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# Vox Sanguinis

## The International Journal of Transfusion Medicine

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# Vox Sanguinis

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# Vox Sanguinis

International Journal of Blood Transfusion

**Aims and Scope**

*Vox Sanguinis* reports on all issues related to transfusion medicine, from donor vein to recipient vein, including cellular therapies. Comments, reviews, original articles, short reports and international fora are published, grouped into 10 main sections:

1. Blood Component Collection and Production: Blood collection methods and devices (including apheresis); blood component preparation; inventory management; collection and storage of tissues; quality management and good manufacturing practice; automation and information technology; plasma fractionation techniques and plasma derivatives;
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5. Immunohaematology and Immunogenetics: autoimmunity in haematology; alloimmunity of blood; pre-transfusion testing; complement in immunohaematology; blood phenotyping and genotyping; genetic markers of blood cells and serum proteins: polymorphisms and function; parentage testing and forensic immunohaematology;
6. International Forum: Section in which topics related to any aspects of the transfusion chain (from technical to scientific, including socioeconomic and ethical facets) are discussed by invited participants and summarized by guest editors
7. Patient Blood Management: Bloodless surgery; preoperative anaemia; minimizing blood loss during surgery; alternatives to blood transfusion;
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REVIEW

# Impact of restrictive red blood cell transfusion strategy on thrombosis-related events: A meta-analysis and systematic review

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## Abstract

**Background and Objectives:** There is an ongoing controversy regarding the risks of restrictive and liberal red blood cell (RBC) transfusion strategies. This meta-analysis assessed whether transfusion at a lower threshold was superior to transfusion at a higher threshold, with regard to thrombosis-related events, that is, whether these outcomes can benefit from a restrictive transfusion strategy is debated.

**Materials and Methods:** We searched PubMed, Cochrane Central Register of Controlled Trials and Scopus from inception up to 31 July 2021. We included randomized controlled trials (RCTs) in any clinical setting that evaluated the effects of restrictive versus liberal RBC transfusion in adults. We used random-effects models to calculate the risk ratios (RRs) and 95% confidence intervals (CIs) based on pooled data.

**Results:** Thirty RCTs involving 17,334 participants were included. The pooled RR for thromboembolic events was 0.65 (95% CI 0.44–0.94; $p = 0.020$; $I^2 = 0.0\%$, very low-quality evidence), favouring the restrictive strategy. There were no significant differences in cerebrovascular accidents (RR $=$ 0.83; 95% CI 0.64–1.09; $p = 0.180$; $I^2 = 0.0\%$, very low-quality evidence) or myocardial infarction (RR $=$ 1.05; 95% CI 0.87–1.26; $p = 0.620$; $I^2 = 0.0\%$, low-quality evidence). Subgroup analyses showed that a restrictive (relative to liberal) strategy reduced (1) thromboembolic events in RCTs conducted in North America and (2) myocardial infarctions in the subgroup of RCTs where the restrictive transfusion threshold was 7 g/dl but not in the 8 g/dl subgroup (with a liberal transfusion threshold of 10 g/dl in both subgroups).

**Conclusions:** A restrictive (relative to liberal) transfusion strategy may be effective in reducing venous thrombosis but not arterial thrombosis.

KEYWORDS

cerebrovascular accidents, myocardial infarction, restrictive, thromboembolism, transfusion strategy

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**Highlights**

- A restrictive red blood cell transfusion strategy significantly reduced the risk of thromboembolic events, although the Grading of Recommendations Assessment, Development and Evaluation quality of evidence was very low.
- For cerebrovascular accidents and myocardial infarction, there were no statistically significant differences between restrictive and liberal transfusion strategies.
- Subgroup analyses showed that the restrictive (relative to liberal) transfusion strategy reduced (1) thromboembolic events in trials conducted in North America and (2) myocardial infarctions in the subgroup of trials where the restrictive transfusion threshold was 7 g/dl but not in the 8 g/dl subgroup (with a liberal transfusion threshold of 10 g/dl in both subgroups).

# INTRODUCTION

Red blood cell (RBC) transfusion can increase or maintain oxygen levels in tissues [1], improving anaemia, which can save lives [2, 3]. However, transfusion is associated with several adverse events, such as thromboembolism [4, 5], cerebrovascular accidents [6] and myocardial infarction [1, 7]. The mechanisms behind these adverse events include increased circulating RBC mass [1], increased oxidative stress [4], reduced nitric oxide and/or increased inflammatory mediators [6]. Consequently, an appropriate transfusion strategy should be used to reduce the adverse events.

Haemoglobin or haematocrit thresholds are commonly used when deciding whether to perform a transfusion. The most commonly used trigger for transfusion in the twentieth century was haemoglobin of 10 g/dl or haematocrit of 30% [8]. However, several transfusion guidelines suggest that a restrictive transfusion strategy (haemoglobin <7 or 8 g/dl) is suitable in most clinical settings [9–11]. Nevertheless, whether a restrictive transfusion strategy reduces thrombosis-related events compared to a liberal one remains controversial, with some reviews indicating no significant differences [11–14] and others indicating that restrictive strategies decrease cerebrovascular accidents but increase myocardial infarction [15–17]. However, most of these reviews included only a few randomized controlled trials (RCTs) or evaluated composite outcomes.

In order to conduct a comprehensive meta-analysis comparing the restrictive and liberal transfusion strategies with regard to thrombosis-related events in adults, we included all available RCTs reporting these outcomes. In addition, we also performed various subgroup analyses, such as comparing outcomes between different restrictive transfusion thresholds (with a fixed liberal transfusion threshold) and among study areas, which were rarely considered in other meta-analyses.

# MATERIALS AND METHODS

## Search strategy

We searched PubMed, Cochrane Central Register of Controlled Trials and Scopus from inception to 31 July 2021, using ([blood transfusion*] OR [red blood cell] or RBC or transfus* or haemoglobin) AND (trigger* OR threshold* OR liberal OR restrict* OR strateg*) AND ([randomized controlled trial*] OR [controlled clinical trial*] OR [clinical trial*] OR [randomized trial*] OR trial*). We checked the references of included RCTs for additional relevant articles. The complete search strategy is provided in the Supplementary Material. After records were imported into the EndNote software, duplicate records were removed. Two reviewers (M.M. and C.X.Z.) independently screened the titles and abstracts of the relevant studies. Thereafter, full-text versions were retrieved to further assess eligibility. Disagreements were settled by discussing with other reviewers (J.G.X., Z.C.Z., H.D.L. and O.C.O.).

## Study selection

The eligibility criteria were (1) RCT, (2) compared liberal and restrictive transfusion strategies, (3) reported thrombosis-related events and (4) patients aged ≥16 years. For RCTs that generated multiple publications, we excluded duplicate patients and outcome data.

## Data extraction

Two authors (M.M. and C.X.Z.) independently extracted information about the first author, year of publication, study area (continent where the patients were recruited from), sample size, transfusion thresholds, demographics, medications and clinical outcomes using a data extraction form. Disagreements were resolved based on reaching a consensus among all authors.

## Outcome definitions

Thrombosis-related events can occur in veins or arteries. For venous thrombosis, we included thromboembolic events comprising deep vein thrombosis, pulmonary embolism and thromboembolism. For arterial thrombosis, we included cerebrovascular accidents (stroke or transient ischaemic attack, cerebrovascular attack or cerebral

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ischaemia) and myocardial infarction (myocardial infarction, acute myocardial infarction, acute myocardial ischaemia or stent thrombosis). Detailed definitions are provided in Table S1.

## Risk of bias and quality

Two authors (M.M. and C.X.Z.) independently assessed the risk of bias (RoB) in the outcomes in the RCTs (categorized as 'low', 'some concerns' or 'high') using the revised Cochrane RoB tool (RoB 2) [18, 19]. As the assessment of each RoB domain for each included outcome in any given RCT was the same, we present the RoB results by RCT instead of by outcome in each RCT. The following domains were assessed: randomization process, deviations from intended interventions, missing outcome data, outcome measurement and selection of the reported result.

We also assessed the overall quality of evidence of each outcome (categorized as 'very low', 'low', 'moderate' or 'high') using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) criteria [20].

## Statistical analysis

Mantel–Haenszel risk ratios (RRs) with 95% confidence intervals (CIs) were calculated based on pooled data. Thereafter, we performed subgroup analyses by clinical setting, transfusion threshold, transfusion timing (intra- and post-operatively, peri-operatively, post-operatively or during the hospital/intensive care unit [ICU] stay), transfusion type (leucocyte-reduced or non-leucocyte-reduced RBCs) and study area (continent where the patients were recruited from). To test for statistical heterogeneity, $I^2$ (50% or 85% indicates moderate or substantial heterogeneity, respectively) and $\chi^2$ ($p < 0.05$ indicates significant heterogeneity) were calculated. Random-effect models were used to account for clinical heterogeneity. Several sensitivity analyses were performed to explore various aspects of the trials and review methodology. First, we excluded each trial one at a time and recalculated the pooled effects. Excluding one trial at a time can help investigate how each individual study affects the overall effect size and identify influential studies. Second, we excluded trials with high RoB. Third, we excluded trials from each clinical setting (ICU treatment, cardiac surgery, orthopaedic surgery, vascular surgery, upper gastrointestinal bleeding treatment and cancer treatment) in turn. Fourth, we limited the analysis to trials using specific transfusion thresholds (restrictive transfusion threshold [RTT] = 7, RTT > 7 g/dl, RTT = 7 or 8 g/dl with liberal transfusion threshold [LTT] = 9 g/dl, RTT = 7 or 8 g/dl with LTT = 10 g/dl). Fifth, we excluded trials that involved transfusion during the hospital/ICU stay without a clear indication of transfusion timing. Sixth, we excluded trials that did not report on transfusion type (i.e., whether the RBC transfusion was leucocyte-reduced) and trials that involved either leucocyte-reduced or non-leucocyte-reduced RBCs. Seventh, we excluded trials from each continent in turn.

Funnel plots and Egger's regression were used to assess publication bias. The analyses were performed in Stata 14.0 and Review Manager 5.3.

# RESULTS

## Included RCTs

The search strategy (Figure S1) identified 2358 articles. After removing duplicates, there were 1750 articles. After screening the titles/abstracts, 119 were selected to be reviewed in full. Thirty RCTs (described in Tables 1 and S2), with 17,334 participants (8576 in restrictive groups and 8637 in liberal groups) were included [21-50]. The RCTs were published between 1992 and 2021. The mean age ranged from 35 to 82.3 years.

Clinical settings varied: 10 involved patients undergoing cardiac surgery [27, 32–35, 37, 40, 47–49], 7 involved patients undergoing orthopaedic surgery [23, 24, 26, 38, 45, 46, 50], 7 involved patients treated in ICUs [21, 22, 25, 28, 29, 39, 42], 3 involved patients undergoing vascular surgery [31, 36, 44], 2 involved patients with upper gastrointestinal bleeding [30, 41] and 1 involved patients undergoing cancer treatment [43]. Additionally, 23 RCTs included cardiovascular disease as a baseline characteristic [21–24, 26–30, 32, 35–38, 40, 42, 44–50], 12 excluded patients with anaemia or bleeding [21, 26–29, 41, 42, 45–49], 1 excluded patients with too high transfusion rate [22] and 1 included patients with high transfusion rates [40].

Regarding RTT and LTT, the haemoglobin RTT ranged from 7.0 to 9.0 g/dl [21–26, 28–30, 33–47, 49, 50], with four additional RCTs specifying haematocrit values of 24% or 25% [27, 31, 32, 48]. The haemoglobin LTT ranged from 8.5 to 10.0 g/dl [21–26, 28–30, 33–47, 49, 50], with four additional RCTs specifying haematocrit values of 28%, 30% or 32% [27, 31, 32, 48]. In four RCTs, transfusion was also permitted for symptoms of anaemia in the restrictive group [45–47, 50]. RCTs were divided into the following pairs of subgroups based on RTT alone or RTT plus LTT: (1) RTT = 7 g/dl versus RTT > 7 g/dl; (2) RTT = 7 g/dl and LTT = 9 g/dl versus RTT = 8 g/dl and LTT = 9 g/dl and (3) RTT = 7 g/dl and LTT = 10 g/dl versus RTT = 8 g/dl and LTT = 10 g/dl.

Specific transfusion timing was reported in 13 RCTs: 3 transfused intra- and post-operatively [35, 40, 44]; 6 transfused post-operatively [31, 33, 36, 37, 45, 46] and 4 transfused peri-operatively [23, 27, 34, 43]. For the remaining RCTs, 12 transfused during the hospital/ICU stay [21, 22, 25, 28, 29, 32, 39, 41, 42, 47–49] and the transfusion timing was not reported by the others [24, 26, 30, 38, 50]. Regarding transfusion type, 12 RCTs used leucocyte-reduced RBCs [21, 22, 26, 29, 36, 39, 41, 42, 46–49], 4 used non-leucocyte-reduced RBCs [23, 25, 27, 28] and the remaining 14 did not provide this information [24, 30–35, 37, 38, 40, 43–45, 50]. Regarding the study area, 12 RCTs were conducted in Europe [23, 24, 26, 29, 30, 34, 36–38, 41, 42, 49], 9 in North America [28, 31, 33, 39, 40, 44, 46–48], 4 in South America [21, 22, 25, 27], 2 in Asia [43, 50] and the remaining 3 recruited patients across several different continents [32, 35, 45].

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**TABLE 1** Characteristics of included randomized controlled trials

| Author (year) | Country | Patient type | Setting | Period | Baseline characteristics | Restrictive threshold (g/dl) | Liberal threshold (g/dl) |
|---|---|---|---|---|---|---|---|
| Almeida (2015) | Brazil | Surgical oncology | ICU | 2012 | Age ≥ 18; CVD; cancer; surgery; tobacco use, hypertension, diabetes (excluded pre-existing coagulopathy or anticoagulation therapy, anaemia, and active bleeding) | 7 | 9 |
| Bergamin (2017) | Brazil | Cancer with septic shock | ICU | 2012–2014 | Age ≥ 18; CVD; cancer; infection; smoking, hypertension, diabetes (excluded patients with too high a transfusion rate) | 7 | 9 |
| Bracey (1999) | USA | Elective primary CABG surgery | Cardiac | 1997 | Surgery | 8 | 9 |
| Bush (1997) | USA | Elective aortic or infrainguinal arterial reconstruction | Vascular | 1995–1996 | CVD; surgery; smoking, hypertension, diabetes | 9 | 10 |
| Carson (1998) | USA, UK | Hip fracture | Orthopaedic | 1996–1997 | Hb < 10 g/dl; CVD; surgery; diabetes (excluded anaemia) | 8 | 10 |
| Carson (2011) | USA, Canada | Hip fracture | Orthopaedic | 2004–2009 | Age ≥ 50; Hb < 10 g/dl; CVD; surgery; tobacco use, hypertension, diabetes (excluded anaemia and active bleeding) | 8 | 10 |
| Carson (2013) | USA | Coronary syndrome or stable coronary artery disease undergoing catheterization | Cardiac | 2010–2012 | Age ≥ 18; Hb < 10 g/dl; CVD; surgery; tobacco use, hypertension, diabetes (excluded anaemia and active bleeding) | 8 | 10 |
| Cooper (2011) | USA | AMI | Cardiac | 2003–2009 | Age ≥ 21; haematocrit ≤ 30%; CVD; surgery; tobacco use, hypertension, diabetes (excluded active bleeding) | 24%[a] | 30% |
| Ducrocq (2021) | France, Spain | AMI and anaemia | Cardiac | 2016–2019 | Age ≥ 18; Hb: 7–10 g/dl; CVD; tobacco use, hypertension, diabetes (excluded massive ongoing bleeding) | 8 | 10 |
| Fan (2014) | China | Total hip replacement | Orthopaedic | 2011–2013 | Age > 65; CVD; surgery; hypertension, diabetes | 8 | 10 |
| Foss (2009) | Denmark | Hip fracture | Orthopaedic | 2004–2006 | Age > 65; CVD; surgery; hypertension, diabetes | 8 | 10 |
| Gillies (2020) | UK | Surgery for fractured neck of femur | Orthopaedic | 2017–2019 | Age ≥ 50; CVD; surgery; hypertension, diabetes | 7 | 9 |
| Gobatto (2019) | Brazil | Moderate or severe traumatic brain injury | ICU | 2014–2016 | Age > 18; Hb < 9 g/dl; trauma | 7 | 9 |
| Grover (2006) | UK | Elective total knee or hip arthroplasty | Orthopaedic | Not mentioned | Age ≥ 55; CVD; surgery; smoking, hypertension, diabetes (excluded anaemia) | 8 | 10 |
| Hajjar (2010) | Brazil | Elective cardiac surgery | Cardiac | 2009–2010 | Age ≥ 18; CVD; surgery; smoking, hypertension, diabetes (excluded anaemia) | 24% | 30% |
| Hebert (1999) | Canada | Critically ill with euvolemia | ICU | 1994–1997 | Age ≥ 16; Hb < 9 g/dl; CVD; trauma; infection (excluded anaemia and active bleeding) | 7 | 10 |

(Continues)

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**TABLE 1** (Continued)

| Author (year) | Country | Patient type | Setting | Period | Baseline characteristics | Restrictive threshold (g/dl) | Liberal threshold (g/dl) |
|---|---|---|---|---|---|---|---|
| Holst (2014) | Denmark, Sweden, Norway, Finland | Septic shock | ICU | 2011–2013 | Age ≥ 18; Hb < 9 g/dl; CVD; surgery; infection (excluded life-threatening bleeding) | 7 | 9 |
| Jairath (2015) | UK | Acute upper gastrointestinal bleeding | Upper gastrointestinal bleeding | 2012–2013 | Age ≥ 18; CVD; acute upper gastrointestinal bleeding; hypertension (excluded exsanguinating haemorrhage) | 8 | 10 |
| Johnson (1992) | USA | Elective operations for myocardial revascularization | Vascular | Not mentioned | Surgery | 25% | 32% |
| Koch (2017) | USA, India | Cardiac surgery | Cardiac | 2007–2014 | Age ≥ 18; CVD; surgery; smoking, hypertension, diabetes | 24% | 28% |
| Laine (2017) | Finland | Elective open-heart surgery | Cardiac | 2014–2015 | Surgery | 8 | 10 |
| Mazer (2017) | 19 countries | CABG and/or valve | Cardiac | 2014–2017 | Age ≥ 18; CVD; surgery; diabetes | 7.5 | 8.5, 9.5 |
| Møller (2019) | Denmark | Elective open infra-renal abdominal aortic aneurysm repair or lower-limb bypass | Vascular | 2015–2016 | Age > 40; CVD; surgery; smoking, hypertension, diabetes | 8 | 9.7 |
| Murphy (2015) | UK | CABG and/or valve or major aortic procedure | Cardiac | 2009–2013 | Age > 16; CVD; surgery; diabetes | 7.5 | 9 |
| Nielsen (2014) | Denmark | Hip revision surgery | Orthopaedic | 2009–2011 | Age ≥ 18; CVD; surgery; smoking, hypertension, diabetes | 7.3 | 8.9 |
| Robertson (2014) | USA | Closed head injury | ICU | 2006–2012 | Trauma; surgery; no comorbidities reported | 7 | 10 |
| Shehata (2012) | Canada | Elective cardiac surgery | Cardiac | 2007–2010 | CVD; surgery; hypertension, diabetes; high transfusion rates | 7, 7.5 | 9.5, 10 |
| Villanueva (2013) | Spain | Upper gastrointestinal bleeding | Upper gastrointestinal bleeding | 2003–2009 | Age > 18; upper gastrointestinal bleeding (excluded massive exsanguinating bleeding, major CVD or a recent history of trauma or surgery) | 7 | 9 |
| Walsh (2013) | UK | Mechanically ventilated | ICU | 2009–2010 | Age ≥ 55; Hb < 9 g/dl; CVD (excluded active bleeding) | 7 | 9 |
| Zhang (2020) | China | Cancer surgery | Oncologic | 2012–2016 | Hb < 10 g/dl; surgery; cancer | 7 | 10 |

Abbreviations: AMI, acute myocardial infarction; CABG, coronary artery bypass graft; CVD, cardiovascular disease; Hb, haemoglobin; ICU, intensive care unit.
[a]Transfusion was indicated by haematocrit value.

