with radioimmunoprecipitation (RIPA) lysis buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

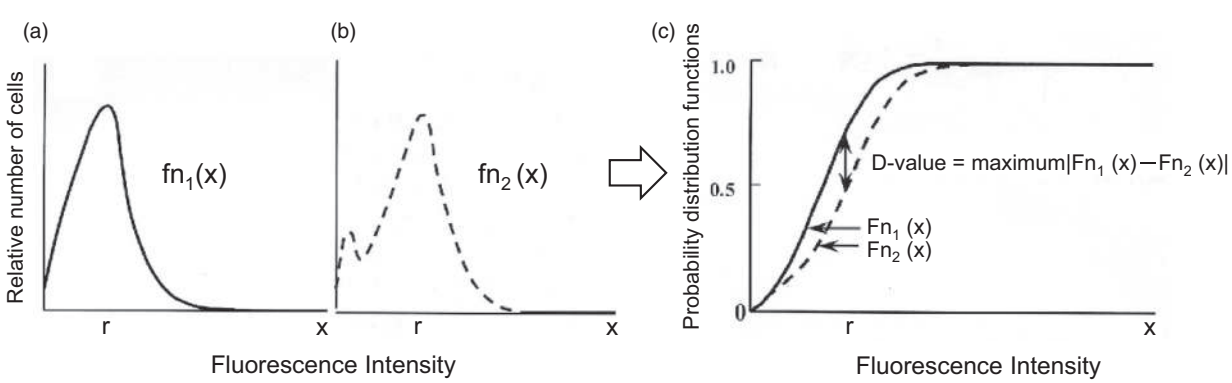

Fig. 3 Calculation of the D-value by the K-S test. Using FABA, CD38 on RBC was quantified from the D-value in the Kolmogorov–Smirnov (K-S) test. Two histograms with respective total cell numbers of n1 (A: CD38-positive) and n2 (B: Control) are assumed to be the function of $fn_1(x)$ and $fn_2(x)$, respectively. When their respective cumulative frequency distribution function is Fn1(x) and Fn2(x), the D-value is the maximum vertical difference between $Fn_1(x)$ and $Fn_2(x)$ (C).

Each lysate containing 30 μg of proteins was loaded into the wells of a 5–20% gradient polyacrylamide gel (ATTO Co., Tokyo, Japan). Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Bio-Rad Laboratories, Inc.). After blocking, the membranes were incubated with a mouse monoclonal anti-CD38 antibody (GeneTex Inc., Irvine, CA) or a rabbit polyclonal anti-β-actin antibody (Cell Signaling Technology, Inc., Beverly, MA), and then incubated with horseradish peroxidase (HRP) secondary antibodies (Cell Signaling Technology, Inc.). The optimum concentration of each antibody was determined according to the manufacturer's recommendations.

Statistical analysis

Paired *t*-test was used to compare the difference of MFIs and the D-value between DTT-treated and untreated RBCs, with significance set at $p < 0{\cdot}05$ by SPSS 25.0 (SPSS Inc, Chicago, IL).

Results

Evaluation of CD38 on RBCs after DTT treatment by conventional FCM and FABA

We evaluated DTT concentrations and treatment times for CD38 inactivation on RBCs by four different methods. The availability of FABA was assessed by comparing with conventional FCM, as well as IAT or WB. In conventional FCM, using FITC-labelled isotype-matched antibody as control, the difference of MFIs between CD38-positive and the control displayed considerable variability in repeated analyses (Fig. 4A). For example, MFI of the control was sometimes higher than that of CD38-positive, and consequently, the difference of MFIs was a minus quantity in the samples after 0·2–0·0025 mol/l DTT treatment.

By contrast, in FABA, using a 100-fold excess concentration of unlabelled anti-human CD38 MoAb as control, the values obtained were more consistent than those obtained using conventional FCM (Fig. 4B). Thus, the D-values were found to be reliable in the analysis of the difference between CD38 on RBCs before and after DTT treatment. The D-value was 0 in the samples after 0·2–0·05 mol/l DTT treatment for 5 min, 0·01 mol/l for 10 min, 0·0075 mol/l for 20 min and 0·0050 mol/l for 25 min (untreated RBCs vs. DTT-treated RBCs; each p value is <0·001). Indeed, the D-value was quantifiable after 0·0025 mol/l DTT treatment for 30 min.