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## Outcomes

### Thromboembolic events

Based on 13 RCTs (3976 participants) reporting on thromboembolic events, the risk was significantly lower in the restrictive group than the liberal group (RR = 0.65; 95% CI 0.44–0.94; $p = 0.020$; Figure 1). Study heterogeneity was not significant ($\chi^2 = 10.79$; degrees of freedom [df] = 12 [$p = 0.55$]; $I^2 = 0.0\%$). Subgroup analyses were then performed. Regarding the clinical setting, there was no significant difference in thromboembolic events between the two transfusion strategies in any clinical setting subgroup assessed (Figure S2).

Regarding transfusion threshold, there was no significant difference in thromboembolic events between the two transfusion strategies in the RTT = 7 g/dl or RTT > 7 g/dl subgroups (Figure S3). However, the risk of thromboembolic events was significantly lower in the restrictive (relative to liberal) transfusion group in the RTT = 7 g/dl and LTT = 10 g/dl subgroup (RR = 0.37; 95% CI 0.17–0.79; Figure S4) but not the RTT = 8 g/dl and LTT = 10 g/dl subgroup; nevertheless, there was only one RCT included in the former subgroup.

Regarding transfusion timing, no significant difference was observed in thromboembolic events between the two transfusion strategies in the intra- and post-operative, peri-operative or post-operative subgroups (Figure S5). Regarding transfusion type, there was no significant difference in thromboembolic events between the two transfusion strategies in the non-leucocyte-reduced or leucocyte-reduced RBC subgroups (Figure S6). Lastly, regarding the study area, there were fewer thromboembolic events in the restrictive (relative to liberal) group in trials conducted in North America (RR = 0.50; 95% CI 0.28–0.87; Figure S7) but not in trials conducted in Europe, South America and Asia.

Sensitivity analysis showed that, after removing the trial by Robertson et al. [39] or Jairath et al. [30], there was no longer a significant difference in thromboembolism between the restrictive and liberal groups. Likewise, there was no longer a significant difference in thromboembolism after removing trials involving ICU treatment, orthopaedic surgery, or upper gastrointestinal bleeding treatment, or after limiting the analysis to trials reporting on transfusion timing (intra- and post-operative, peri-operative or post-operative), trials reporting on transfusion type (leucocyte-reduced or non-leucocyte-reduced RBCs) and non-North American trials. Limiting the analysis to trials involving 'low'/'some concerns' RoB; RTT = 7 or 8 g/dl with LTT = 10 g/dl; and RTT = 7 g/dl and LTT = 10 g/dl maintained the significant decrease in thromboembolism for the restrictive (relative to liberal) strategy.

### Cerebrovascular accidents

Based on 21 RCTs (14,509 participants) reporting on cerebrovascular accidents, the risk did not differ by restrictive versus liberal strategy (RR = 0.83; 95% CI 0.64–1.09; $p = 0.180$) (Figure 2). Study heterogeneity was not significant ($\chi^2 = 13.47$; df = 20 [$p = 0.860$]; $I^2 = 0.0\%$). There were no differences in cerebrovascular accidents between the transfusion strategies in any of the subgroup analyses (Figures S8–S14).

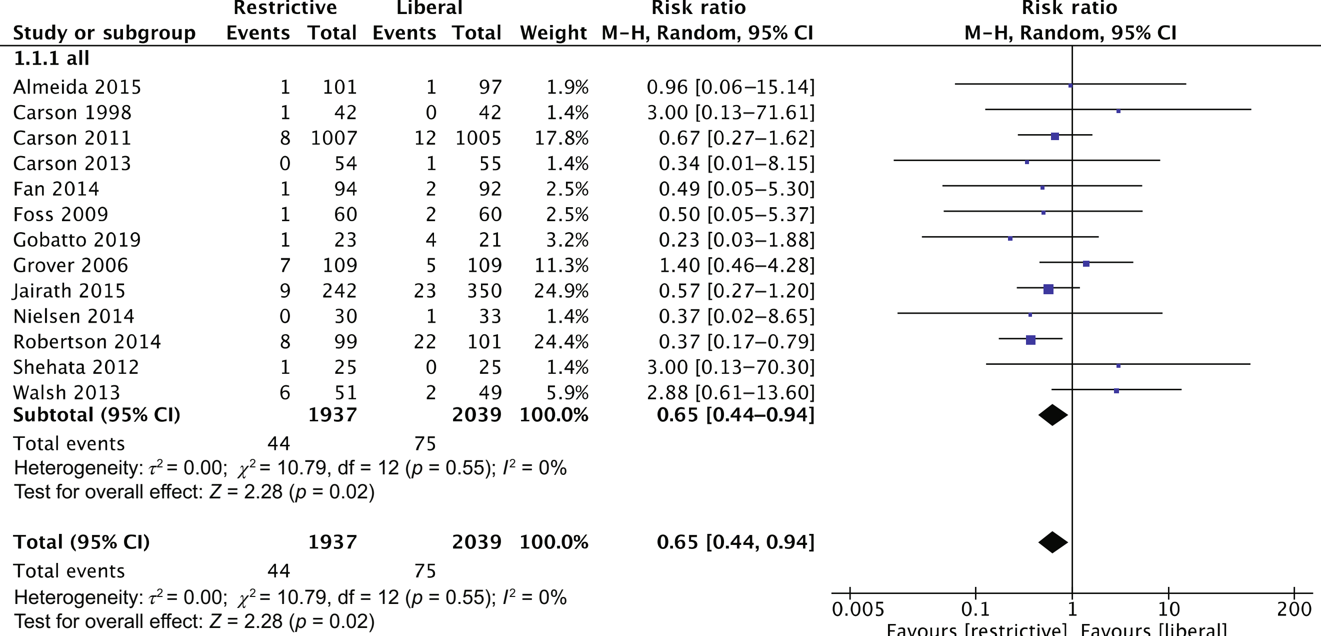


**FIGURE 1** Comparison of thromboembolic events between restrictive and liberal transfusion strategies in randomized controlled trials (RCTs). Size of squares for risk ratio reflects weight of RCT in pooled analysis. Horizontal bars represent 95% confidence intervals (CIs). Risk ratio >1.0 favours liberal transfusion strategy. df, degrees of freedom; M–H, Mantel–Haenszel; Random, random-effects model

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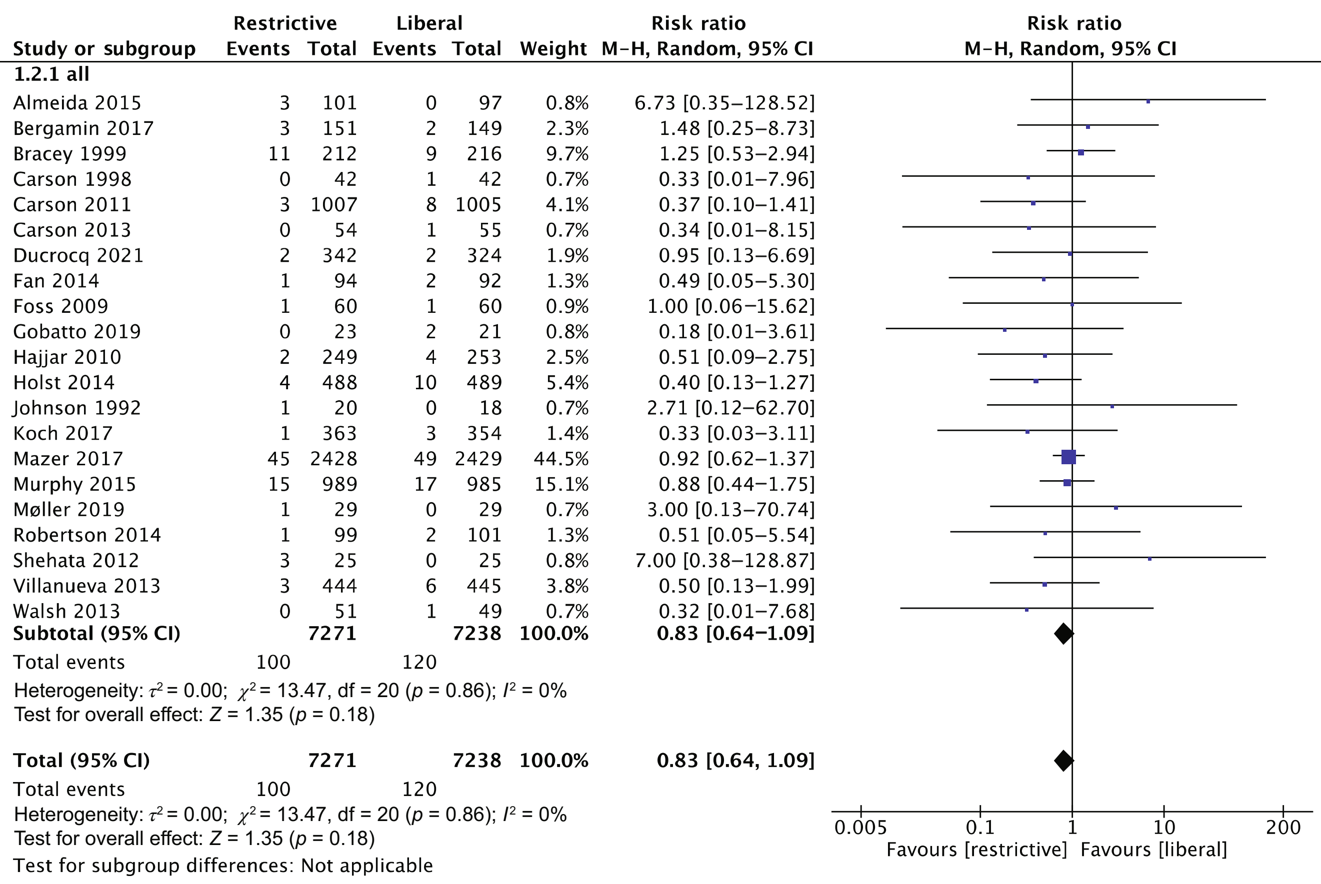


**FIGURE 2** Comparison of cerebrovascular accidents between restrictive and liberal transfusion strategies in randomized controlled trials (RCTs). Size of squares for risk ratio reflects weight of RCT in pooled analysis. Horizontal bars represent 95% confidence intervals (CIs). Risk ratio >1.0 favours liberal transfusion strategy. df, degrees of freedom; M–H, Mantel–Haenszel; Random, random-effects model

The risk of cerebrovascular accidents became significantly lower in the restrictive (relative to liberal) group when limiting that analysis to trials that reported on transfusion type (leucocyte-reduced or non-leucocyte-reduced RBCs); however, removing trials involving either leucocyte-reduced RBCs or non-leucocyte-reduced RBCs resulted in no significant difference. Other sensitivity analyses for cerebrovascular accidents did not differ from the overall pooled results.

## Myocardial infarction

Based on 25 RCTs (14,829 participants) reporting on myocardial infarction, the risk did not differ by restrictive versus liberal strategy (RR = 1.05; 95% CI 0.87–1.26; $p = 0.620$) (Figure 3). Study heterogeneity was not significant ($\chi^2 = 21.13$; df = 22 [$p = 0.510$]; $I^2 = 0\%$). The risk of myocardial infarction was significantly lower in the restrictive (relative to liberal) transfusion group in the RTT = 7 g/dl and LTT = 10 g/dl subgroup (RR = 0.32; 95% CI 0.11–0.93) but not in the RTT = 8 g/dl and LTT = 10 g/dl subgroup (Figure S18). Regarding the other subgroup analyses, there were no differences in myocardial infarction between the transfusion strategies (Figures S15–S17 and S19–S21).

The difference in myocardial infarction between the restrictive and liberal groups was still non-significant when limiting the analysis to trials involving RTT = 7 or 8 g/dl with LTT = 10 g/dl, but further limiting the analysis to trials involving RTT = 7 g/dl and LTT = 10 g/dl showed that the restrictive (relative to liberal) group had a significantly reduced risk of myocardial infarction. Other sensitivity analyses for myocardial infarction did not show differences from the overall pooled results.

## RoB and quality

Twenty trials (66.7%) had 'some concerns' or 'high' RoB [21, 23, 24, 26, 29–33, 35–38, 40, 41, 43, 46, 47, 49, 50] (Figures 4 and 5). The main category for some concerns and high RoB was deviations from intended interventions, which included lack of blinding of participants, caregivers or outcome assessors (as the nature of blood transfusion makes it hard to blind them) and insufficient information provided about the appropriateness of the analysis. The GRADE quality of evidence was judged to be 'very low' for thromboembolic events and cerebrovascular accidents, and 'low' for myocardial infarction (Figure 6). The reasons included inadequate blinding, large variation in effect and the small number of events.

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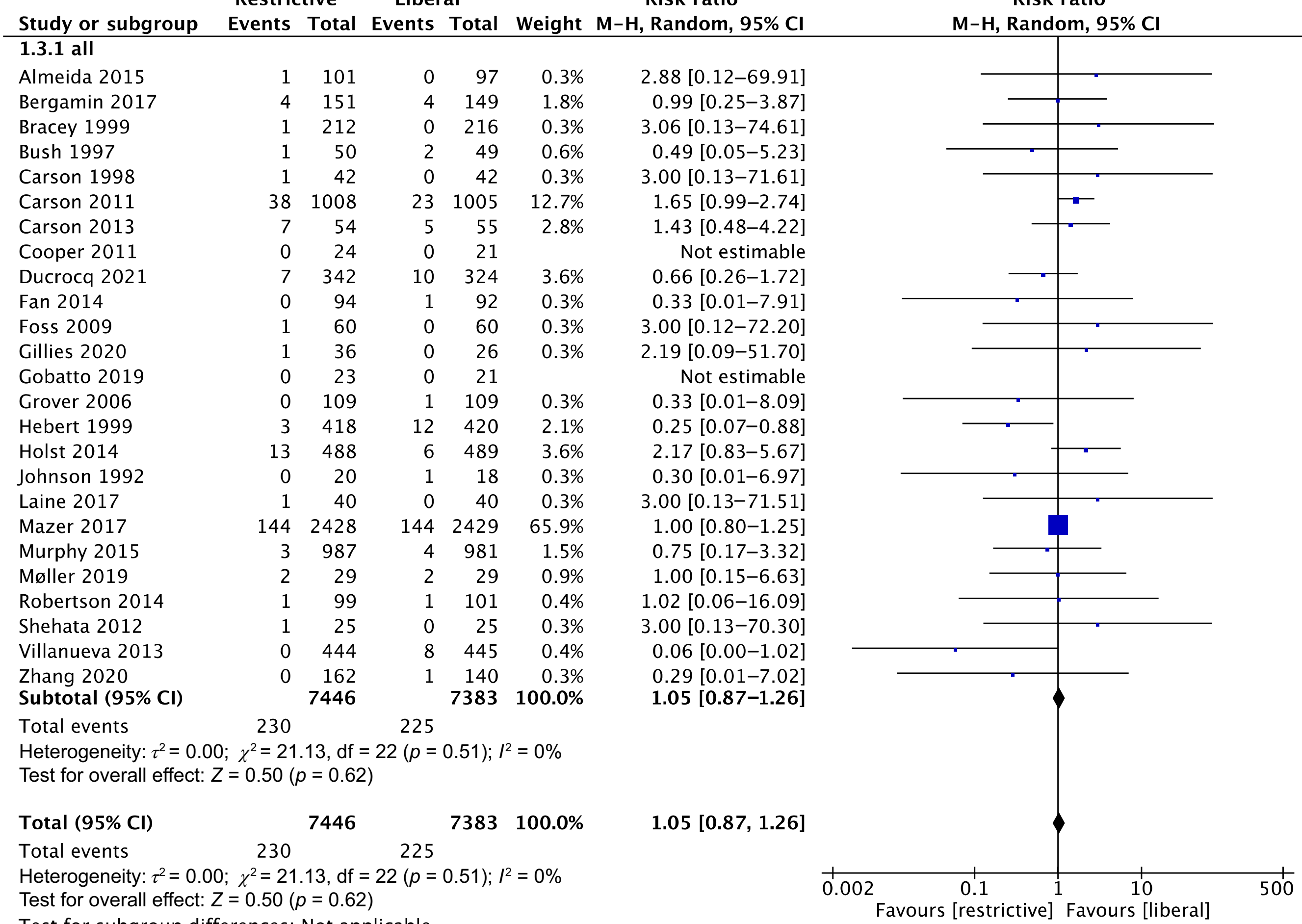


**FIGURE 3** Comparison of myocardial infarction between restrictive and liberal transfusion strategies in randomized controlled trials (RCTs). Size of squares for risk ratio reflects weight of RCT in pooled analysis. Horizontal bars represent 95% confidence intervals (CIs). Risk ratio >1.0 favours liberal transfusion strategy. df, degrees of freedom; M–H, Mantel–Haenszel; Random, random-effects model

## Publication bias

Regarding thromboembolic events and cerebrovascular accidents, no publication bias was found according to the funnel plots or Egger's test (Figures S22 and S23). However, the funnel plot for myocardial infarction showed slight asymmetry (Figure S24), suggesting publication bias. Nevertheless, Egger's test for myocardial infarction was not significant ($p = 0.578$). Overall, the publication bias regarding this outcome appears to be small.

## DISCUSSION

Our meta-analysis of 30 RCTs compared thrombosis-related complications between restrictive and liberal transfusion strategies. The incidence of thromboembolic events was lower in the restrictive (relative to liberal) transfusion group, but there were no differences in cerebrovascular accidents or myocardial infarction.

A 2016 Cochrane review reported that restrictive transfusion strategies decrease the proportion of transfused patients across many clinical settings without worsening clinical outcomes [51]. Similar statements were made by Brunskill et al. [15] and Shehata et al. [52] in their systematic reviews of transfusion thresholds for patients with hip fractures and patients undergoing cardiac surgery, respectively. The findings of these reviews suggested that restrictive transfusion strategies can also effectively reduce adverse events, such as mortality and infections.

Our meta-analysis focused on the effects of transfusion strategies on thrombosis-related events. Several meta-analyses have assessed the effects of different transfusion strategies on thromboembolic events in various clinical settings [12, 15, 51, 53–55]. For example, one found no difference in venous thromboembolism between transfusion strategies in adult and paediatric patients (RR = 0.76; 95% CI 0.41–1.41; $p = 0.920$) [12]. A study of hip fracture patients also reported no significant difference in thromboembolism between transfusion strategies (RR = 1.15; 95% CI 0.56–2.37; $p = 0.710$) (based on low-quality evidence) [15]. Another study on hip fracture patients similarly reported no difference in thromboembolic events between transfusion strategies (RR = 0.71; 95% CI 0.34–1.45; $p = 0.350$) [53]. However, these reviews included studies other than

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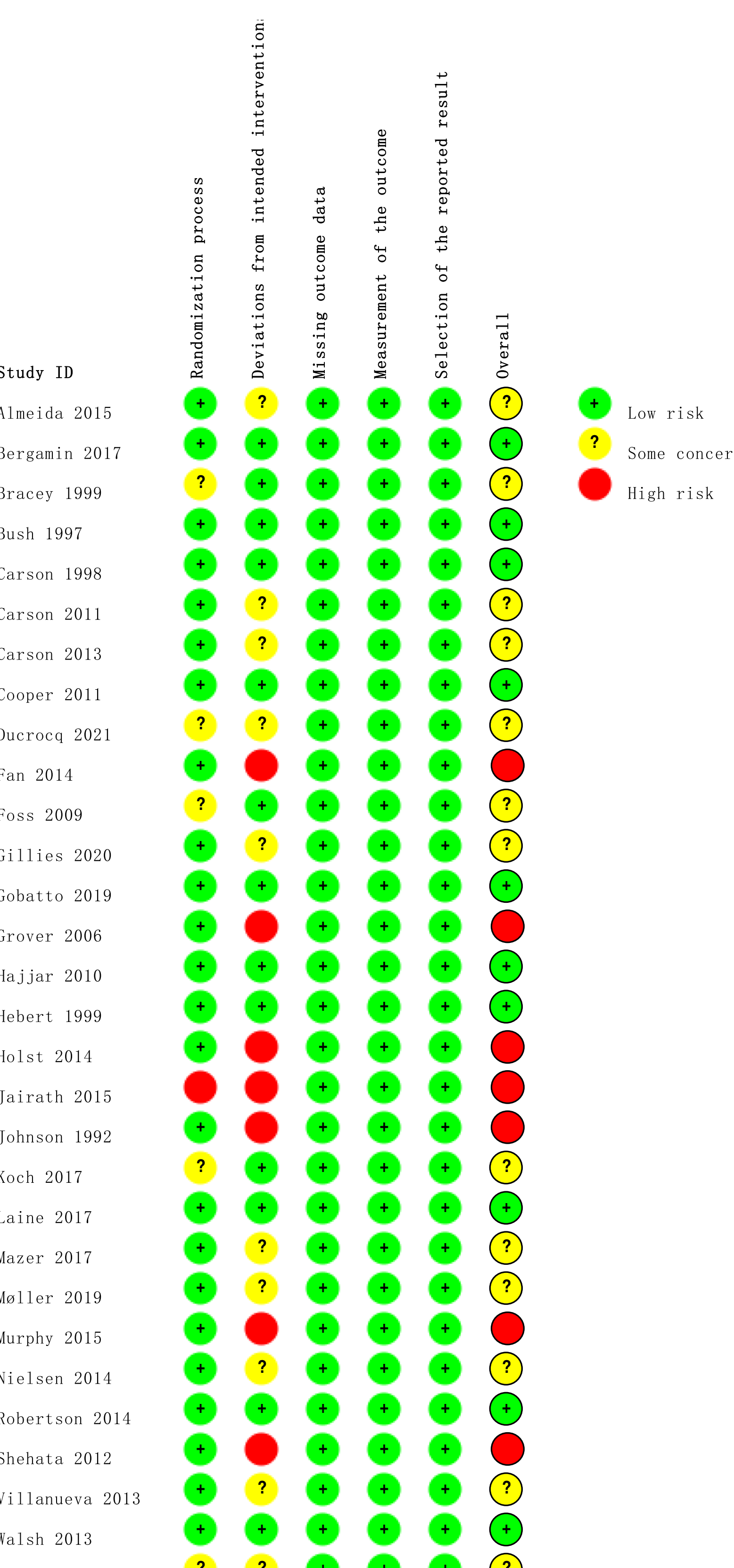


**FIGURE 4** Risk of bias assessment of included randomized controlled trials

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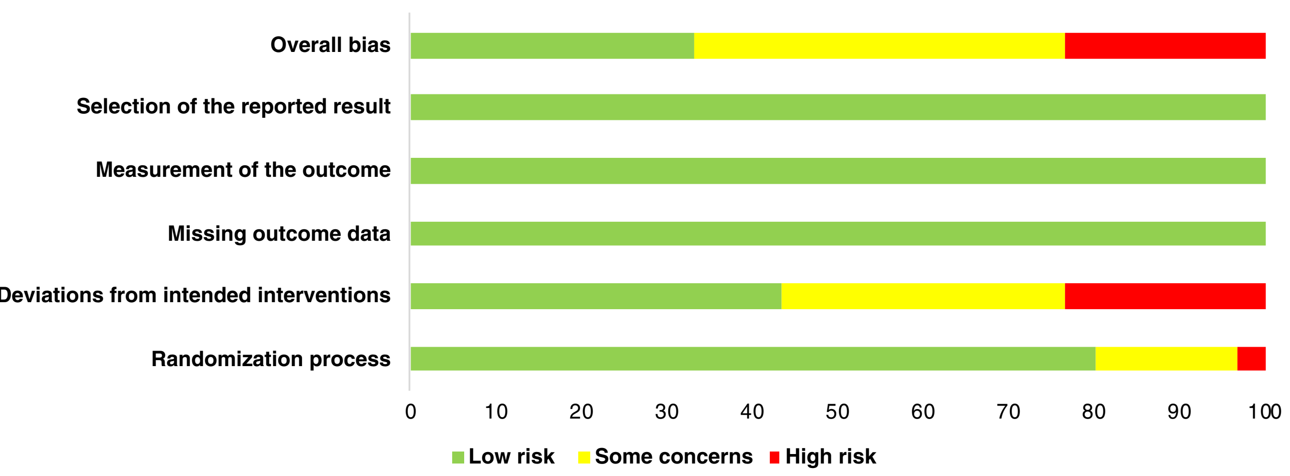


FIGURE 5 Risk of bias summary

**Summary of findings:**

**Restrictive transfusion strategy compared to liberal transfusion strategy in adult patients**

**Patient or population**: adult patients
**Setting**: inpatient
**Intervention**: restrictive transfusion strategy
**Comparison**: liberal transfusion strategy

| Outcomes | Anticipated absolute effects* (95% CI) | | Relative effect (95% CI) | No. of participants (studies) | Certainty of the evidence (GRADE) | Comments |
|---|---|---|---|---|---|---|
| | Risk with liberal transfusion strategy | Risk with restrictive transfusion strategy | | | | |
| Thromboembolic events | 37 per 1000 | **24 per 1,000** (16–35) | **RR 0.65** (0.44–0.94) | 3976 (13 RCTs) | ⊕◯◯◯ VERY LOW a,b,c | |
| Cerebrovascular accidents | 17 per 1000 | **14 per 1,000** (11–18) | **RR 0.83** (0.64–1.09) | 14,509 (21 RCTs) | ⊕◯◯◯ VERY LOW a,b,c | |
| Myocardial infarction | 30 per 1000 | **32 per 1,000** (27–38) | **RR 1.05** (0.87–1.26) | 14,829 (25 RCTs) | ⊕⊕◯◯ LOW a,b | |

***The risk in the intervention group** (and its 95% CI) is based on the assumed risk in the comparison group and the **relative effect** of the intervention (and its 95% CI).

**CI,** confidence interval; **RR,** risk ratio

**GRADE Working Group grades of evidence**
**High certainty:** We are very confident that the true effect lies close to that of the estimate of the effect
**Moderate certainty:** We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different
**Low certainty:** Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect
**Very low certainty:** We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect

**Explanations**

a. Most studies were not adequately blinded (including participants, personnel, and outcome assessors).
b. Point estimates varied widely across studies.
c. The small number of events occurred in two transfusion groups.

FIGURE 6 Summary of findings (including Grading of Recommendations Assessment, Development and Evaluation [GRADE] quality of evidence) in included randomized controlled trials

RCTs, and some of the included RCTs did not report specific haemoglobin- or haematocrit-based transfusion thresholds. We found a lower risk of thromboembolic events with the restrictive strategy (RR = 0.65; 95% CI 0.44–0.94; $p$ = 0.020). RBC transfusions may result in thrombosis by altering the rheologic variables and due to the infusion of pro-inflammatory and pro-thrombotic microparticles [56]; thus, lowering the transfusion threshold may reduce the risk. Subgroup analysis also showed that a restrictive (relative to liberal)

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strategy reduced thromboembolic events in RCTs conducted in North America (RR = 0.50; 95% CI 0.28–0.87; $p = 0.010$). The incidence of venous thrombosis varies among ethnic groups, with lower rates in Asians, Pacific Islanders and Hispanics than in Whites in the United States [57]. However, the association between transfusion strategies and ethnicity needs further investigation. Nevertheless, we should be cautious when interpreting the effect of a restrictive strategy on thromboembolic events. Sensitivity analyses showed that there was no longer a difference in thromboembolism between the restrictive and liberal groups after excluding the trial by Robertson et al. [39] (conducted in the United States) or Jairath et al. [30] (conducted in the United Kingdom).

We found no significant difference in cerebrovascular accidents between the restrictive and liberal strategies (RR = 0.83; 95% CI 0.64–1.09; $p = 0.180$). Curley et al. [58] reported that transfusion threshold was not associated with the risk of stroke among five RCTs (RR = 1.15; 95% CI 0.57–2.32; $p = 0.510$). Likewise, there was no significant difference in cerebrovascular accidents between restrictive and liberal strategies in cardiac patients [59] (RR = 0.97; 95% CI 0.72–1.30; $p = 0.840$); however, the review included only seven RCTs and used neurological complications as the cerebrovascular accident outcome. In contrast to other reviews, a review by Chong et al. [17] found that restrictive transfusion strategies were associated with fewer cerebrovascular accidents in critically ill patients based on six included RCTs (OR = 0.63; 95% CI 0.40–0.99; $p = 0.040$).

We also found that a restrictive strategy did not significantly affect myocardial infarction (RR = 1.05; 95% CI 0.87–1.26; $p = 0.620$), which is supported by previous meta-analyses [52, 59, 60]. Chen et al. [59] demonstrated that there was no difference in acute myocardial infarction between restrictive and liberal strategies in patients undergoing cardiac surgery. Similarly, Simon et al. [60] found no significant difference in myocardial infarction between the two transfusion strategies in older patients. However, Yao et al. [61] found that the incidence of myocardial infarction was lower with a restrictive (relative to liberal) transfusion strategy in ICU patients (OR = 0.54; 95% CI 0.30–0.98; $p = 0.040$). One of their included RCTs, conducted by Villanueva et al. [41], had only a small percentage of patients who were admitted to the ICU, despite the study having the greatest weight in the meta-analysis. After removing this study, the significant positive effect of the restrictive transfusion strategy on myocardial infarction in the review [61] became non-significant, indicating the instability of the effect.

Regarding transfusion type, we found no difference in the risk of thrombosis-related events between the transfusion strategies in either the leucocyte-reduced or non-leucocyte-reduced RBC subgroups. Regarding study area, the restrictive (relative to liberal) transfusion strategy reduced the risk of thromboembolic events in North America. This may have occurred because different areas have different ethnic groups who had varying levels of thrombosis risks after transfusion [57], different transfusion guidelines were employed in different regions and/or the perception of the risk of transfusion varied across areas [62–65].

Our meta-analysis has several strengths. First, we conducted a comprehensive search for RCTs that reported on thrombosis-related events (thromboembolic events, cerebrovascular accidents and myocardial infarction), which have not been fully analysed in previous meta-analyses [17, 55]. Second, we included the five most recent RCTs (published in 2019–2021) conducted in patients with traumatic brain injury [25], patients undergoing vascular surgery [36], patients in orthopaedic units [24], patients with acute myocardial infarction and anaemia [49] and patients undergoing cancer treatment [43]. Lastly, we used the latest Cochrane RoB tool, RoB 2, to evaluate RoB.

Our meta-analysis also has several limitations. First, the included RCTs had different RTTs; most were based on haemoglobin level, while some were based on haematocrit level. Furthermore, the RTTs varied among RCTs, even though they were based on haemoglobin level. Most trials used RTTs of 7–8 g/dl, but others used higher RTTs, potentially causing clinical heterogeneity. Second, the participants came from various clinical settings, leading to different tolerances for transfusion strategies. Third, thrombosis-related complications (such as thromboembolic events) were pre-specified as outcomes in only 25 of the 30 RCTs. Fourth, the definitions and follow-up time of each outcome varied across trials. Lastly, the transfusion timing (intra-operative, post-operative and during hospital/ICU stay) differed among the RCTs.

In conclusion, this meta-analysis demonstrated that restrictive transfusion strategies had a lower risk of thromboembolic events. The incidences of cerebrovascular accidents and myocardial infarction were unaffected by the transfusion strategy. Subgroup analyses indicated that restrictive (relative to liberal) strategies led to (1) fewer thromboembolic events in RCTs conducted in North America and (2) fewer myocardial infarctions in the RTT = 7 g/dl and LTT = 10 g/dl subgroup but not in the RTT = 8 g/dl and LTT = 10 g/dl subgroup. Restrictive (relative to liberal) transfusion strategies may be effective at reducing venous thrombosis but not arterial thrombosis. Other interventions are needed to reduce the incidence of thrombosis-related complications.

## ACKNOWLEDGEMENTS

M.M. performed the literature search, collected and analyzed the data and wrote the first draft of the manuscript; C.X.Z. collected the data and wrote the first draft of the manuscript; J.G.X. and Z.C.Z. supervised the research and reviewed and edited the manuscript; H.D.L. and O.C.O. verified the methodology and edited the manuscript.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## SUPPORTING INFORMATION

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REVIEW

# Impact of restrictive red blood cell transfusion strategy on thrombosis-related events: A meta-analysis and systematic review

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## Abstract

**Background and Objectives:** There is an ongoing controversy regarding the risks of restrictive and liberal red blood cell (RBC) transfusion strategies. This meta-analysis assessed whether transfusion at a lower threshold was superior to transfusion at a higher threshold, with regard to thrombosis-related events, that is, whether these outcomes can benefit from a restrictive transfusion strategy is debated.

**Materials and Methods:** We searched PubMed, Cochrane Central Register of Controlled Trials and Scopus from inception up to 31 July 2021. We included randomized controlled trials (RCTs) in any clinical setting that evaluated the effects of restrictive versus liberal RBC transfusion in adults. We used random-effects models to calculate the risk ratios (RRs) and 95% confidence intervals (CIs) based on pooled data.

**Results:** Thirty RCTs involving 17,334 participants were included. The pooled RR for thromboembolic events was 0.65 (95% CI 0.44–0.94; $p = 0.020$; $I^2 = 0.0\%$, very low-quality evidence), favouring the restrictive strategy. There were no significant differences in cerebrovascular accidents (RR = 0.83; 95% CI 0.64–1.09; $p = 0.180$; $I^2 = 0.0\%$, very low-quality evidence) or myocardial infarction (RR = 1.05; 95% CI 0.87–1.26; $p = 0.620$; $I^2 = 0.0\%$, low-quality evidence). Subgroup analyses showed that a restrictive (relative to liberal) strategy reduced (1) thromboembolic events in RCTs conducted in North America and (2) myocardial infarctions in the subgroup of RCTs where the restrictive transfusion threshold was 7 g/dl but not in the 8 g/dl subgroup (with a liberal transfusion threshold of 10 g/dl in both subgroups).

**Conclusions:** A restrictive (relative to liberal) transfusion strategy may be effective in reducing venous thrombosis but not arterial thrombosis.

**KEYWORDS**
cerebrovascular accidents, myocardial infarction, restrictive, thromboembolism, transfusion strategy

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**Highlights**

- A restrictive red blood cell transfusion strategy significantly reduced the risk of thromboembolic events, although the Grading of Recommendations Assessment, Development and Evaluation quality of evidence was very low.
- For cerebrovascular accidents and myocardial infarction, there were no statistically significant differences between restrictive and liberal transfusion strategies.
- Subgroup analyses showed that the restrictive (relative to liberal) transfusion strategy reduced (1) thromboembolic events in trials conducted in North America and (2) myocardial infarctions in the subgroup of trials where the restrictive transfusion threshold was 7 g/dl but not in the 8 g/dl subgroup (with a liberal transfusion threshold of 10 g/dl in both subgroups).

## INTRODUCTION

Red blood cell (RBC) transfusion can increase or maintain oxygen levels in tissues [1], improving anaemia, which can save lives [2, 3]. However, transfusion is associated with several adverse events, such as thromboembolism [4, 5], cerebrovascular accidents [6] and myocardial infarction [1, 7]. The mechanisms behind these adverse events include increased circulating RBC mass [1], increased oxidative stress [4], reduced nitric oxide and/or increased inflammatory mediators [6]. Consequently, an appropriate transfusion strategy should be used to reduce the adverse events.

Haemoglobin or haematocrit thresholds are commonly used when deciding whether to perform a transfusion. The most commonly used trigger for transfusion in the twentieth century was haemoglobin of 10 g/dl or haematocrit of 30% [8]. However, several transfusion guidelines suggest that a restrictive transfusion strategy (haemoglobin <7 or 8 g/dl) is suitable in most clinical settings [9–11]. Nevertheless, whether a restrictive transfusion strategy reduces thrombosis-related events compared to a liberal one remains controversial, with some reviews indicating no significant differences [11–14] and others indicating that restrictive strategies decrease cerebrovascular accidents but increase myocardial infarction [15–17]. However, most of these reviews included only a few randomized controlled trials (RCTs) or evaluated composite outcomes.

In order to conduct a comprehensive meta-analysis comparing the restrictive and liberal transfusion strategies with regard to thrombosis-related events in adults, we included all available RCTs reporting these outcomes. In addition, we also performed various subgroup analyses, such as comparing outcomes between different restrictive transfusion thresholds (with a fixed liberal transfusion threshold) and among study areas, which were rarely considered in other meta-analyses.

## MATERIALS AND METHODS

### Search strategy

We searched PubMed, Cochrane Central Register of Controlled Trials and Scopus from inception to 31 July 2021, using ([blood transfusion*] OR [red blood cell] or RBC or transfus* or haemoglobin) AND (trigger* OR threshold* OR liberal OR restrict* OR strateg*) AND ([randomized controlled trial*] OR [controlled clinical trial*] OR [clinical trial*] OR [randomized trial*] OR trial*). We checked the references of included RCTs for additional relevant articles. The complete search strategy is provided in the Supplementary Material. After records were imported into the EndNote software, duplicate records were removed. Two reviewers (M.M. and C.X.Z.) independently screened the titles and abstracts of the relevant studies. Thereafter, full-text versions were retrieved to further assess eligibility. Disagreements were settled by discussing with other reviewers (J.G.X., Z.C.Z., H.D.L. and O.C.O.).

### Study selection

The eligibility criteria were (1) RCT, (2) compared liberal and restrictive transfusion strategies, (3) reported thrombosis-related events and (4) patients aged ≥16 years. For RCTs that generated multiple publications, we excluded duplicate patients and outcome data.

### Data extraction

Two authors (M.M. and C.X.Z.) independently extracted information about the first author, year of publication, study area (continent where the patients were recruited from), sample size, transfusion thresholds, demographics, medications and clinical outcomes using a data extraction form. Disagreements were resolved based on reaching a consensus among all authors.