Evaluation of CD38 and K on RBCs after DTT treatment by IAT tube testing

In PEG-IAT tube testing, we detected agglutination due to residual CD38 on RBCs after DTT treatment (Fig. 5). The reaction strength was 0 in the samples after 0·2–-0·05 mol/l DTT treatment for 10-30 min, 0·01 mol/l for 20-30 min, and 0·0075 mol/l for 30 min, but 1+ in the untreated RBCs (Table 1A). By contrast, reaction strength was w+ in samples after 0·01 mol/l DTT treatment for 10 min, 0·0075 mol/l for 20 min and 0·0050 mol/l for 20–30 min, and 1+ after 0·0075–0·0050 mol/l for 10 min and 0·0025–0·0001 mol/l even for 10–30 min.

Additionally, we evaluated K on RBCs after DTT treatment by IAT tube testing. The reaction strength was 2+ in the samples after 0·1 mol/l DTT treatment for 10–20 min and 0·05–0·001 mol/l for 10–30 min, while it was 3+ in the untreated RBCs (Table 1B). By contrast, reaction strength was 1+ in samples after 0·2 mol/l DTT treatment for 10 min and 0·1 mol/l for 30 min, and 0 after 0·2 mol/l for 20–30 min. The antigenicity of K on RBCs was preserved after 0·0075 mol/l DTT for 30 min, which is the lowest concentration of DTT that disrupts CD38. However, the reactivity with anti-K decreased 1:16–1:4 (Table 2).

Evaluation of CD38 on RBCs after DTT treatment by WB

We also assessed the optimum DTT concentrations and treatment times by WB, which enabled the detection of a low level of antigens. WB determined CD38 in the samples after 0·01–0·001 mol/l DTT treatment for 10–30 min, but CD38 was denatured in those after 0·2 mol/l DTT treatment for 10–30 min (Fig. 6).

Discussion

Anti-CD38 MoAbs have shown promising results in the treatment of refractory and relapsed MM [8]. Unfortunately, anti-CD38 MoAbs interfere with pretransfusion testing for up to six months after treatment, which often leads to delays in the provision of RBC transfusions. Among previously described approaches to negate the interference [15,17–19], DTT treatment has emerged as one of the most useful methods and has been widely adopted [14]. However, the optimum concentration and treatment time for DTT have not been well defined. Here, in order to resolve these problems, we have used the FABA technique [29–32]. The major advantage of FABA is to quantitatively detect low levels of cell surface receptors with high specificity and sensitivity. Therefore, we

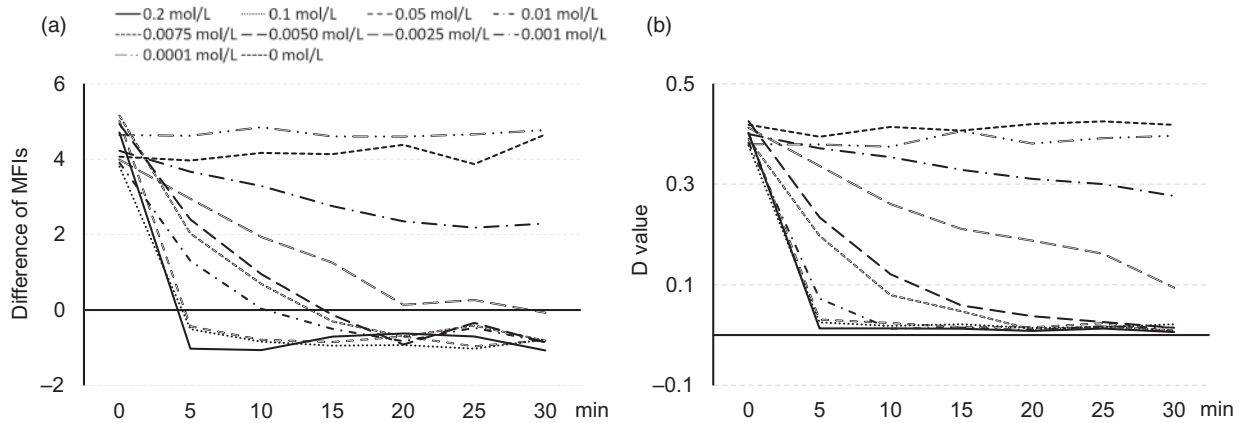

Fig. 4 Evaluation of dissociation of CD38-positive and control histograms after DTT treatment by conventional FCM (A) and FABA (B). (A) In conventional FCM, we adopted the difference of mean fluorescence intensities (MFIs) between CD38-positive and control histograms (mean, $n = 3$) as the amount of CD38 on RBCs. Vertical and horizontal lines show the difference of MFIs and the treatment time of RBCs with DTT, respectively. (B) In FABA, we adopted a D-value as the amount of CD38 on RBCs. The D-value is the maximum vertical difference between two cumulative frequency distributions for the histograms of CD38-positive and control samples. Values were calculated using the K-S test (mean, $n = 3$). Vertical and horizontal lines show D-value and the treatment time of RBCs with DTT, respectively. Details of the method are described in the main text.