### Outcome definitions

Thrombosis-related events can occur in veins or arteries. For venous thrombosis, we included thromboembolic events comprising deep vein thrombosis, pulmonary embolism and thromboembolism. For arterial thrombosis, we included cerebrovascular accidents (stroke or transient ischaemic attack, cerebrovascular attack or cerebral

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ischaemia) and myocardial infarction (myocardial infarction, acute myocardial infarction, acute myocardial ischaemia or stent thrombosis). Detailed definitions are provided in Table S1.

## Risk of bias and quality

Two authors (M.M. and C.X.Z.) independently assessed the risk of bias (RoB) in the outcomes in the RCTs (categorized as 'low', 'some concerns' or 'high') using the revised Cochrane RoB tool (RoB 2) [18, 19]. As the assessment of each RoB domain for each included outcome in any given RCT was the same, we present the RoB results by RCT instead of by outcome in each RCT. The following domains were assessed: randomization process, deviations from intended interventions, missing outcome data, outcome measurement and selection of the reported result.

We also assessed the overall quality of evidence of each outcome (categorized as 'very low', 'low', 'moderate' or 'high') using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) criteria [20].

## Statistical analysis

Mantel–Haenszel risk ratios (RRs) with 95% confidence intervals (CIs) were calculated based on pooled data. Thereafter, we performed subgroup analyses by clinical setting, transfusion threshold, transfusion timing (intra- and post-operatively, peri-operatively, post-operatively or during the hospital/intensive care unit [ICU] stay), transfusion type (leucocyte-reduced or non-leucocyte-reduced RBCs) and study area (continent where the patients were recruited from). To test for statistical heterogeneity, $I^2$ (50% or 85% indicates moderate or substantial heterogeneity, respectively) and $\chi^2$ ($p < 0.05$ indicates significant heterogeneity) were calculated. Random-effect models were used to account for clinical heterogeneity. Several sensitivity analyses were performed to explore various aspects of the trials and review methodology. First, we excluded each trial one at a time and recalculated the pooled effects. Excluding one trial at a time can help investigate how each individual study affects the overall effect size and identify influential studies. Second, we excluded trials with high RoB. Third, we excluded trials from each clinical setting (ICU treatment, cardiac surgery, orthopaedic surgery, vascular surgery, upper gastrointestinal bleeding treatment and cancer treatment) in turn. Fourth, we limited the analysis to trials using specific transfusion thresholds (restrictive transfusion threshold [RTT] = 7, RTT > 7 g/dl, RTT = 7 or 8 g/dl with liberal transfusion threshold [LTT] = 9 g/dl, RTT = 7 or 8 g/dl with LTT = 10 g/dl). Fifth, we excluded trials that involved transfusion during the hospital/ICU stay without a clear indication of transfusion timing. Sixth, we excluded trials that did not report on transfusion type (i.e., whether the RBC transfusion was leucocyte-reduced) and trials that involved either leucocyte-reduced or non-leucocyte-reduced RBCs. Seventh, we excluded trials from each continent in turn.

Funnel plots and Egger's regression were used to assess publication bias. The analyses were performed in Stata 14.0 and Review Manager 5.3.

# RESULTS

## Included RCTs

The search strategy (Figure S1) identified 2358 articles. After removing duplicates, there were 1750 articles. After screening the titles/abstracts, 119 were selected to be reviewed in full. Thirty RCTs (described in Tables 1 and S2), with 17,334 participants (8576 in restrictive groups and 8637 in liberal groups) were included [21-50]. The RCTs were published between 1992 and 2021. The mean age ranged from 35 to 82.3 years.

Clinical settings varied: 10 involved patients undergoing cardiac surgery [27, 32–35, 37, 40, 47–49], 7 involved patients undergoing orthopaedic surgery [23, 24, 26, 38, 45, 46, 50], 7 involved patients treated in ICUs [21, 22, 25, 28, 29, 39, 42], 3 involved patients undergoing vascular surgery [31, 36, 44], 2 involved patients with upper gastrointestinal bleeding [30, 41] and 1 involved patients undergoing cancer treatment [43]. Additionally, 23 RCTs included cardiovascular disease as a baseline characteristic [21–24, 26–30, 32, 35–38, 40, 42, 44–50], 12 excluded patients with anaemia or bleeding [21, 26–29, 41, 42, 45–49], 1 excluded patients with too high transfusion rate [22] and 1 included patients with high transfusion rates [40].

Regarding RTT and LTT, the haemoglobin RTT ranged from 7.0 to 9.0 g/dl [21–26, 28–30, 33–47, 49, 50], with four additional RCTs specifying haematocrit values of 24% or 25% [27, 31, 32, 48]. The haemoglobin LTT ranged from 8.5 to 10.0 g/dl [21–26, 28–30, 33–47, 49, 50], with four additional RCTs specifying haematocrit values of 28%, 30% or 32% [27, 31, 32, 48]. In four RCTs, transfusion was also permitted for symptoms of anaemia in the restrictive group [45–47, 50]. RCTs were divided into the following pairs of subgroups based on RTT alone or RTT plus LTT: (1) RTT = 7 g/dl versus RTT > 7 g/dl; (2) RTT = 7 g/dl and LTT = 9 g/dl versus RTT = 8 g/dl and LTT = 9 g/dl and (3) RTT = 7 g/dl and LTT = 10 g/dl versus RTT = 8 g/dl and LTT = 10 g/dl.

Specific transfusion timing was reported in 13 RCTs: 3 transfused intra- and post-operatively [35, 40, 44]; 6 transfused post-operatively [31, 33, 36, 37, 45, 46] and 4 transfused peri-operatively [23, 27, 34, 43]. For the remaining RCTs, 12 transfused during the hospital/ICU stay [21, 22, 25, 28, 29, 32, 39, 41, 42, 47–49] and the transfusion timing was not reported by the others [24, 26, 30, 38, 50]. Regarding transfusion type, 12 RCTs used leucocyte-reduced RBCs [21, 22, 26, 29, 36, 39, 41, 42, 46–49], 4 used non-leucocyte-reduced RBCs [23, 25, 27, 28] and the remaining 14 did not provide this information [24, 30–35, 37, 38, 40, 43–45, 50]. Regarding the study area, 12 RCTs were conducted in Europe [23, 24, 26, 29, 30, 34, 36–38, 41, 42, 49], 9 in North America [28, 31, 33, 39, 40, 44, 46–48], 4 in South America [21, 22, 25, 27], 2 in Asia [43, 50] and the remaining 3 recruited patients across several different continents [32, 35, 45].

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**TABLE 1** Characteristics of included randomized controlled trials

| Author (year) | Country | Patient type | Setting | Period | Baseline characteristics | Restrictive threshold (g/dl) | Liberal threshold (g/dl) |
|---|---|---|---|---|---|---|---|
| Almeida (2015) | Brazil | Surgical oncology | ICU | 2012 | Age ≥ 18; CVD; cancer; surgery; tobacco use, hypertension, diabetes (excluded pre-existing coagulopathy or anticoagulation therapy, anaemia, and active bleeding) | 7 | 9 |
| Bergamin (2017) | Brazil | Cancer with septic shock | ICU | 2012–2014 | Age ≥ 18; CVD; cancer; infection; smoking, hypertension, diabetes (excluded patients with too high a transfusion rate) | 7 | 9 |
| Bracey (1999) | USA | Elective primary CABG surgery | Cardiac | 1997 | Surgery | 8 | 9 |
| Bush (1997) | USA | Elective aortic or infrainguinal arterial reconstruction | Vascular | 1995–1996 | CVD; surgery; smoking, hypertension, diabetes | 9 | 10 |
| Carson (1998) | USA, UK | Hip fracture | Orthopaedic | 1996–1997 | Hb < 10 g/dl; CVD; surgery; diabetes (excluded anaemia) | 8 | 10 |
| Carson (2011) | USA, Canada | Hip fracture | Orthopaedic | 2004–2009 | Age ≥ 50; Hb < 10 g/dl; CVD; surgery; tobacco use, hypertension, diabetes (excluded anaemia and active bleeding) | 8 | 10 |
| Carson (2013) | USA | Coronary syndrome or stable coronary artery disease undergoing catheterization | Cardiac | 2010–2012 | Age ≥ 18; Hb < 10 g/dl; CVD; surgery; tobacco use, hypertension, diabetes (excluded anaemia and active bleeding) | 8 | 10 |
| Cooper (2011) | USA | AMI | Cardiac | 2003–2009 | Age ≥ 21; haematocrit ≤ 30%; CVD; surgery; tobacco use, hypertension, diabetes (excluded active bleeding) | 24%[a] | 30% |
| Ducrocq (2021) | France, Spain | AMI and anaemia | Cardiac | 2016–2019 | Age ≥ 18; Hb: 7–10 g/dl; CVD; tobacco use, hypertension, diabetes (excluded massive ongoing bleeding) | 8 | 10 |
| Fan (2014) | China | Total hip replacement | Orthopaedic | 2011–2013 | Age > 65; CVD; surgery; hypertension, diabetes | 8 | 10 |
| Foss (2009) | Denmark | Hip fracture | Orthopaedic | 2004–2006 | Age > 65; CVD; surgery; hypertension, diabetes | 8 | 10 |
| Gillies (2020) | UK | Surgery for fractured neck of femur | Orthopaedic | 2017–2019 | Age ≥ 50; CVD; surgery; hypertension, diabetes | 7 | 9 |
| Gobatto (2019) | Brazil | Moderate or severe traumatic brain injury | ICU | 2014–2016 | Age > 18; Hb < 9 g/dl; trauma | 7 | 9 |
| Grover (2006) | UK | Elective total knee or hip arthroplasty | Orthopaedic | Not mentioned | Age ≥ 55; CVD; surgery; smoking, hypertension, diabetes (excluded anaemia) | 8 | 10 |
| Hajjar (2010) | Brazil | Elective cardiac surgery | Cardiac | 2009–2010 | Age ≥ 18; CVD; surgery; smoking, hypertension, diabetes (excluded anaemia) | 24% | 30% |
| Hebert (1999) | Canada | Critically ill with euvolemia | ICU | 1994–1997 | Age ≥ 16; Hb < 9 g/dl; CVD; trauma; infection (excluded anaemia and active bleeding) | 7 | 10 |

(Continues)

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**TABLE 1** (Continued)

| Author (year) | Country | Patient type | Setting | Period | Baseline characteristics | Restrictive threshold (g/dl) | Liberal threshold (g/dl) |
|---|---|---|---|---|---|---|---|
| Holst (2014) | Denmark, Sweden, Norway, Finland | Septic shock | ICU | 2011–2013 | Age ≥ 18; Hb < 9 g/dl; CVD; surgery; infection (excluded life-threatening bleeding) | 7 | 9 |
| Jairath (2015) | UK | Acute upper gastrointestinal bleeding | Upper gastrointestinal bleeding | 2012–2013 | Age ≥ 18; CVD; acute upper gastrointestinal bleeding; hypertension (excluded exsanguinating haemorrhage) | 8 | 10 |
| Johnson (1992) | USA | Elective operations for myocardial revascularization | Vascular | Not mentioned | Surgery | 25% | 32% |
| Koch (2017) | USA, India | Cardiac surgery | Cardiac | 2007–2014 | Age ≥ 18; CVD; surgery; smoking, hypertension, diabetes | 24% | 28% |
| Laine (2017) | Finland | Elective open-heart surgery | Cardiac | 2014–2015 | Surgery | 8 | 10 |
| Mazer (2017) | 19 countries | CABG and/or valve | Cardiac | 2014–2017 | Age ≥ 18; CVD; surgery; diabetes | 7.5 | 8.5, 9.5 |
| Møller (2019) | Denmark | Elective open infra-renal abdominal aortic aneurysm repair or lower-limb bypass | Vascular | 2015–2016 | Age > 40; CVD; surgery; smoking, hypertension, diabetes | 8 | 9.7 |
| Murphy (2015) | UK | CABG and/or valve or major aortic procedure | Cardiac | 2009–2013 | Age > 16; CVD; surgery; diabetes | 7.5 | 9 |
| Nielsen (2014) | Denmark | Hip revision surgery | Orthopaedic | 2009–2011 | Age ≥ 18; CVD; surgery; smoking, hypertension, diabetes | 7.3 | 8.9 |
| Robertson (2014) | USA | Closed head injury | ICU | 2006–2012 | Trauma; surgery; no comorbidities reported | 7 | 10 |
| Shehata (2012) | Canada | Elective cardiac surgery | Cardiac | 2007–2010 | CVD; surgery; hypertension, diabetes; high transfusion rates | 7, 7.5 | 9.5, 10 |
| Villanueva (2013) | Spain | Upper gastrointestinal bleeding | Upper gastrointestinal bleeding | 2003–2009 | Age > 18; upper gastrointestinal bleeding (excluded massive exsanguinating bleeding, major CVD or a recent history of trauma or surgery) | 7 | 9 |
| Walsh (2013) | UK | Mechanically ventilated | ICU | 2009–2010 | Age ≥ 55; Hb < 9 g/dl; CVD (excluded active bleeding) | 7 | 9 |
| Zhang (2020) | China | Cancer surgery | Oncologic | 2012–2016 | Hb < 10 g/dl; surgery; cancer | 7 | 10 |

Abbreviations: AMI, acute myocardial infarction; CABG, coronary artery bypass graft; CVD, cardiovascular disease; Hb, haemoglobin; ICU, intensive care unit.
[a]Transfusion was indicated by haematocrit value.

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## Outcomes

### Thromboembolic events

Based on 13 RCTs (3976 participants) reporting on thromboembolic events, the risk was significantly lower in the restrictive group than the liberal group (RR = 0.65; 95% CI 0.44–0.94; $p = 0.020$; Figure 1). Study heterogeneity was not significant ($\chi^2 = 10.79$; degrees of freedom [df] = 12 [$p = 0.55$]; $I^2 = 0.0\%$). Subgroup analyses were then performed. Regarding the clinical setting, there was no significant difference in thromboembolic events between the two transfusion strategies in any clinical setting subgroup assessed (Figure S2).

Regarding transfusion threshold, there was no significant difference in thromboembolic events between the two transfusion strategies in the RTT = 7 g/dl or RTT > 7 g/dl subgroups (Figure S3). However, the risk of thromboembolic events was significantly lower in the restrictive (relative to liberal) transfusion group in the RTT = 7 g/dl and LTT = 10 g/dl subgroup (RR = 0.37; 95% CI 0.17–0.79; Figure S4) but not the RTT = 8 g/dl and LTT = 10 g/dl subgroup; nevertheless, there was only one RCT included in the former subgroup.

Regarding transfusion timing, no significant difference was observed in thromboembolic events between the two transfusion strategies in the intra- and post-operative, peri-operative or post-operative subgroups (Figure S5). Regarding transfusion type, there was no significant difference in thromboembolic events between the two transfusion strategies in the non-leucocyte-reduced or leucocyte-reduced RBC subgroups (Figure S6). Lastly, regarding the study area, there were fewer thromboembolic events in the restrictive (relative to liberal) group in trials conducted in North America (RR = 0.50; 95% CI 0.28–0.87; Figure S7) but not in trials conducted in Europe, South America and Asia.

Sensitivity analysis showed that, after removing the trial by Robertson et al. [39] or Jairath et al. [30], there was no longer a significant difference in thromboembolism between the restrictive and liberal groups. Likewise, there was no longer a significant difference in thromboembolism after removing trials involving ICU treatment, orthopaedic surgery, or upper gastrointestinal bleeding treatment, or after limiting the analysis to trials reporting on transfusion timing (intra- and post-operative, peri-operative or post-operative), trials reporting on transfusion type (leucocyte-reduced or non-leucocyte-reduced RBCs) and non-North American trials. Limiting the analysis to trials involving 'low'/'some concerns' RoB; RTT = 7 or 8 g/dl with LTT = 10 g/dl; and RTT = 7 g/dl and LTT = 10 g/dl maintained the significant decrease in thromboembolism for the restrictive (relative to liberal) strategy.

### Cerebrovascular accidents

Based on 21 RCTs (14,509 participants) reporting on cerebrovascular accidents, the risk did not differ by restrictive versus liberal strategy (RR = 0.83; 95% CI 0.64–1.09; $p = 0.180$) (Figure 2). Study heterogeneity was not significant ($\chi^2 = 13.47$; df = 20 [$p = 0.860$]; $I^2 = 0.0\%$). There were no differences in cerebrovascular accidents between the transfusion strategies in any of the subgroup analyses (Figures S8–S14).

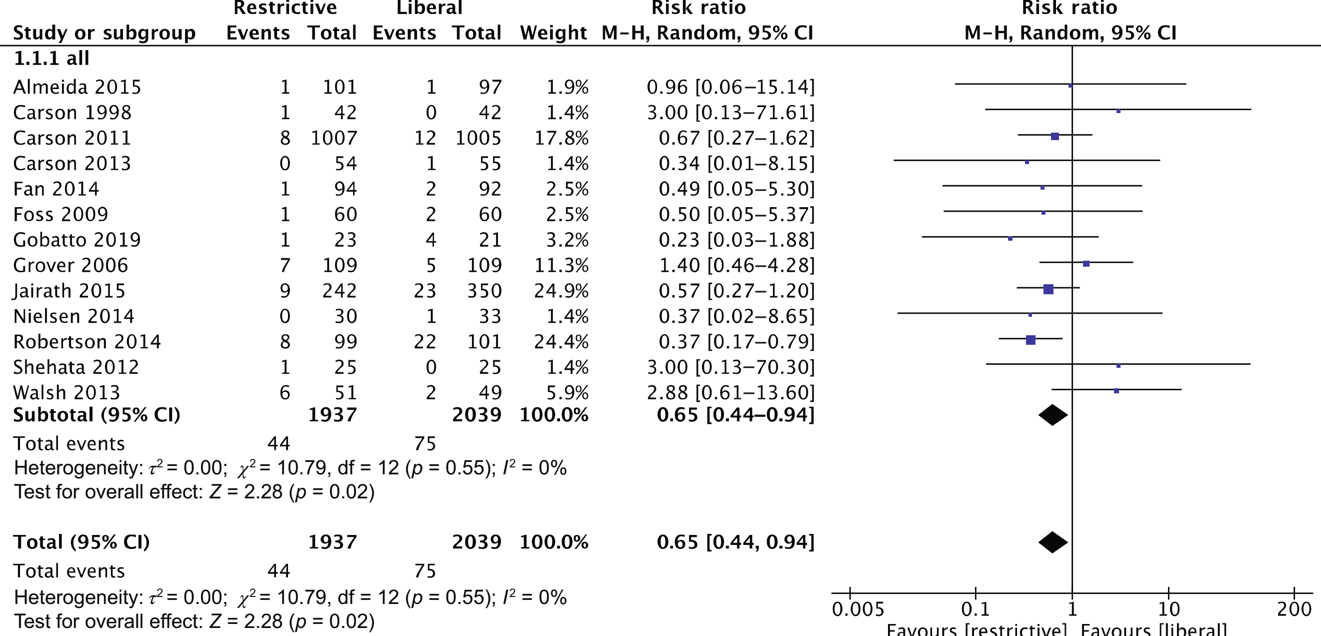


**FIGURE 1** Comparison of thromboembolic events between restrictive and liberal transfusion strategies in randomized controlled trials (RCTs). Size of squares for risk ratio reflects weight of RCT in pooled analysis. Horizontal bars represent 95% confidence intervals (CIs). Risk ratio >1.0 favours liberal transfusion strategy. df, degrees of freedom; M–H, Mantel–Haenszel; Random, random-effects model

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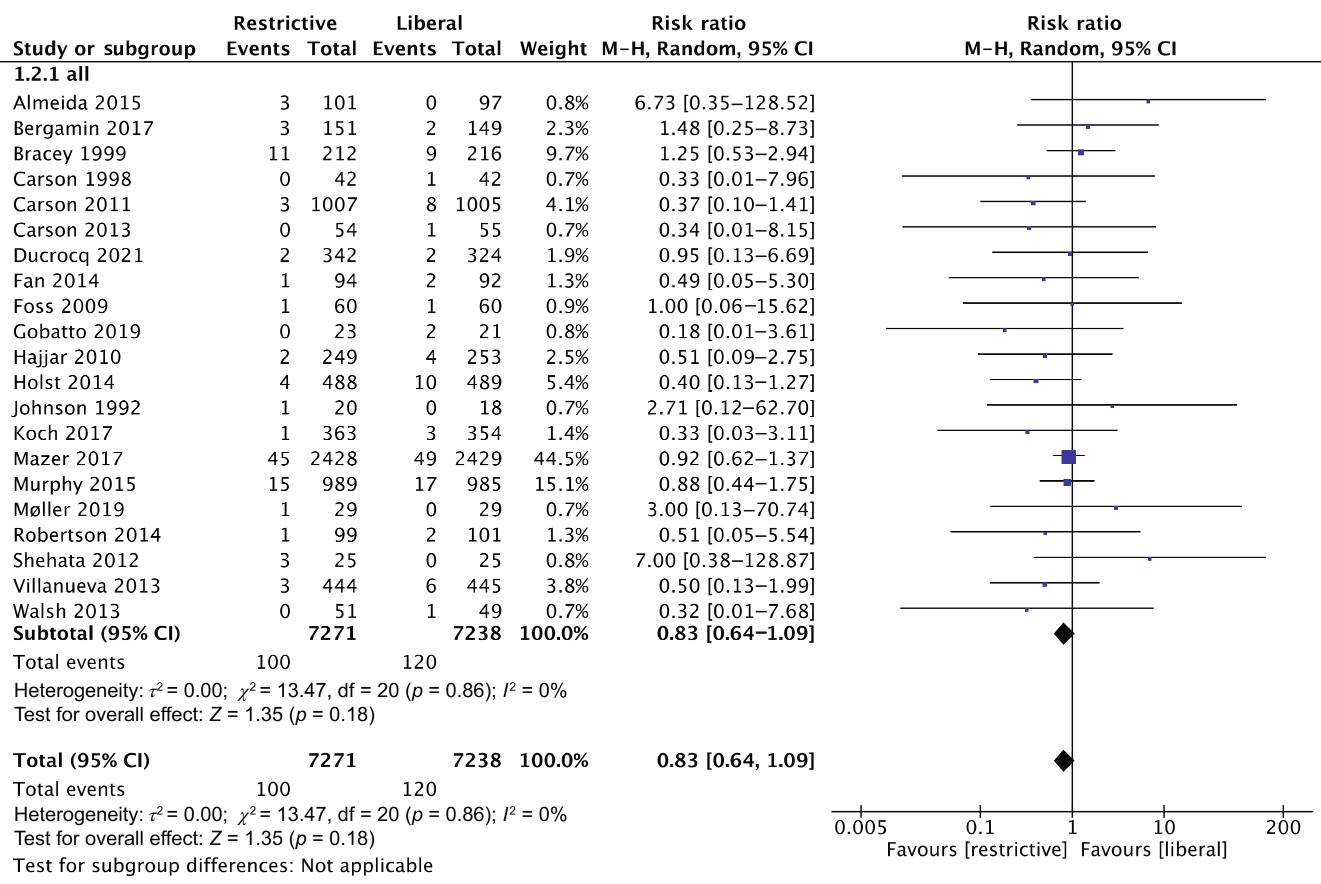


**FIGURE 2** Comparison of cerebrovascular accidents between restrictive and liberal transfusion strategies in randomized controlled trials (RCTs). Size of squares for risk ratio reflects weight of RCT in pooled analysis. Horizontal bars represent 95% confidence intervals (CIs). Risk ratio >1.0 favours liberal transfusion strategy. df, degrees of freedom; M–H, Mantel–Haenszel; Random, random-effects model

The risk of cerebrovascular accidents became significantly lower in the restrictive (relative to liberal) group when limiting that analysis to trials that reported on transfusion type (leucocyte-reduced or non-leucocyte-reduced RBCs); however, removing trials involving either leucocyte-reduced RBCs or non-leucocyte-reduced RBCs resulted in no significant difference. Other sensitivity analyses for cerebrovascular accidents did not differ from the overall pooled results.

## Myocardial infarction

Based on 25 RCTs (14,829 participants) reporting on myocardial infarction, the risk did not differ by restrictive versus liberal strategy (RR = 1.05; 95% CI 0.87–1.26; $p = 0.620$) (Figure 3). Study heterogeneity was not significant ($\chi^2 = 21.13$; df = 22 [$p = 0.510$]; $I^2 = 0\%$). The risk of myocardial infarction was significantly lower in the restrictive (relative to liberal) transfusion group in the RTT = 7 g/dl and LTT = 10 g/dl subgroup (RR = 0.32; 95% CI 0.11–0.93) but not in the RTT = 8 g/dl and LTT = 10 g/dl subgroup (Figure S18). Regarding the other subgroup analyses, there were no differences in myocardial infarction between the transfusion strategies (Figures S15–S17 and S19–S21).

The difference in myocardial infarction between the restrictive and liberal groups was still non-significant when limiting the analysis to trials involving RTT = 7 or 8 g/dl with LTT = 10 g/dl, but further limiting the analysis to trials involving RTT = 7 g/dl and LTT = 10 g/dl showed that the restrictive (relative to liberal) group had a significantly reduced risk of myocardial infarction. Other sensitivity analyses for myocardial infarction did not show differences from the overall pooled results.

## RoB and quality

Twenty trials (66.7%) had 'some concerns' or 'high' RoB [21, 23, 24, 26, 29–33, 35–38, 40, 41, 43, 46, 47, 49, 50] (Figures 4 and 5). The main category for some concerns and high RoB was deviations from intended interventions, which included lack of blinding of participants, caregivers or outcome assessors (as the nature of blood transfusion makes it hard to blind them) and insufficient information provided about the appropriateness of the analysis. The GRADE quality of evidence was judged to be 'very low' for thromboembolic events and cerebrovascular accidents, and 'low' for myocardial infarction (Figure 6). The reasons included inadequate blinding, large variation in effect and the small number of events.

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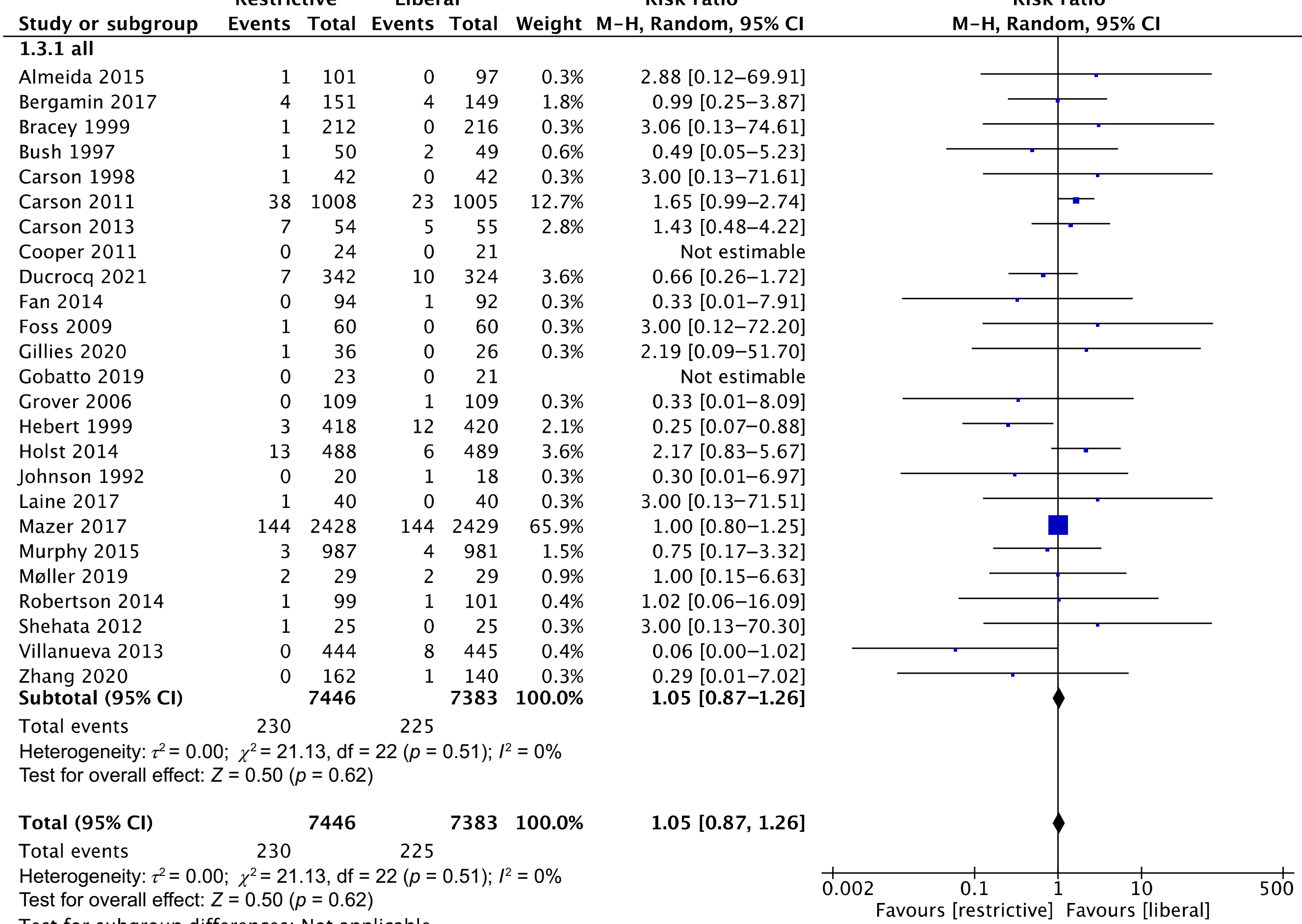


**FIGURE 3** Comparison of myocardial infarction between restrictive and liberal transfusion strategies in randomized controlled trials (RCTs). Size of squares for risk ratio reflects weight of RCT in pooled analysis. Horizontal bars represent 95% confidence intervals (CIs). Risk ratio >1.0 favours liberal transfusion strategy. df, degrees of freedom; M–H, Mantel–Haenszel; Random, random-effects model

## Publication bias

Regarding thromboembolic events and cerebrovascular accidents, no publication bias was found according to the funnel plots or Egger's test (Figures S22 and S23). However, the funnel plot for myocardial infarction showed slight asymmetry (Figure S24), suggesting publication bias. Nevertheless, Egger's test for myocardial infarction was not significant ($p = 0.578$). Overall, the publication bias regarding this outcome appears to be small.

## DISCUSSION

Our meta-analysis of 30 RCTs compared thrombosis-related complications between restrictive and liberal transfusion strategies. The incidence of thromboembolic events was lower in the restrictive (relative to liberal) transfusion group, but there were no differences in cerebrovascular accidents or myocardial infarction.

A 2016 Cochrane review reported that restrictive transfusion strategies decrease the proportion of transfused patients across many clinical settings without worsening clinical outcomes [51]. Similar statements were made by Brunskill et al. [15] and Shehata et al. [52] in their systematic reviews of transfusion thresholds for patients with hip fractures and patients undergoing cardiac surgery, respectively. The findings of these reviews suggested that restrictive transfusion strategies can also effectively reduce adverse events, such as mortality and infections.

Our meta-analysis focused on the effects of transfusion strategies on thrombosis-related events. Several meta-analyses have assessed the effects of different transfusion strategies on thromboembolic events in various clinical settings [12, 15, 51, 53–55]. For example, one found no difference in venous thromboembolism between transfusion strategies in adult and paediatric patients (RR = 0.76; 95% CI 0.41–1.41; $p = 0.920$) [12]. A study of hip fracture patients also reported no significant difference in thromboembolism between transfusion strategies (RR = 1.15; 95% CI 0.56–2.37; $p = 0.710$) (based on low-quality evidence) [15]. Another study on hip fracture patients similarly reported no difference in thromboembolic events between transfusion strategies (RR = 0.71; 95% CI 0.34–1.45; $p = 0.350$) [53]. However, these reviews included studies other than

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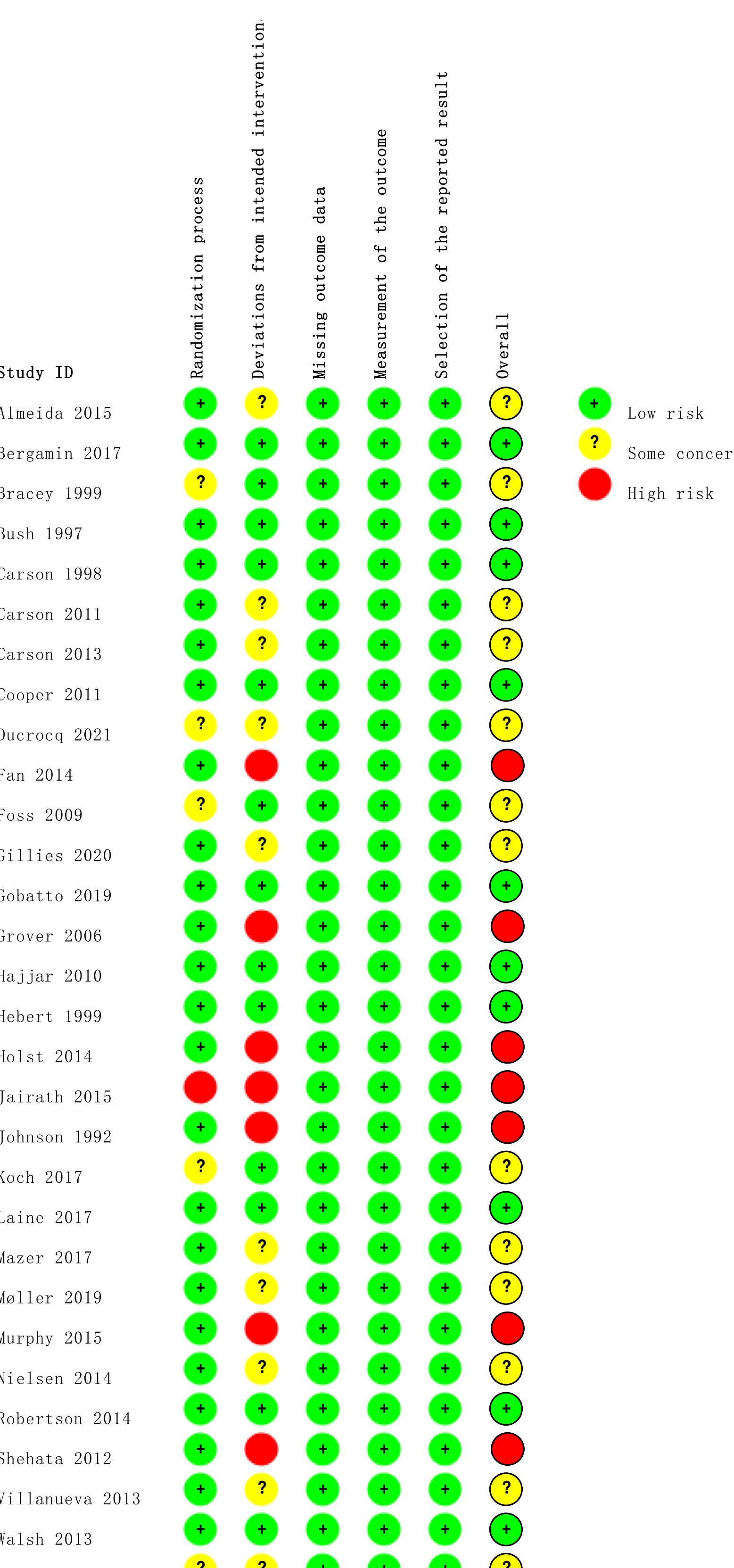


**FIGURE 4** Risk of bias assessment of included randomized controlled trials

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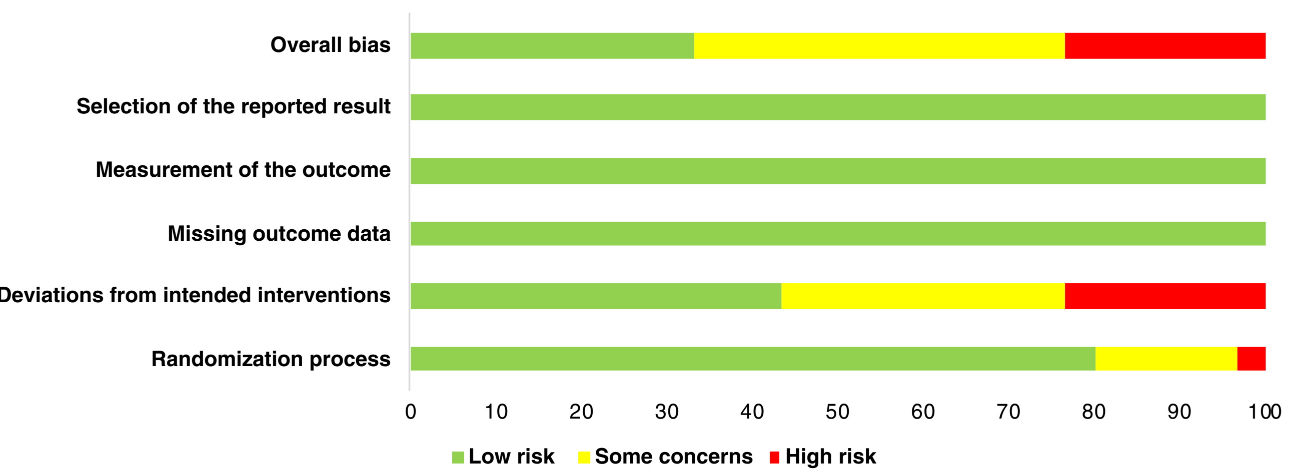


**FIGURE 5** Risk of bias summary

**Summary of findings:**

**Restrictive transfusion strategy compared to liberal transfusion strategy in adult patients**

**Patient or population**: adult patients
**Setting**: inpatient
**Intervention**: restrictive transfusion strategy
**Comparison**: liberal transfusion strategy

| Outcomes | Anticipated absolute effects* (95% CI) | | Relative effect (95% CI) | No. of participants (studies) | Certainty of the evidence (GRADE) | Comments |
|---|---|---|---|---|---|---|
| | **Risk with liberal transfusion strategy** | **Risk with restrictive transfusion strategy** | | | | |
| Thromboembolic events | 37 per 1000 | **24 per 1,000** (16–35) | **RR 0.65** (0.44–0.94) | 3976 (13 RCTs) | ⊕◯◯◯ VERY LOW a,b,c | |
| Cerebrovascular accidents | 17 per 1000 | **14 per 1,000** (11–18) | **RR 0.83** (0.64–1.09) | 14,509 (21 RCTs) | ⊕◯◯◯ VERY LOW a,b,c | |
| Myocardial infarction | 30 per 1000 | **32 per 1,000** (27–38) | **RR 1.05** (0.87–1.26) | 14,829 (25 RCTs) | ⊕⊕◯◯ LOW a,b | |

*__The risk in the intervention group__ (and its 95% CI) is based on the assumed risk in the comparison group and the **relative effect** of the intervention (and its 95% CI).