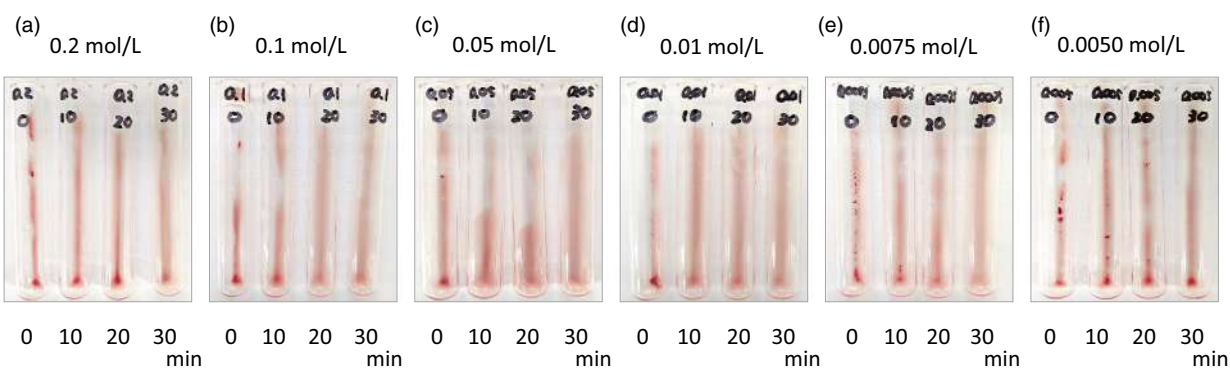

Fig. 5 Agglutination reactions for CD38 on RBCs after treatment with various concentrations of DTT (0·2–0·0050 mol/l) by PEG-IAT. Free CD38 on untreated or DTT-treated RBCs ($n = 3$) was quantitated by PEG-IAT tube testing. Reaction strength (0, w+, 1+, 2+, 3+ and 4+) was assessed with agglutination reactions [34]. Agglutination reactions are shown for various DTT concentrations and treatment times. DTT concentrations were (A) 0·2 mol/l, (B) 0·1 mol/l, (C) 0·05 mol/l, (D) 0·01 mol/l, (E) 0·0075 mol/l and (F) 0·0050 mol/l. Treatment times were 0, 10, 20 and 30 min. Representative results from three independent experiments are shown.

reasoned that FABA could be applied to detect CD38 on RBCs. Here, we objectively quantified CD38 on RBCs before and after DTT treatment by adopting our newly devised assay, and assessed the optimum DTT treatment conditions for CD38 inactivation on RBCs.

In FABA, the dissociation of CD38-positive and control histograms was more stable and consistent than using conventional FCM. In addition, we used the D-value to evaluate dissociation of two histograms, which is a suitable method of quantifying a small number of receptors. In developing this approach, we consistently detected subtle changes in CD38 on RBCs before and after DTT treatment. DARA used as the control in FABA can be obtained from residual liquid attached in the vial used in the clinical treatment of DARA after obtaining permission from each institutional review board.

We compared the results from FABA with those from PEG-IAT or WB. The results from FABA correlated with the results of PEG-IAT tube testing. Nonetheless, the results from FABA were more consistent and quantitative by comparison to the PEG-IAT tube testing. WB is generally superior for the detection of a low level of proteins [36], although it might recognize limited epitopes. Indeed, in this study, a low level of CD38 was detectable by WB. However, WB was too sensitive to allow evaluation of DTT treatment for CD38 on RBCs.

Table 1 (A) Summary of reaction strengths for CD38 using plasma samples containing anti-CD38 monoclonal antibody and RBCs treated with DTT at different concentrations and times by PEG-IAT. (B) Summary of reaction strengths for the K antigen using anti-K sera and reagent cells treated with DTT at different concentrations and times by IAT.

(A)

		DTT conc. (mol/l)									
Incubation	**RBC**	**0·2**	**0·1**	**0·05**	**0·01**	**0·0075**	**0·0050**	**0·0025**	**0·001**	**0·0001**	**0**
30 min	#1	0	0	0	0	0	w+	1+	1+	1+	1+
	#2	0	0	0	0	0	0	1+	1+	1+	1+
	#3	0	0	0	0	0	0	w+	1+	1+	1+
Representative results		0	0	0	0	0	w+	1+	1+	1+	1+
20 min	#1	0	0	0	0	w+	w+	1+	1+	1+	1+
	#2	0	0	0	0	w+	w+	1+	1+	1+	1+
	#3	0	0	0	0	0	w+	1+	1+	1+	1+
Representative results		0	0	0	0	w+	w+	1+	1+	1+	1+
10 min	#1	0	0	0	w+	1+	1+	1+	1+	1+	1+
	#2	0	0	0	w+	1+	1+	1+	1+	1+	1+
	#3	0	0	0	w+	w+	1+	1+	1+	1+	1+
Representative results		0	0	0	w+	1+	1+	1+	1+	1+	1+
0 min	#1	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+
	#2	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+
	#3	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+
Representative results		1+	1+	1+	1+	1+	1+	1+	1+	1+	1+

(B)

		DTT conc. (mol/l)									
Incubation	**Reagent cell**	**0·2**	**0·1**	**0·05**	**0·01**	**0·0075**	**0·0050**	**0·0025**	**0·01**	**0·0001**	**0**
30 min	#1	0	1+	2+	2+	2+	2+	2+	2+	3+	3+
	#2	0	1+	2+	2+	2+	2+	2+	2+	3+	3+
	#3	0	w+	1+	2+	2+	2+	2+	2+	3+	3+
Representative results		0	1+	2+	2+	2+	2+	2+	2+	3+	3+
20 min	#1	0	2+	2+	2+	2+	2+	2+	2+	3+	3+
	#2	0	2+	2+	2+	2+	2+	2+	2+	3+	3+
	#3	0	1+	2+	2+	2+	2+	2+	2+	3+	3+
Representative results		0	2+	2+	2+	2+	2+	2+	2+	3+	3+
10 min	#1	1+	2+	2+	2+	2+	2+	2+	3+	3+	3+
	#2	1+	2+	2+	2+	2+	2+	2+	2+	3+	3+
	#3	w+	2+	2+	2+	2+	2+	2+	2+	3+	3+
Representative results		1+	2+	2+	2+	2+	2+	2+	2+	3+	3+
0 min	#1	3+	2+	3+	3+	3+	3+	3+	3+	3+	3+
	#2	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
	#3	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
Representative results		3+	3+	3+	3+	3+	3+	3+	3+	3+	3+

Agglutination was assessed based on the AABB technical manual [34]. The most frequent reaction strengths from three independent experiments are shown as representative results. However, for the reaction strength of CD38 in 0·0050 mol/l DTT treatment 30 min, w + was adopted because all results from three independent experiments were not negative.

Chapuy *et al.* [15] reported that 0·2 mol/l DTT treatment of RBCs was a robust method to negate anti-CD38 MoAbs interference. However, 0·2 mol/l DTT is reported to disrupt several RBC antigens in the KEL, DO, IN and JMH blood group systems [14]. Notably, anti-K is commonly encountered and clinically significant, but

Table 2 Summary of reaction strengths for the K antigen using each serial twofold dilution of anti-K sera and reagent cells before and after DTT treatment by IAT.

		Anti-K titre						
	Reagent cell	**×1**	**×2**	**×4**	**×8**	**×16**	**×32**	**×64**
0 mol/l (untreated)	#1	3+	3+	2+	2+	1+	w+	0
	#2	3+	3+	2+	1+	1+	w+	0
	#3	3+	2+	2+	1+	1+	w+	0
	Representative results	3+	2+	2+	1+	1+	w+	0
0·0075 mol/l DTT, 30 min	#1	2+	1+	1+	w+	0	0	0
	#2	2+	1+	1+	w+	0	0	0
	#3	2+	1+	w+	w+	0	0	0
	Representative results	2+	1+	1+	w+	0	0	0

Agglutination was assessed based on the AABB technical manual [34]. The most frequent reaction strengths from three independent experiments are shown as 'Representative results'.