**CI,** confidence interval; **RR,** risk ratio

**GRADE Working Group grades of evidence**
**High certainty:** We are very confident that the true effect lies close to that of the estimate of the effect
**Moderate certainty:** We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different
**Low certainty:** Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect
**Very low certainty:** We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect

**Explanations**

a. Most studies were not adequately blinded (including participants, personnel, and outcome assessors).
b. Point estimates varied widely across studies.
c. The small number of events occurred in two transfusion groups.

**FIGURE 6** Summary of findings (including Grading of Recommendations Assessment, Development and Evaluation [GRADE] quality of evidence) in included randomized controlled trials

RCTs, and some of the included RCTs did not report specific haemoglobin- or haematocrit-based transfusion thresholds. We found a lower risk of thromboembolic events with the restrictive strategy (RR = 0.65; 95% CI 0.44–0.94; $p$ = 0.020). RBC transfusions may result in thrombosis by altering the rheologic variables and due to the infusion of pro-inflammatory and pro-thrombotic microparticles [56]; thus, lowering the transfusion threshold may reduce the risk. Subgroup analysis also showed that a restrictive (relative to liberal)

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strategy reduced thromboembolic events in RCTs conducted in North America (RR = 0.50; 95% CI 0.28–0.87; $p = 0.010$). The incidence of venous thrombosis varies among ethnic groups, with lower rates in Asians, Pacific Islanders and Hispanics than in Whites in the United States [57]. However, the association between transfusion strategies and ethnicity needs further investigation. Nevertheless, we should be cautious when interpreting the effect of a restrictive strategy on thromboembolic events. Sensitivity analyses showed that there was no longer a difference in thromboembolism between the restrictive and liberal groups after excluding the trial by Robertson et al. [39] (conducted in the United States) or Jairath et al. [30] (conducted in the United Kingdom).

We found no significant difference in cerebrovascular accidents between the restrictive and liberal strategies (RR = 0.83; 95% CI 0.64–1.09; $p = 0.180$). Curley et al. [58] reported that transfusion threshold was not associated with the risk of stroke among five RCTs (RR = 1.15; 95% CI 0.57–2.32; $p = 0.510$). Likewise, there was no significant difference in cerebrovascular accidents between restrictive and liberal strategies in cardiac patients [59] (RR = 0.97; 95% CI 0.72–1.30; $p = 0.840$); however, the review included only seven RCTs and used neurological complications as the cerebrovascular accident outcome. In contrast to other reviews, a review by Chong et al. [17] found that restrictive transfusion strategies were associated with fewer cerebrovascular accidents in critically ill patients based on six included RCTs (OR = 0.63; 95% CI 0.40–0.99; $p = 0.040$).

We also found that a restrictive strategy did not significantly affect myocardial infarction (RR = 1.05; 95% CI 0.87–1.26; $p = 0.620$), which is supported by previous meta-analyses [52, 59, 60]. Chen et al. [59] demonstrated that there was no difference in acute myocardial infarction between restrictive and liberal strategies in patients undergoing cardiac surgery. Similarly, Simon et al. [60] found no significant difference in myocardial infarction between the two transfusion strategies in older patients. However, Yao et al. [61] found that the incidence of myocardial infarction was lower with a restrictive (relative to liberal) transfusion strategy in ICU patients (OR = 0.54; 95% CI 0.30–0.98; $p = 0.040$). One of their included RCTs, conducted by Villanueva et al. [41], had only a small percentage of patients who were admitted to the ICU, despite the study having the greatest weight in the meta-analysis. After removing this study, the significant positive effect of the restrictive transfusion strategy on myocardial infarction in the review [61] became non-significant, indicating the instability of the effect.

Regarding transfusion type, we found no difference in the risk of thrombosis-related events between the transfusion strategies in either the leucocyte-reduced or non-leucocyte-reduced RBC subgroups. Regarding study area, the restrictive (relative to liberal) transfusion strategy reduced the risk of thromboembolic events in North America. This may have occurred because different areas have different ethnic groups who had varying levels of thrombosis risks after transfusion [57], different transfusion guidelines were employed in different regions and/or the perception of the risk of transfusion varied across areas [62–65].

Our meta-analysis has several strengths. First, we conducted a comprehensive search for RCTs that reported on thrombosis-related events (thromboembolic events, cerebrovascular accidents and myocardial infarction), which have not been fully analysed in previous meta-analyses [17, 55]. Second, we included the five most recent RCTs (published in 2019–2021) conducted in patients with traumatic brain injury [25], patients undergoing vascular surgery [36], patients in orthopaedic units [24], patients with acute myocardial infarction and anaemia [49] and patients undergoing cancer treatment [43]. Lastly, we used the latest Cochrane RoB tool, RoB 2, to evaluate RoB.

Our meta-analysis also has several limitations. First, the included RCTs had different RTTs; most were based on haemoglobin level, while some were based on haematocrit level. Furthermore, the RTTs varied among RCTs, even though they were based on haemoglobin level. Most trials used RTTs of 7–8 g/dl, but others used higher RTTs, potentially causing clinical heterogeneity. Second, the participants came from various clinical settings, leading to different tolerances for transfusion strategies. Third, thrombosis-related complications (such as thromboembolic events) were pre-specified as outcomes in only 25 of the 30 RCTs. Fourth, the definitions and follow-up time of each outcome varied across trials. Lastly, the transfusion timing (intra-operative, post-operative and during hospital/ICU stay) differed among the RCTs.

In conclusion, this meta-analysis demonstrated that restrictive transfusion strategies had a lower risk of thromboembolic events. The incidences of cerebrovascular accidents and myocardial infarction were unaffected by the transfusion strategy. Subgroup analyses indicated that restrictive (relative to liberal) strategies led to (1) fewer thromboembolic events in RCTs conducted in North America and (2) fewer myocardial infarctions in the RTT = 7 g/dl and LTT = 10 g/dl subgroup but not in the RTT = 8 g/dl and LTT = 10 g/dl subgroup. Restrictive (relative to liberal) transfusion strategies may be effective at reducing venous thrombosis but not arterial thrombosis. Other interventions are needed to reduce the incidence of thrombosis-related complications.

## ACKNOWLEDGEMENTS

M.M. performed the literature search, collected and analyzed the data and wrote the first draft of the manuscript; C.X.Z. collected the data and wrote the first draft of the manuscript; J.G.X. and Z.C.Z. supervised the research and reviewed and edited the manuscript; H.D.L. and O.C.O. verified the methodology and edited the manuscript.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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REVIEW

# Low-dose prophylaxis and its impact on the health of haemophilia patients

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## Abstract

**Background and Objectives:** There is convincing evidence to show that low-dose prophylaxis (LDP) results in reduction in annualized bleeding rate (ABR) and better health-related quality of life (HRQoL) compared with on-demand or episodic treatment (ET) in haemophilia patients. The aim is to review various LDP protocols practised for the treatment of haemophilia, specifically in resource-limited countries.

**Methods:** A literature survey was made of articles published in English language in PubMed and EMBASE without any time limit using keywords 'low dose', 'prophylaxis' and 'haemophilia' in different combinations.

**Results:** A total of 19 reports involving LDP in patients with haemophilia were included in this review. Almost all studies reported reduction in ABR, improvement in joint function, pain and HRQoL compared with ET, but this did not fully translate into significant improvement in structural arthropathy already caused by earlier bleeds, suggesting that LDP may be less or ineffective in either stopping or reversing the damage. Individualized dose escalation protocols based on pharmacokinetic (PK) or clinical parameters were found to be superior to fixed LDP protocols and cost-effective compared with standard dose protocols.

**Conclusion:** The developing countries can initiate LDP as the first step of prophylaxis, but certainly this should not be the final goal of the health care system in any country. Due to the complex pathophysiological mechanisms underlying haemophilic arthropathy, long-term data on LDP in haemophilia patients are warranted.

**KEYWORDS**
haemophilia, low dose, prophylaxis

## Highlights

- Low-dose prophylaxis reduces overall bleeding and joint bleeding rates in haemophilia patients compared with episodic treatment.
- Treatment should be individualized by understanding bleeding triggers and pharmacokinetic profiles.
- Long-term data on sub-clinical bleeding and its impact on joint health, the actual factor VIII trough levels required to prevent spontaneous joint bleed and well-standardized outcome parameters are important factors to be revisited and reviewed.

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## INTRODUCTION

The characteristic clinical manifestation of severe haemophilia is joint bleeding and progressive, irreversible joint damage resulting in permanent disability, despite being on-demand treatment. Due to the exorbitant cost of factors, the majority of patients are treated on-demand in developing countries and significantly large number of bleeds are left untreated or treated with suboptimal doses.

Prophylaxis is universally recognized as the treatment of choice for persons with haemophilia. Early prophylaxis is found to be superior to episodic treatment (ET) in reducing the risk of overall bleeding and improving joint health and quality of life (QoL) [1]. It is classified as primary, secondary and tertiary prophylaxis based on the time at which it is initiated. Primary prophylaxis is the regularly scheduled prophylaxis started before 3 years of age in the absence of any documented joint disease and before the second clinically evident joint bleed. These patients are less likely to have arthropathy. Secondary prophylaxis commences after two or more joint bleeds before the onset of joint disease. Tertiary prophylaxis is the initiation of prophylaxis after the onset of joint disease and it can be started at any age. The aim of tertiary prophylaxis is to slow the deterioration of joints, reduce pain and maintain mobility, specifically in adult haemophilia patients [2].

The objective of prophylaxis is to transition a person with severe haemophilia (factor VIII/factor IX [FVIII/FIX] <1 IU/dl) to mild or moderate haemophilia by maintaining factor levels above 1 IU/dl [3]. The major barrier in implementing this clinically effective therapy worldwide is the huge cost incurred on factors. The standard high-dose prophylactic regimen requires factor dosage of 25–40 IU/kg, thrice weekly, which is not feasible in majority of the developing countries where the per capita FVIII/IX use is below 0.1 IU, against a mean global per capita FVIII and FIX usage of 2.551 and 0.485 IU, respectively [4].

Different regimens are used by different groups for the initiation of prophylaxis therapy.

The long-term data on intermediate dosage protocols (IDP) from the Netherlands (median 2100 IU/kg per year, interquartile range 1400–2900 IU/kg/year) and dose escalation protocols from Canada (mean 3656 IU/kg/year) have been successful with slightly reduced bleed rates compared with standard dose prophylaxis (SDP) [5, 6]. With limited access to factors for majority of the patients globally, assessment of different dosages/protocols, specifically low-dose prophylaxis (LDP) protocols and pharmacokinetic (PK)-guided dose escalation protocols, are becoming increasingly important. Few reports from developing countries do support the superiority of LDP over ET in terms of annualized bleeding rate (ABR), annual joint bleed rate (AJBR) and the joint scores [7–25]. However, there is high heterogeneity in these reports in terms of demographics of patients, inclusion criteria, dosages, type of products and the outcome measurement parameters.

This review gives a summary of different LDP protocols and their impact on the overall short-term and long-term well-being of haemophilia patients in terms of ABR, AJBR, joint health and health-related QoL and also critically reviews some of the gaps that exist in the interpretation of the outcome data.

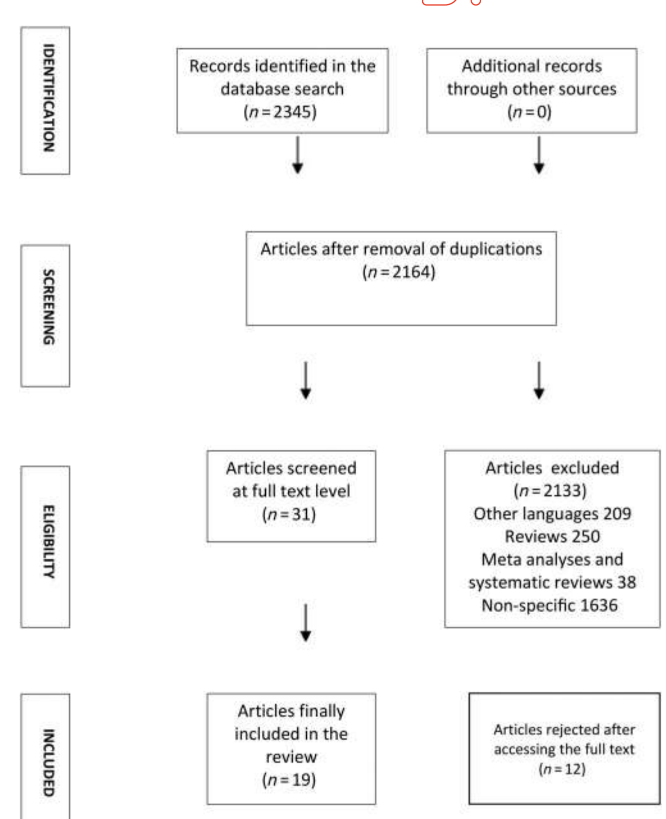


**FIGURE 1** Flow chart of the study selection process

## METHODS

The review is based on a comprehensive literature search of electronic databases, such as the MEDLINE and the EMBASE, until 25 October 2021. The following keywords were used: low dose, prophylaxis and haemophilia. The electronic databases were screened to cover dosage, duration of prophylaxis, study groups, type of product and outcome parameters. The bibliographies in all review articles, meta-analyses and in all included articles were hand searched and reviewed by two authors (S.S. and S.K.) to include any additional studies (Figure 1).

All publications were examined against pre-set inclusion and exclusion criteria. For inclusion, the studies had to meet the following criteria: (1) randomized and controlled clinical trials; (2) observational, original, retrospective, prospective and cross-sectional studies; (3) interventional and non-interventional studies; (4) cohort studies using both standard and extended half-life factor products but using low dose and a comparison has been made with either standard dose prophylaxis or ET. Excluded were as follows: (1) immune tolerance induction protocols using LDP; (2) studies on prophylaxis using non-factor products; (3) reviews; (4) studies where comparison has been made between standard half-life and extended half-life product; (5) conference abstracts published in journals; and (6) studies published in other languages.

The study selection and data extraction of all publications were performed in the first step by S.S., and in case of any ambiguity, the

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**TABLE 1** Published studies comparing low-dose prophylaxis and episodic treatment in the same cohort of patients

| | | Pre-prophylaxis period | | | Low-dose prophylaxis (LDP) period | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Reference | Country | No. of patients (haemophilia type) | Duration | Inclusion criteria | Type of prophylaxis | Protocol | Duration | Inclusion criteria | Outcome |
| [7] | China | 34 (HA 28, HB 6) | 12 weeks | 2–18 years; presence of clinical joint disease | Secondary and tertiary | FVIII 10 IU/kg/twice weekly<br>FIX 20 IU/kg/twice weekly | 12 weeks | ≥3 bleeds during observation period | AJBR reduced by 83% compared with ET; moderate improvement in joint function, daily activity, sports participation and school attendance |
| [8] | China | 66 (severe HA 34, moderate HA 32) | 8 weeks | 2–18 years; clinical joint disease | Tertiary | FVIII 10 IU/kg/twice weekly | 6–12 weeks | Same as ET | Reduction in ABR by 68.9% and AJBR by 78.8% between ET and LDP period; improved daily activities; no increase in factor consumption in LDP period compared with optimal regimen ET. Daily activities as measured by BCH scale upgraded in 31% and 60% improvement in FISH score |
| [9] | China | 33 (severe HA) | 20.0 ± 11.7 months | Adult severe haemophilia; age ≥18 years | Tertiary | 5–10 IU/kg/twice or thrice weekly | 20.8 + 9.9 months | Same as ET | ABR reduced by 71% during LDP; no change in the Pettersson score; significant improvement in FISH score<br>Scores for self-care and mobility both improved significantly |

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**TABLE 1** (Continued)

| | | Pre-prophylaxis period | | | Low-dose prophylaxis (LDP) period | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Reference | Country | No. of patients (haemophilia type) | Duration | Inclusion criteria | Type of prophylaxis | Protocol | Duration | Inclusion criteria | Outcome |
| [10] | India | 11 (severe HA and HB) | 6 months | >20 ED; having clinical joint disease | Secondary | FVIII 10–20 IU/kg/twice weekly<br>FIX 25–40 IU/kg/week | 6 months | Same as ET | Significant reduction in ABR; differences in HJHS and FISH scores were not statistically significant; mean hospitalization rate decreased from 12.45 to 2.36 |
| [11] | India | 70 (HA 58, HB 12; severe 21, moderate 49) | 12 weeks | Patients on ET; having clinical joint disease with ≥3 joint bleed into single joint | Tertiary | FVIII 10 IU/kg/ twice weekly<br>FIX 20 IU/kg/ once a week | 12 weeks | Same as ET | ABR reduced by 69% and 65% in moderate and severe haemophilia, respectively, during LDP; 60% reduction in hospitalization rate and 54% reduction in absenteeism from school/work |
| [12] | India | 30 HA | 6 months | Age ≤12 years | Secondary and tertiary | *10 IU/kg/twice weekly | 1 year | Same as ET | AJBR reduced by 85.76% and school absenteeism by 86%; 93% improvement in HJHS score; reduction in mean hospitalization rate from 8.7 to 1.1. The improvement in SAPS was mild in 57% of patients and moderate in 38%. No further change was seen in 5% of patients |

(Continues)

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**TABLE 1** (Continued)

| Reference | Country | Pre-prophylaxis period: No. of patients (haemophilia type) | Duration | Inclusion criteria | Low-dose prophylaxis (LDP) period: Type of prophylaxis | Protocol | Duration | Inclusion criteria | Outcome |
|---|---|---|---|---|---|---|---|---|---|
| [13] | Ivory Coast | 25 (HA 21, HB 4; severe 24, moderate 1) | NA | NA | Primary 5 and secondary 20 | *HA 20 IU/kg/ once a week HB 20 IU/kg/ every 10 days | 12 months LDP + 12 months follow-up | Age <10 years; treated on demand with SHL FVIII or IX, limited functional joint impairment; (AJBR) ≥5 | Reduction in AJBR by 87.6%; marginal decrease in HJHS scores and remained unchanged in 12 patients; 12.5% positive for inhibitors |

Abbreviations: ABR, annual bleed rate; AJBR, annual joint bleed rate; BCH, beijing childrens hospital; ED, exposure day; ET, episodic treatment; FVIII, factor VIII; FIX, factor IX; FISH, Functional Independence Score in Haemophilia; HA, haemophilia A; HB, haemophilia B; HJHS, Haemophilia Joint Health Score; SAPS, School Activity Participation Score; SHL, standard half-life.
*Extended half-life clotting factor concentrates (EHL-CFC).

second author (S.B.) was consulted. Studies with ongoing results were excluded. Duplicate publications were further screened and data were extracted only once.

The data were classified into four categories based on the study groups and treatment protocols: (1) studies where the outcome comparison has been made between ET and LDP in the same cohort of patients; (2) studies where comparison of the outcomes is made between ET and LDP in different patient cohorts; (3) long-term retrospective analysis of patients on LDP; and (4) studies with dose/frequency escalation based on certain clinical criteria, thus following individualized treatment protocols.