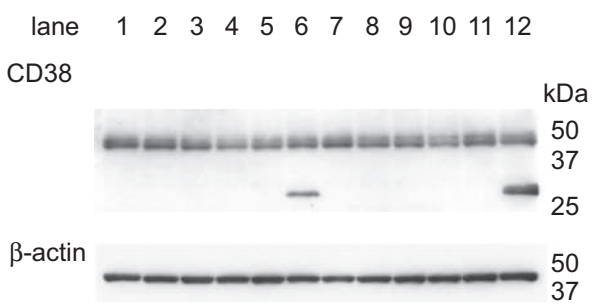

Fig. 6 WB for CD38 expression after DTT treatment. Pellets of RBC membranes were lysed with RIPA lysis buffer and separated on a 5–20% gradient gel by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. CD38 was detected by a mouse anti-CD38 antibody. DTT concentration; Lane 1, 7: 0 mol/l, Lane 2, 8: 0·001 mol/l, Lane 3, 9: 0·0025 mol/l, Lane 4, 10: 0·0050 mol/l, Lane 5, 11: 0·01 mol/l, Lane 6, 12: 0·2 mol/l. Treatment time; Lane 1–6: 10 min, Lane 7–12: 30 min.

antibodies to DTT-sensitive antigens other than K are rare. Therefore, particular care should be taken with K on reagent RBCs in DTT treatment.

Disbro [37] reported excessive haemolysis three days after 0·2 mol/l DTT treatment. Lally *et al.* [38] reported that 0·2 mol/l DTT decreased agglutination strength of RBCs for several alloantibodies by one to two grades in the antibody detection and identification during nine days of storage. In general, reagent RBCs gradually deteriorate during transportation and storage, and become more sensitive to enzyme or chemical modification. Several antigens, such as Fy^a, Fy^b and M, are more susceptible to undergoing antigenic variation [34]. Previous reports suggest that if marked haemolysis occurs after 0·2 mol/l DTT treatment, the procedure should be repeated with fresher reagent RBCs and a smaller volume of DTT [39]. However, these reports did not give details of variations in DTT treatment. Izaguirre *et al.* [24] reported that 0·04 mol/l DTT treatment for 15 min eliminated the DARA interference while preventing haemolysis and maintaining the ability to detect the majority of blood group antibodies using the gel testing. Similar results were reported by Lorenzen *et al.* [40]. Hosokawa *et al.* [25] reported that 0·01 mol/l DTT treatment for 30 min negate the DARA interference and partially preserved K using conventional FCM. These studies have suggested various DTT concentrations and treatment times for CD38 inactivation. The cause of this variation was derived from the difference of evaluation methods for the detection of CD38 on RBCs, such as IAT (tube or gel column testing) and conventional FCM. FABA improved sensitivity and instability in these conventional methods. Here, we used FABA, which enables the detection of subtle changes in CD38 on RBCs. We demonstrated that treatment with 0·0075 mol/l DTT for 30 min is the lowest concentration of DTT that disrupts CD38 on RBCs, while at the same time negating DARA interference and preserving K antigenicity and minimizing haemolysis. Furthermore, we show that the concentration and treatment time of DTT can be configured to each institution by introduction of our methodology.

In conclusion, our newly devised flow cytometric antibody binding assay, FABA, facilitates the detection of RBC antigens present at low levels, including CD38. Furthermore, FABA displays improved reliability over conventional FCM, IAT and WB for these types of analyses. Using this assay, we will be able to determine an appropriate DTT concentration and treatment time for the inactivation of CD38 on RBCs in each institution. Thus, FABA will be a clinically useful assay in the area of transfusion.

Conflict of interest

The authors declare no competing conflicts of interest related to the work described.

Author contributions

HF and AT developed the study design. HF acquired the data. HF and AT analysed the data that were generated. All authors interpreted the data, drafted and reviewed the report, gave their final approval for publication, and agreed to be accountable for all aspects of the work.