## RESULTS

The search strategy yielded 183 publications. After screening titles and abstracts, and deleting duplicates and other language articles, 19 publications were retained (Figure 1). Among these 19 reports, 7 were on retrospective analysis of patients on LDP. Five studies were long-term studies extending beyond 2 years, including the follow-up period. Five studies were on dose/frequency escalation protocols based on different clinical criteria and PK parameters, and four were randomized controlled trials. The LDP dosage used in majority of the studies was 10–15 IU/kg body weight/twice weekly.

### LDP versus ET in the same patient cohort

Seven studies have reported comparison of LDP treatment with ET in the same patient cohort, three each from China and India, and one from Ivory Coast in Africa (Table 1). Wu et al. reported data on LDP in 34 moderate and severe children with haemophilia A and B with joint disease (secondary and tertiary prophylaxis). Though the study period was short, that is, 12 weeks observation and 12 weeks prophylaxis period, the authors have shown 83% reduction in AJBR and 67% improvement in joint scores with a factor consumption of only 900–1000 IU/kg/year [7]. These findings were further confirmed in another multicentre study from China where 191 children with haemophilia were enrolled: only 66 patients completed the LDP protocol. The number of bleeding events was 166 on ET against 35 on LDP protocol. All these positive benefits were without any increase in factor consumption, when the comparison was made between the optimal on-demand therapy group and LDP group [8]. The joint scores did not show much improvement in another cohort of severe haemophilia A patients above 18 years of age, where the prophylaxis period extended up to 2 years. But the joints did not deteriorate further during this period requiring any medical intervention. This was in contrast to 16 procedural interventions during ET in the same cohort prior to prophylactic period [9]. This is the first long-term study, where it was shown that even in adults with joint arthropathy, LDP has substantial benefit in terms of reduction in ABR, hospitalization and improvement in QoL. Three studies from India on LDP have shown significant improvement in QoL indicators besides reduction in

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**TABLE 2** Studies comparing low-dose prophylaxis (LDP) with episodic treatment (ET) in different groups of patients

| | | Observation period (ET) | | | LDP | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Reference | Country | No. of patients | Duration | Inclusion criteria | Type of prophylaxis | No. of patients | Protocol | Duration | Inclusion criteria | Outcome |
| [14] | India | 10 | 11.5 months | Age 1–10 years; severe HA | Primary and secondary | 11 | 10 IU/kg/twice weekly | 11.5 months | Same as ET | ABR, AJBR and school absenteeism reduced significantly in LDP group compared with ET group. Children <3 years of age had zero bleeds |
| [15] | Indonesia | 25 | 12 months + follow-up period 12.8 ± 0.86 months | previously treated patients, 4–18 years, FVIII <1%, on ET | Tertiary | 25 | 10–15 IU/kg/ twice or thrice weekly | 12 months + follow-up period 12.3 ± 0.54 | Same as ET | AJBR reduced during LDP period; joint status improved; no difference in HEAD-US scores between 6- and 12-month observation |
| [16] | Thailand | 35 | 3 months | HA and HB patients; severe and moderate; on ET or LDP | Secondary and tertiary | 50 | 500 IU/twice weekly | 3 months | Same as ET | Patients on LDP had 40% target joints compared with 74% in ET group; none required hospitalization in the LDP group |
| [17] | China | 18 severe HA | 6 years | FVIII <1%; age 4–18 years | Tertiary | 16 Medium term 9 Long term 7 | 10–15 IU/kg/ twice or thrice weekly | LDP Medium term 6–18 months Long term 19–30 months + 6-year follow-up | Same as ET | Both in medium-term and long-term LDP groups, ABR and AJBR decreased; HJHS improved in both groups but significant in long-term prophylaxis group |

Abbreviations: ABR, annual bleed rate; AJBR, annual joint bleed rate; FVIII, factor VIII; HA, haemophilia A; HB, haemophilia B; HEAD-US, haemophilia early arthropathy detection ultrasonography; HJHS, Haemophilia Joint Health Score.

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**TABLE 3** Long-term retrospective data on LDP in haemophilia patients

| Reference | Country | Duration of LDP | Duration of follow-up | Inclusion criteria | Total no. of patients | Prophylaxis type | Dosage IU/kg/week mean (range) | Outcome |
|---|---|---|---|---|---|---|---|---|
| [18] | China | 28 patients with <24 weeks of LDP treatment 29 patients with >24 weeks of LDP treatment | 6 years | FVIII <1%, LDP 10–30 IU/kg/ twice weekly for ≥12 weeks | 57 | Primary 3, secondary 21, tertiary 33 | 10–30 IU/kg/ twice weekly | Significant ABR/AJBR reduction in primary, secondary and tertiary groups; the efficacy of the primary prophylaxis was better than the secondary and tertiary prophylaxes; no significant difference in AJBR between secondary and tertiary groups |
| [19] | China | Median no. of weeks on LDP per year: 19.62 (1.5–52) | 7.95 (6–10) years | FVIII <1%; age <18 years; ≥12 weeks on LDP protocol of 5–15 IU/kg/ once, twice or thrice weekly | 21 | Primary 1, secondary 14, tertiary 6 | Median 22.9 IU/kg/ week | Total IPSG MRI and HJHS scores in 21 patients ranged between 2–24 and 2–27, respectively; decrease in target joint numbers; joint scores had a positive correlation with the age at initiation of LDP and inverse correlation with factor dosage; QoL scores improved significantly in comparison with ET, but inferior to that of full dose prophylaxis; none required wheelchair or other accessory devices for walking |

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TABLE 3 (Continued)

| Reference | Country | Duration of LDP | Duration of follow-up | Inclusion criteria | Total no. of patients | Prophylaxis type | Dosage IU/kg/week mean (range) | Outcome |
|---|---|---|---|---|---|---|---|---|
| [20] | China | Median 6 (range 3–13.3) years | - | Moderate or severe HA and HB; age 11–41 years; previously received ET | HA 10, HB 5; moderate 5, severe 10 | Tertiary | 9.1–25.0 IU/kg twice weekly for HA and 10.0–20.0 IU/kg/once or twice weekly for HB | Significantly less number of patients with multiple joint damage in LDP group compared with ET group |

Abbreviations: ABR, annual bleed rate; AJBR, annual joint bleed rate; ED, exposure day; ET, episodic treatment; FVIII, factor VIII; HA, haemophilia A; HB, haemophilia B; HJHS, Haemophilia Joint Health Score; IPSG, International Prophylaxis Study Group; LDP, low-dose prophylaxis; MRI, magnetic resonance imaging; QoL, quality of life.

ABR [10–12]. Although the majority of the patients (70%) were of moderate phenotype in the cohort reported by Singh et al., there was not much difference in the outcome parameters between the severe and moderate groups. Gulshan et al. have presented similar outcome data from Eastern India on LDP treatment in 30 moderate and severe haemophilic children using extended half-life products. In a prospective analysis of 25 persons with haemophilia by Lambert et al. on LDP using Fc fusion recombinant factor VIII (Fc-rFVIII) and Fc-rFIX, a reduction of around 88% in AJBR and marginal decrease in Haemophilia Joint Health Scores (HJHS) was observed [13].

## LDP versus ET in different groups of patients

Table 2 shows studies where a comparison of LDP is made with ET in different groups of patients. A randomized study from India involving LDP (10 IU/kg/twice weekly) in 21 children (11 LDP and 10 ET), <10 years of age, has shown a marked reduction in ABR, AJBR and marginally better joint function. What is significant in this study is the occurrence of zero joint bleeds in children <3 years of age, compared with 11 joint bleeds in children >3 years of age. [14]. Almost similar results were observed in the study by Chozie et al. in 25 patients each on LDP and ET regimens using plasma-derived FVIII over a 12-month duration [15]. The report by Chuansumrit et al. on a short-term prospective analysis of 50 patients showed excellent results with a fixed dose of 500 IU/kg bodyweight given twice weekly. Twenty-four patients had zero bleeds at the end of treatment [16]. Liu et al. in a 6-year follow-up study on medium-term (6–18 months) versus long-term prophylaxis (19–30 months) showed significant reduction in both ABR and AJBR in medium-term as well as long-term prophylactic groups, but significant differences in HJHS scores were observed only in the long-term prophylaxis group and not in the medium-term group [17].

There are no reports on the comparison of clinical efficacy between LDP and SDP; however, there are few reports on comparison between IDP and SDP protocols. The IDP utilizes 15–25 IU/kg, thrice weekly, whereas SDP regimen utilizes 25–40 IU FVIII/kg, thrice weekly, with minor variations. In a prospective study with 20–30 years of follow-up involving Swedish patients (SDP) and Dutch patients (IDP), it was shown that at the median age of 24 years, no significant arthropathy is observed in 54% of patients on IDP and 89% of patients on SDP; QoL was almost similar in both groups with 66% increase in factor cost for patients on SDP [26]. In an earlier retrospective analysis on comparison of clinical outcome among on-demand, IDP and SDP, the Pettersson score was almost similar for IDP and SDP (6.0 and 6.5), but was much higher for patients on ET (18.8), though the factor consumption was more or less similar between ET and IDP regimen [27].

## Long-term retrospective analysis of LDP protocols

Although LDP is effective in the short term, it is not clear whether, in the long term, there is a substantial reduction in joint deformity and arthropathy in children with haemophilia. In the retrospective study in Chinese pediatric hemophilia a patients with rFVIII contained regular prophylaxis study, significant progress was observed in the primary prophylaxis group compared with secondary and tertiary prophylaxis groups [18]. The median ABR of the primary prophylaxis group was 0, suggesting that there was a good control of bleeding in this group. Though there was a significant difference in ABR between secondary and tertiary prophylactic groups, it was not the same for AJBR. Wu et al. in their retrospective analysis of 21 children with haemophilia with a follow-up period ranging from 6 to 10 years (mean 7.95 years) reported that none of the children had any prominent joint mobility disability. When the factors affecting the joint scores were analysed, age at initiation of LDP, duration and dosage of prophylaxis were found to be strongly associated with joint health [19]. Liu et al. have compared LDP with ET over a period ranging between 3 and 13.3 years (median 6 years) and have reported significant reduction in joint damage in the LDP group compared with patients on ET. Significantly better outcomes were observed in the knee and elbow joints but not in the ankle joints [20].

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**TABLE 4** Low-dose prophylaxis with dose/frequency escalation (personalized) protocols

| | | Maintenance period | | | Dose/frequency escalation period | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Reference | Country | No. of patients and type of haemophilia | Duration | Inclusion criteria | Type of prophylaxis | No. of patients | Escalation parameters | Escalation dosage | Duration | Median factor consumption (IU/kg/y) | Outcome |
| [21] | Tunisia | HA 42<br>HB 9 | NA | Age <15 years | Secondary and tertiary | 31 | Monthly evaluation of bleeds | HA 10–30 IU/kg/once or twice weekly<br>HB 15–35 IU/kg/once or twice weekly | 1 year + follow-up period of median 5 (range 1–9 years) | NA | 21 children did not need frequency escalation; median ABR reduced from 7 to 0.5. HJHS and FISH scores remained stable during study period; all parameters of HaemoQoL were found to be satisfactory |
| [22] | Iran | 33 severe HA | NA | FVIII <1 IU/dl; PUP; age <3 years | Primary | 2 | Occurrence of three episodes of haemarthrosis or four soft tissue bleed or life-threatening bleed in the first 3 months | 25 IU/kg/once a week to 25 IU/kg/twice weekly | 1 year | NA | Mean ABR after prophylaxis was 1.08 ± 2.21 episodes per year; five patients became positive for inhibitors (15.1%) |
| [23] | Iran | HA 20<br>HB 5 | 6 months | FVIII/IX <1 IU/dl; at least 6 months on ET; <15 years of age | Secondary or tertiary | 6; 5 HA and 1 HB | Three joint bleeds, four soft tissue bleeds or one life-threatening bleed without | HA 25 IU/kg/once weekly to 25 IU/kg/twice weekly | 3 years | 1754 | Mean no. of target joints reduced from 0.24 to 0.16; 76% of the patients did not need |

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**TABLE 4** (Continued)

| | | Maintenance period | | | Dose/frequency escalation period | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Reference | Country | No. of patients and type of haemophilia | Duration | Inclusion criteria | Type of prophylaxis | No. of patients | Escalation parameters | Escalation dosage | Duration | Median factor consumption (IU/kg/y) | Outcome |
| | | | | | | | specific trauma | HB 30–50 IU/kg once weekly to 30–50 IU/kg twice weekly | | | escalation from once a week regimen. The mean ABR was reduced to 1.86 from 5.6 during ET |
| [24] | China | 15 severe HA | 6 months | Severe HA; age 5–16 years; >50 ED; ≥1 target joint | Tertiary | 8 severe HA | PK studies indicating total time below 1 IU/dl of more than 30 h per week | 10 IU/kg/twice weekly to 10 IU/kg/every day | 6 months | 2402 | AJBR reduced from 7.8 to 1.4; factor consumption increased approximately by 50% |
| [25] | China | HA 33; severe 26, moderate 7 | 12 weeks | FVIII <2 IU/dl; on ET or LDP 10–15 IU/kg; >50 ED | Secondary | HA 33; severe 26, moderate 7 | Index joint bleed; persistent joint swelling | 10–15 IU/kg twice weekly to 20–25 IU/kg thrice weekly | 1 year | 1786 | Significant reduction in ABR and AJBR; no progression in arthropathy; resolution of 40% of the target joints; subjects with zero index joint bleed increased from 51.5% to 81.8% |

Abbreviations: ABR, annual bleed rate; AJBR, annual joint bleed rate; ED, exposure day; ET, episodic treatment; FVIII, factor VIII; FIX, factor IX; FISH, Functional Independence Score in Haemophilia; HA, haemophilia A; QoL, quality of life; HB, haemophilia B; HEAD-US, haemophilia early arthropathy detection ultrasonography; HJHS, Haemophilia Joint Health Score; LDP, low-dose prophylaxis; NA, not available; SAPS, School Activity Participation Score; SHL, standard half-life.

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Table 3 shows the summary of studies on long-term retrospective analysis of patients on LDP regimen.

## Studies with personalized and frequency escalation protocols

Individualized LDP protocols, based on periodical assessment of bleeding rate and joint status or PK parameters, are reported to help in the escalation of factor dosage and frequency in children with haemophilia, thus resulting in significant reduction in ABR, AJBR and improvement in joint health along with reduced use of factor concentrates.

For tailoring of dosage of factors to a certain trough level, PK must be determined in the individual patient. A study on 21 HA patients on PK-based prophylaxis showed a higher mean trough level (2.2 IU/dl) compared with SDP (0.9 IU/dl) along with reduced FVIII usage (mean 85,000 vs. 124,000 IU) in 6-month analysis [28]. Although a trough level of 1 IU/dl is often recommended for patients on prophylaxis, in many clinical situations, this does not seem to be appropriate. Very few data are available on accurate trough levels relevant to individual patients or to a specific clinical situation. Epidemiologic data in a Dutch cohort showed that an FVIII level of 1% may still have more than five annual joint bleeds, while levels of 10%–12% or more have zero joint bleeds [29]. Studies have also shown that daily prophylaxis of 5 IU/kg achieved double the FVIII trough levels compared with 10 IU/kg every alternate day with same amount of factor consumption [30].

An analysis by Gouider et al. in 55 children with haemophilia using an escalation dose and frequency protocol based on periodical clinical evaluation of bleeding data over a median period of 5 years (range 1–9 years) showed a drastic reduction of ABR from 7 during ET to 0.5 during LDP. The HJHS also showed significant improvement with a median of 4 (range 0–24), with 41 of 55 children showing a score less than 10 [21]. Two studies from Iran have shown a significant reduction in ABR using varied escalation criteria. In both studies, the frequency of infusion was increased to two or three times a week based on the number of episodes of joint bleed (three episodes) or soft tissue bleed (four episodes) as well as non-traumatic gastrointestinal bleeding or intracranial haemorrhage, within 3 months. Karimi et al. in a 1-year prospective study in 33 previously untreated patients (PUPs) with haemophilia A used an LDP protocol of once a week infusion of 25 IU/kg body weight, thus reducing the frequency of infusion with excellent outcome [22]. Eshghi et al. included severe HA and HB patients <15 years of age in their study and showed almost similar results [23]. Patients were followed up for 1–2 years after the initiation of prophylaxis.

Li et al. subjected 15 severe haemophilia A children to an observatory LDP regimen using the standard protocol of 10 IU/kg body weight, twice weekly for 6 months and then divided into two groups based on pharmacokinetic parameters, which mainly involved the total time per week with trough level of FVIII <1 IU/dl. All those children ($n = 8$) with less than 30 h with FVIII levels <1 IU/dl were put on PK-tailored prophylactic regimen. Remaining seven children were continued with the same standard LDP protocol for the next 6 months. The follow-up period in this study was just 6 months for both the maintenance and prophylactic groups. The annual FVIII consumption under this PK-tailored protocol was increased by approximately 50%, but there was a significant reduction in AJBR compared with the maintenance group [24].

Wu et al. in a prospective analysis of 33 boys with moderate and severe HA in a 1-year multicentre study (a pre-prophylaxis observation period of 3 months followed by a 1-year prophylaxis period) used an individualized treatment protocol based on index joint bleeding, joint swelling and ultrasound examination of index joints assessed serially throughout the prophylactic period. This secondary prophylaxis study used four escalating LDP regimens ranging from 10–15 IU/kg bi-weekly to 20–25 IU/kg daily. Compared with the pre-prophylactic observation period, a reduction of 43% in total bleeding events, 53% reduction in index joint bleeds and 70% reduction in target joint bleeds were observed. The percentage of children with zero bleeds increased from 52% to 82%. Except six children, remaining were on different steps of dose/frequency escalation regimen, suggesting that there is a high heterogeneity among haemophilia patients [25].

Table 4 presents the details of studies with dose/frequency escalation protocols.

# DISCUSSION

Prophylaxis aims to convert patients with haemophilia from a severe to a moderate clinical phenotype by regular infusion of FVIII. The concept is based on the fact that haemophilia patients with >1 IU/dl factor levels have lesser bleeding episodes and seldom have arthropathy. Thus, moderate haemophilia is considered as a 'natural evidence' for prophylaxis in haemophilia patients. Different protocols are used, with different dosages and frequencies of factor infusions, but there is no definition of an optimum protocol. Besides cost, the universal use of prophylaxis is limited by several factors, which include age, lifestyle, joint status, dosing intervals and adherence to treatment.

The standard prophylaxis regimen requires 25–40 U/kg every 48 h to maintain FVIII trough levels at more than 1 IU/dl. The concept of trough level as an important predictor of bleeding is supported by the fact that the time per week with FVIII/IX levels less than 1 IU/dl is associated with an increased rate of bleeding [31]. However, in a cohort of 34 children on primary prophylaxis, 27 (79%) had a trough level of <1 IU/dl versus 7 (21%) with a trough level of >1%; 16 of 27 (59%) patients with trough level of <1% had no clinical evidence of haemarthrosis during 1-year follow-up, and there was no difference in the number of bleeds between the two groups [32]. All these children were in the preschool age and were on standard dosage prophylactic regimen, that is, 20–40 IU FVIII or FIX, 3–4 times weekly for haemophilia A and 2–3 times weekly for haemophilia B. One important point to note from this study is that in very young children, the half-life of the factors being the lowest, even with the standard dose prophylactic regimen, very often, the trough levels do not go above 1%. It may only be interpreted that longer the patient spends with a

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low FVIII, the higher is their risk of bleeding, along with the understanding that the ABR depends on many other factors, like patients' physical activity, joint status and several other patient-related factors.