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References

1 Röllig C, Knop S, Bornhäuser M: Multiple myeloma. *Lancet* 2015; **385**:2197–208
2 Bianchi G, Richardson PG, Anderson KC: Promising therapies in multiple myeloma. *Blood* 2015; **126**:300–10
3 Lonial S, Boise LH, Kaufman J: How I treat high-risk myeloma. *Blood* 2015; **126**:1536–43
4 Sonneveld P: Management of multiple myeloma in the relapsed/refractory patient. *Hematol Am Soc Hematol Educ Program* 2017; **2017**:508–17
5 Varga C, Maglio M, Ghobrial IM, *et al.*: Current use of monoclonal antibodies in the treatment of multiple myeloma. *Br J Haematol* 2018; **181**:447–59
6 Attal M, Richardson PG, Rajkumar SV, *et al.*: Isatuximab plus pomalidomide and low-dose dexamethasone versus pomalidomide and low-dose dexamethasone in patients with relapsed and refractory multiple myeloma (ICARIA-MM): a randomised, multicentre, open-label, phase 3 study. *Lancet* 2019; **394**:2096–107
7 Petrucci MT, Vozella F: The anti-CD38 antibody therapy in multiple myeloma. *Cells* 2019; **8**:1629
8 van de Donk N, Richardson PG, Malavasi F: CD38 antibodies in multiple myeloma: back to the future. *Blood* 2018; **131**:13–29
9 Varga C, Laubach JP, Anderson KC, *et al.*: Investigational agents in immunotherapy: a new horizon for the treatment of multiple myeloma. *Br J Haematol* 2018; **181**:433–46
10 Deaglio S, Mehta K, Malavasi F: Human CD38: a (r)evolutionary story of enzymes and receptors. *Leuk Res* 2001; **25**:1–12
11 Mehta K, Shahid U, Malavasi F: Human CD38, a cell-surface protein with multiple functions. *FASEB J* 1996; **10**:1408–17
12 Lin P, Owens R, Tricot G, *et al.*: Flow cytometric immunophenotypic analysis of 306 cases of multiple myeloma. *Am J Clin Pathol* 2004; **121**:482–8
13 van de Donk NW, Janmaat ML, Mutis T, *et al.*: Monoclonal antibodies targeting CD38 in hematological malignancies and beyond. *Immunol Rev* 2016; **270**:95–112
14 Chapuy CI, Aguad MD, Nicholson RT, *et al.*: International validation of a dithiothreitol (DTT)-based method to resolve the daratumumab interference with blood compatibility testing. *Transfusion* 2016; **56**:2964–72
15 Chapuy CI, Nicholson RT, Aguad MD, *et al.*: Resolving the daratumumab interference with blood compatibility testing. *Transfusion* 2015; **55**:1545–54
16 Yamada C, Takeshita A, Ohto H, *et al.*: A Japanese multi-institutional collaborative study of antigen-positive red blood cell (RBC) transfusions in patients with corresponding RBC antibodies. *Vox Sang* 2020; **115**:456–65
17 Hannon JL, Clarke G: Transfusion management of patients receiving daratumumab therapy for advanced plasma cell myeloma. *Transfusion* 2015; **55**:2770
18 Oostendorp M, Lammerts van Bueren JJ, Doshi P, *et al.*: When blood transfusion medicine becomes complicated due to interference by monoclonal antibody therapy. *Transfusion* 2015; **55**:1555–62
19 Schmidt AE, Kirkley S, Patel N, *et al.*: An alternative method to dithiothreitol treatment for antibody screening in patients receiving daratumumab. *Transfusion* 2015; **55**:2292–3
20 Cushing MM, DeSimone RA, Goel R, *et al.*: The impact of Daratumumab on transfusion service costs. *Transfusion* 2019; **59**:1252–8
21 Reid ME, Lomas-Francis C, Olsson ML: *The blood group antigen factsbook*, 3rd edn. Oxford: Academic Press, imprint of Elsevier, 2012
22 Quach H, Benson S, Haysom H, *et al.*: Considerations for pre-transfusion immunohaematology testing in patients receiving the anti-CD38 monoclonal antibody daratumumab for the treatment of multiple myeloma. *Intern Med J* 2018; **48**:210–20
23 Wagner FF: Antibody testing in patients treated with anti-CD38: there is still room for improvement. *Blood Transfus* = Trasfusione del sangue 2020; **18**:244-6
24 Izaguirre EC, Del Mar Luis-Hidalgo M, González LL, *et al.*: New method for overcoming the interference produced by anti-CD38 monoclonal antibodies in compatibility testing. *Blood Transfus* = Trasfusione del sangue 2020; **18**:290–4
25 Hosokawa M, Kashiwagi H, Nakayama K, *et al.*: Distinct effects of daratumumab on indirect and direct antiglobulin tests: a new method employing 0.01 mol/L dithiothreitol for negating the daratumumab interference with preserving K antigenicity (Osaka method). *Transfusion* 2018; **58**:3003–13
26 de Vooght KMK, Lozano M, Bueno JL, *et al.*: Vox Sanguinis International Forum on typing and matching strategies in patients on anti-CD38 monoclonal therapy: summary. *Vox Sang* 2018; **113**:492–8

27 Ikegaya N, Yamamoto T, Takeshita A, *et al.*: Elevated erythropoietin receptor and transforming growth factor-beta1 expression in stenotic arteriovenous fistulae used for hemodialysis. *J Am Soc Nephrol* 2000; **11**:928–35

28 Kawasaki T, Takeshita A, Souda K, *et al.*: Serum thrombopoietin levels in patients with chronic hepatitis and liver cirrhosis. *Am J Gastroenterol* 1999; **94**:1918–22

29 Shinjo K, Takeshita A, Nakamura S, *et al.*: Serum thrombopoietin levels in patients correlate inversely with platelet counts during chemotherapy-induced thrombocytopenia. *Leukemia* 1998; **12**:295–300

30 Shinjo K, Takeshita A, Ohnishi K, *et al.*: Expression of granulocyte colony-stimulating factor receptor increases with differentiation in myeloid cells by a newly-devised quantitative flow-cytometric assay. *Br J Haematol* 1995; **91**:783–94

31 Takeshita A, Shinjo K, Higuchi M, *et al.*: Quantitative expression of erythropoietin receptor (EPO-R) on acute leukaemia cells: relationships between the amount of EPO-R and CD phenotypes, in vitro proliferative response, the amount of other cytokine receptors and clinical prognosis. Japan Adult Leukaemia Study Group. *Br J Haematol* 2000; **108**:55–63

32 Takeshita A, Shinjo K, Izumi M, *et al.*: Quantitative expression of thrombopoietin receptor on leukaemia cells from patients with acute myeloid leukaemia and acute lymphoblastic leukaemia. *Br J Haematol* 1998; **100**:283–90

33 Young IT: Proof without prejudice: use of the Kolmogorov-Smirnov test for the analysis of histograms from flow systems and other sources. *J Histochem Cytochem* 1977; **25**:935–41

34 Fung MK, Eder AF, Spitalnik SL, *et al.*: *Technical manual*, 19th edn. Bethesda, MD: American Association of Blood Banks, 2017

35 Schwoch G, Passow H: Preparation and properties of human erythrocyte ghosts. *Mol Cell Biochem* 1973; **2**:197–218

36 Falk BW, Elliott C: Fluorescent monitoring of proteins during sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. *Anal Biochem* 1985; **144**:537–41

37 Disbro WL: Stability guidelines for dithiothreitol-treated red blood cell reagents used for antibody detection methods in patients treated with daratumumab. *Immunohematology* 2017; **33**:105–9

38 Lally K, Hayes C, His R, *et al.*: Extended use of DTT-treated reagent cells in the era of anti-CD38 therapy. *Transfusion* 2016; **56**:136A

39 Youssef M, Arnesen C, Arledge C, *et al.*: Validation and cost-effectiveness of an in-house dithiothreitol (DTT) treatment protocol for daratumumab patients in a large tertiary care hospital provides gateway for implementation in smaller community hospitals. *Transfus Apher Sci* 2019; **58**:152–5

40 Lorenzen H, Lone Akhtar N, Nielsen M, *et al.*: Thirty-three-day storage of dithiothreitol-treated red blood cells used to eliminate daratumumab interference in serological testing. *Vox Sang* 2018; **113**:686–93

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LETTER TO THE EDITOR

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Universal pathogen reduction in blood components is a close perspective

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Previously, we published the results of our clinical study, which included the determination of quality indicators [1] and clinical utilization of pathogen-reduced red blood cell suspension for transfusions in paediatric patients with various oncological and haematological diseases [2].

In general, pathogen reduction technology is actively used in routine clinical practice to ensure the safety of transfusions [3]. Moreover, the use of pathogen-reduced technologies makes it possible to increase the shelf life of pathogen-reduced platelets up to 7 days, as well as to release pathogen-inactivated plasma immediately without having to quarantine it.

After the initial publication, we carried out an additional retrospective analysis of the case histories of the patients participating in the study and found that two patients received exclusively pathogen-reduced blood products, that is plasma, red blood cell and platelet concentrates during one interval between chemotherapy courses. All blood products have been processed by

Table 1 Characteristics of patients and transfusions

	Gender	Age, years	Weight, kg	Diagnosis	Number of RBC transfusions	Number of PLT transfusions	Number of plasma transfusions	Transfusion-dependent period duration, days	The number of days between the next course of chemotherapy
Patient 1	Male	1	8	Nephroblastoma	1	3	2	5	28
Patient 2	Male	4	16	Rhabdoid tumour	1	3	1	7	21

Table 2 Transfusion efficacy

RBC transfusion efficacy

	Hb before transfusion, g/l	Hb after transfusions, g/l	Hb increment, g/l	Ht before transfusion, %	Ht after transfusions, %	Ht increment, %
Patient 1	80	93	13	22·7	26·3	3·6
Patient 2	77	104	27	22·2	29·5	7·3

PLT transfusion efficacy

	PLT before transfusion	PLT after transfusions	PLT increment
Patient 1	5; 11; 27	34; 27; 49	22·3 ± 6·5
Patient 2	17; 20; 15	45; 47; 54	31·3 ± 6·6

Plasma transfusion efficacy

	Fbn before transfusion, g/l	Fbn after transfusion, g/l	Fbn increment, g/l	PT before transfusion, s	PT after transfusion, s	Decrease in PT, s
Patient 1	1; 1·15	2·15; 1.9	0·95 ± 0·3	22; 18·2	18·6; 16·1	2·75 ± 1
Patient 2	1·5	2·5	1	15	13·8	1·2
	TT before transfusion. s	TT after transfusions. s	Decrease in TT. s	aPTT before transfusion. s	aPTT after transfusions. s	Decrease in aPTT. s
Patient 1	36·1; 31·2	31·2; 30·1	3 ± 2·7	47·7; 42·6	42·6; 35	6·25±1·6
Patient 2	27·6	25·6	2	38·5	37·5	1

Hb, haemoglobin; Ht, haematocrit; RBCs, red blood cells; PLTs, platelets; Fbn, fibrinogen; PT, prothrombin time; aPTT, activated partial thromboplastin time; TT, thrombin time.

pathogen reduction technology that acts on the combined action of riboflavin and ultraviolet light (Mirasol PRT; Terumo BCT, Lakewood, CO, USA). Patient characteristics and the number of transfusions during the interval between chemotherapy courses are presented in Table 1.

Patients received transfusion support according to institutional protocol in standard dosages (for RBC—10 ml/kg, FFP—15 ml/kg, PLT—5 ml/kg). The mean duration of the transfusion-dependent period (the number of days from the first to the last transfusion) in these patients was 4 ± 1 (3–5) days, while the mean interval between chemotherapy courses was 24·5 ± 5 (21–28) days. All transfusions were effective (Table 2), and patients did not have any transfusion adverse effects. Thus, we believe this approach for transfusion support of patients can be used in clinical practice. However, clinical trials are needed to determine the effectiveness and safety of this approach. We plan to conduct a clinical study of pathogen-reduced blood products transfusion versus standard transfusion practice in children with oncological and malignant haematological diseases to determine the clinical and cost-effectiveness of this approach.

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None.

Conflict of interest

All authors declare no conflicts of interest.

References

1 Kumukova I, Trakhtman P, Starostin N, Borsakova D, Ignatova A, Bayzyanova Y. Quality assessment of red blood cell suspensions derived from pathogen-reduced whole blood. Vox Sang 2020; https://doi.org/10.1111/vox.13039
2 Trakhtman P, Kumukova I, Starostin N, et al. The pathogen-reduced red blood cell suspension: single centre study of clinical safety and efficacy in children with oncological and haematological diseases. Vox Sang 2019;114:223–31.
3 Alter H. Pathogen reduction: a precautionary principle paradigm. Transfus Med Rev 2008;22:97–102.

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See also http://www.isbtweb.org/congresses/

4.5.2021	IPFA/PEI – The International Workshop on Surveillance and Screening of Blood-borne Pathogens
13–15.5.2021	The Canadian Society for Transfusion Medicine (CSTM) are holding their annual scientific conference virtually in 2021.
26–27.05.21	21st Congress of the European Society for Hemapheresis
5–9.6.2021	ISBT In Focus, the 31st regional congress of the ISBT, will be a virtual event in 2021
17.9.2021	11th BIC International Conference – Advances in Haemostasis and Bleeding Disorders
22–24.9.2021	Deutsche Gesellschaft für Transfusionsmedizin und Immunhämatologie e.V.
23–26.9.2021	16th International Congress on Myelodysplastic Syndromes (MDS 2021)
13–16.11.2021	32nd Regional congress of ISBT, Brisbane, Australia