In the study by Verma et al. [14], FVIII trough levels were estimated just before the infusion at 6 months and at the end of the study. Median FVIII trough levels at both time points were <1% (range <1%–2%). Only three (27%) children were having FVIII trough levels of >1% at both time points. Mean number of overall bleeds (2 vs. 2.2) and mean joint bleeds (0.9 vs. 1.3) were not statistically different in patients with factor levels >1% and <1%, respectively. FVIII recovery levels were done in all children in prophylaxis group at the end of the study. Median recovery level 1 h after infusion was 8% (range 1%–12%). Using low doses, they proved that patients with factor levels <1% do not necessarily bleed more at all times than those with levels >1%, similar to the study of Petrini et al. [32]. Early prophylaxis also has better clinical outcome, as shown in three studies [14, 18, 22], wherein children on primary prophylaxis showed zero bleeds even on LDP protocol, compared with those on secondary prophylaxis.

Li et al. divided patients according to FVIII activity <1 IU/dl for more than 30 h per week into a PK-tailored group and a maintenance group. However, PK was not completely indicative of bleeding phenotype; the time (FVIII activity <1 IU/dl, more than 30 h per week) in three patients was longer than 30 h but with low AJBR, whereas in three other patients, the trough level was higher than 1 IU/dl despite more frequent haemorrhage [24].

Except one study involving PUPs, where the inhibitors occurred in 15.1% of the patients [22], inhibitor occurrence was not a major concern. There are contradictory reports about the incidence of inhibitors in PUPs on prophylaxis. A pilot study by Kurnik et al. showed that 47% of PUPs on SDP developed inhibitors versus 4% in the LDP group [33]. However, research of determinants of Inhibitor development among previously untreated patients with hemophilia study showed that during the first 20 exposure days, prophylaxis and ET posed similar risk for inhibitor development: the dose and frequency of prophylaxis were not associated with inhibitor development [34].

In conclusion, the benefits of LDP over ET in improving the standard of living of haemophilia patients in developing countries with almost similar cost are evident. It is unreasonable to deny the benefits of prophylaxis to 70%–80% of the world haemophilia population. The studies clearly indicate that it is not enough, but it is better than ET. This should not be the goal of haemophilia care in any country; LDP may be the optimum care until HDP becomes economically feasible for resource-constrained countries.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REVIEW

# The history of buffy coat platelet concentrates: The Dutch story

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None.

## Abstract

The buffy coat method as a source for platelet concentrates was developed in the 1970s and is still used in many blood centres around the world. Development of the method sparked various technological advances in blood collection, processing and storage. At the time, the need for platelet concentrates sharply increased because of better treatment regimens for (onco)haematological diseases, which forced blood centres to standardize and automate their production processes as much as the technology would allow. In this review, a historical overview of the Dutch experiences is provided in the context of the international developments.

KEYWORDS
buffy coat method, platelet concentrates, platelet storage

## Highlights

- The increased demand for platelet concentrates necessitated the development of a streamlined component preparation process, which included isolation of platelets from buffy coats.
- The buffy coat method sparked developments, such as automated component separators, bottom-and-top blood collection systems and 1,4-butanediol cooling plates.
- Nowadays, buffy-coat-derived platelet concentrates are a pre-storage-pooled, leukocyte-reduced, off-the-shelf blood product, screened for the presence of bacteria, with a 7-day outdating, containing sufficient platelets to treat an adult patient.

## INTRODUCTION

The buffy coat method for the preparation of platelet concentrates is applied in many countries. The concept of buffy coat removal from red cell concentrates was known since the 1960s [1]. The use of buffy coats as a source for platelet concentrates was developed in the 1970s, but it was not until the mid-1980s that the buffy coat could reliably be used in a large blood bank setting for routine production of platelet concentrates. In this review, we describe the history of the buffy coat method and how it was developed at the former Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB) and at the Red Cross Blood Bank in Amsterdam, currently merged into Sanquin Blood Supply in The Netherlands.

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### The beginning

Initially, in the Amsterdam Red Cross Blood Bank, blood was drawn in glass bottles, but since the mid-1970s, blood was collected in plastic bags (for a timeline, see Table 1). The collection bag was connected to satellite bags with plastic tubing, which allowed the separation of whole blood into components. Until 1974, unprocessed whole blood was the predominant transfusion product (Figure 1a). In 1975, the blood bank started removing the buffy coat as a standard practice to prevent

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**TABLE 1** Timeline of the development of the buffy coat method in The Netherlands

| Year | Development | References |
|---|---|---|
| 1975 | Introduction of removal of buffy coat from whole blood as standard practice | Annual report |
| 1976 | Development of the buffy coat method for platelet preparation | [2] |
| | First prototype of the Compomat | D. de Korte and H. Loos, personal communication |
| 1977 | Move from glass bottles to plastic blood collection systems | Annual report |
| 1980 | First version of the Compomat | D. de Korte and H. Loos, personal communication |
| 1983 | Radiolabelling studies of cold-stored buffy coat platelets | [3] |
| 1984 | Development of room-temperature-stored, buffy-coat-derived platelets in a closed system | [4] |
| | Radiolabelling studies of room-temperature-stored buffy coat platelets | [4] |
| 1986 | Development of the special centrifuge inserts | [5] |
| | Clinical evaluation of 5-day-stored buffy coat platelets | [6] |
| 1987 | Room temperature storage of platelet concentrates | Annual report |
| | Extension of platelet storage from 3 to 5 days | Annual report |
| 1988 | Development and introduction of 1,4-butanediol cooling plates | [7] |
| 1995 | Compomat generation 3 in use | Annual report |
| | Introduction of bottom-and-top collection systems | Annual report |
| | Introduction of pre-storage pooling of buffy coats | Annual report |
| 1996 | Compomat generation 4 in use | Annual report |
| | Extension of platelet storage from 5 to 7 days | P.F. van der Meer, personal communication |
| 2001 | Introduction of bacterial screening of all platelet concentrates | [8] |
| 2010 | Compomat generation 5 in use | G. Mast, personal communication |

*Note*: Dates are approximate, and have been derived from the indicated references, annual reports of the Amsterdam blood centre, or from memory.

micro-aggregate formation during storage due to the presence of a large number of leukocytes and platelets. Hence, from 1978 onward, buffy-coat-reduced red cell concentrates became the main transfusion product. From about 15% of the donations the buffy coat was not removed, as these units were used for the production of platelet-rich plasma (PRP)-derived platelet concentrates (Figure 1b). In the PRP method, whole blood is softly centrifuged, and the PRP is expressed to a satellite container (see Figure 2; for more detail, see Figure S1). This container undergoes a second hard spin, and the platelet-poor plasma is expressed to a third container. The pelleted platelets are resuspended in about 50 ml of the remaining plasma to obtain a platelet concentrate. This was done on demand, that is, when required for a patient, and two, four, or six PRP-derived concentrates were aseptically pooled for an adult patient shortly before transfusion. Because of the open system, the storage time was limited to 6 h at room temperature.

## The buffy coat method

In 1976, Peter Prins (Senior Scientist, Department of Cell Chemistry, CLB) and Hans Loos (Head, Department of Cell Chemistry, CLB) experimented with isolating platelets using the buffy coat method [2]. They first determined the physical properties of the various blood cells and found, in experiments with the IBM2991 cell processor with circular bags, that the dynamic behaviour of cells during centrifugation could be described according to the Svedberg formula [9]. This experience was used to determine a process with two consecutive centrifugation steps for the isolation of platelets from whole blood [9, 10] using a triple plastic bag system fitting in regular centrifuge holders. First, a hard-spin centrifugation step was developed to lead to a buoyant density equilibrium, with red cells at the bottom followed by a layer of granulocytes, lymphocytes, monocytes, platelets and plasma on top. A plasma clamp was used for extraction of the cell-'free' plasma to one container, and then the centrifuged bag was clamped with a surgical stomach clamp to ensure that the buffy coat layer did not mix with the red cells, followed by expression of the buffy coat to another container (see Figure 2; for more detail, see Figure S2). This buffy coat had an average volume of 55 ml, and contained more than 80% of the platelets and 60% of the leukocytes [2]. As a consequence, there was a 'considerable reduction' in the number and size of micro-aggregates in these buffy-coat-reduced red cells during 3 weeks of storage [2]. After dilution with a small amount of plasma, this buffy coat of about 110 ml and a haematocrit of approximately 20% was centrifuged again under differential centrifugation conditions (soft speed), in which only the platelets were small enough to be pushed upwards in the centripetal streaming plasma [10]. This made it possible to end up with a platelet concentrate with $72 \pm 19 \times 10^9$ platelets and only $17 \pm 21 \times 10^6$ leukocytes [4].

After validation of the process, the Red Cross Blood Bank Amsterdam introduced this method for their routine production of platelet concentrates in 1987. The buffy coat was stored at 4°C as an intermediate blood product for up to 48 h. Two, four, or eight buffy coats were pooled and, after the soft-speed centrifugation step, the platelet-containing supernatant was expressed to a satellite container using a plasma clamp. Also here, storage of the pooled product was limited to 24 h at 4°C as a result of opening the system at the time of pooling. In 1988, already 80% of the platelet concentrates was

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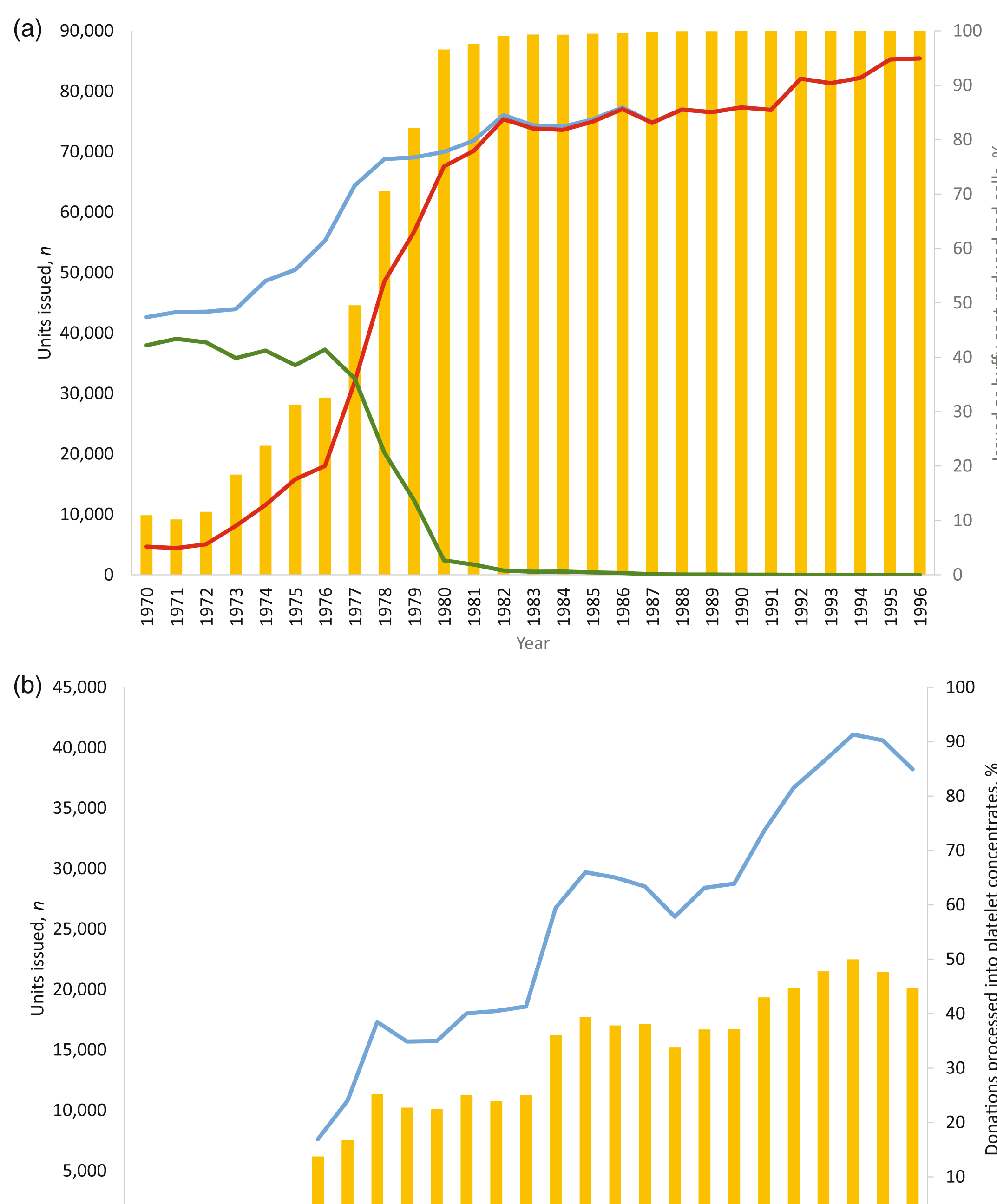


**FIGURE 1** (a) Total red cell products issued (blue), total issued as whole blood (green), total issued as buffy-coat-reduced red cells (red), and percent issued as buffy-coat-reduced red cells (yellow) for the Amsterdam Blood Bank between 1970 and 1996. Data obtained from annual reports. (b) Total platelet products issued (blue), and percent of donations processed into platelet products (yellow) for the Amsterdam Blood Bank between 1976 and 1996. Data obtained from annual reports

processed in this way; this was 89% in 1989, and further increased to 100% of whole-blood-derived platelets being produced as such in 1993.

The criticism, especially from experts in the United States, was that the buffy coat method was 'terribly cumbersome' [11], presumably because of the many process steps, including careful clamping of the whole-blood container. Another comment was that the buffy coats were stored at 4°C before platelet separation, which was known to have a negative impact on platelet survival [12]. The reason for the initial selected 4°C storage of the buffy coats was that platelets retained their adenosine diphosphate (ADP) response for 3 days [11], in contrast to room-temperature-stored, PRP-derived platelets [3]. Storage of buffy coats at room temperature was problematic, because the red cells and leukocytes produced a lot of lactate, resulting in a

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**FIGURE 2** Process steps of the platelet-rich plasma (PRP) method and the buffy coat (BC) method. In the PRP method, whole blood undergoes a soft-spin centrifugation step. The red cells are sedimented, but the platelets remain in suspension and are expressed to a separate container. The PRP is then hard-spun, most of the plasma is removed to another container, and the platelet 'button' is resuspended in the remaining plasma, giving a platelet concentrate. In the BC method, whole blood is hard-spun, and divided into a red cell concentrate, a BC and a unit plasma. The BC is then soft-spun, keeping the platelets in suspension, and the platelet-containing supernatant is expressed to a container. The residue is discarded. For detailed information and evolutions in the BC method, see the main text

pH <6.5 within 24 h, which impaired platelet functionality and survival. This severely limited the storage time, an impractical consequence for blood bank logistics [4].

With the ADP response still present, at the time it was believed that buffy coat platelets stored at 4°C would have a normal survival. Additionally, unpublished observations by Hans Loos had shown that buffy coat platelets had a hypotonic shock response and serotonin uptake similar to that of fresh platelets after 4°C storage (these finding were published much later [3]). Therefore, it was postulated that cold-stored buffy coat platelets would have better survival than room-temperature-stored PRP platelets. However, a radiolabelling study in volunteers showed that recovery after transfusion was only around 30% (requirement: 40%–45%), and moreover, the survival was 2.3 days (requirement: 6–7 days) [3], disputing the hypothesis. In contrast, the room-temperature-stored PRP platelets had a recovery of about 40% and a survival of 7 days. Thus, room temperature storage needed to be explored, but the problem was that the plastic bag system needed to remain closed, as opening it would limit the storage

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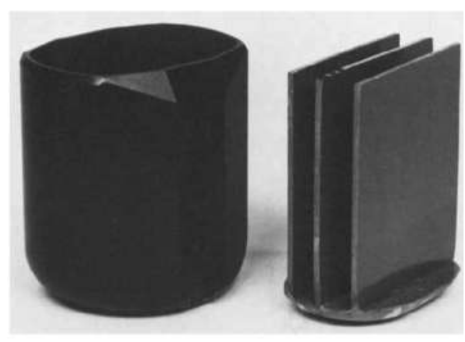
time to 6 h. This problem was solved by the development of a new quadruple-bag configuration, in which the whole blood could be collected and processed to plasma, red cell concentrate and platelet concentrate in a closed system [9]. Meanwhile, in 1984, the demand for platelets further increased because of the introduction of autologous bone marrow transplantations and the improved treatment of haematological malignancies in general. Consequently, by now, a little over a third of all donations was used to produce platelet concentrates from buffy coats in the Amsterdam Blood Bank.

## Room temperature storage

Ruby Pietersz (Deputy Medical Director, Red Cross Blood Bank Amsterdam) was the first to apply the new bag configuration for producing platelet concentrates in a closed system, which allowed storage at room temperature. Whole blood was centrifuged, and the plasma and buffy coat were subsequently removed. A saline–adenine–glucose–mannitol (SAGM) storage solution was added to the red cells, and the trick was to re-centrifuge the buffy coat with a soft spin, and express the platelets to the empty SAGM container (see Figure S3) [4]. These platelets could be stored at room temperature for 72 h: pH remained above 7, and the osmotic reversal reaction remained present, almost at the value of fresh platelets. More important was that platelet recovery after transfusion was on average 43% (requirement: 40%–45%) and survival was averaging 6.8 days (requirement: 6–7 days). An advantage was that the buffy coat platelets contained only about 2% of the leukocytes originally present in the whole blood, versus 10%–20% of platelet concentrates from PRP [4], reducing the risk for human leukocyte antigen alloimmunization. Two, four, or six platelet concentrates were pooled before shipment to the hospital.

In 1986, the hospitals requested approximately 30,000 (single) platelet concentrates. To give a sense of the scale of the demand, one platelet concentrate was produced from one donor unit, and in that year around 77,000 whole-blood units were collected. This illustrates the need for a standardized and automated process to handle so many units of whole blood. In the process up till then, the buffy coat was expressed to a 100-ml container, which was completely filled and did not fold or form creases during centrifugation, preventing red cell contamination of the platelet concentrates when taken from the centrifuge bucket. However, only six units could be centrifuged at the same time, one per centrifuge bucket. Development of an insert to hold four buffy coats in one centrifuge bucket made this process more efficient (Figure 3a) [5]. Blunt-tipped safety pins were put through the top seals of the buffy coat containers to keep them suspended on top of the 'plates' that were part of the insert, thereby keeping them upright during centrifugation (Figure 3b). Individual units could easily be removed after centrifugation. One operator could process 18 platelets concentrates in 1 h [5]. This new process was introduced in the Amsterdam Red Cross Blood Bank in 1987. At the same time, the storage time could be extended from 3 to 5 days, after a clinical trial had shown that the platelets gave sufficient increments in the patients and were able to stop bleeding tendencies [6].

(a)



(b)

FIGURE 3 (a) Oval centrifuge cup and the special insert intended to keep buffy coat bags upright during centrifugation. The insert is made of PVC. (b) Centrifuge cup with the insert to hold four sets of bags. Blunt-tipped safety pins, as shown on the foreground, are used to keep the bags upright. Both photos from Pietersz et al. [5], reproduced with permission

## Cooling plates and overnight hold

Blood was processed, without cooling, into components within 6–8 h after collection. To ease logistics, particularly for collections in the evenings and from mobile sites, overnight hold at room temperature prior to component preparation was investigated. That way, all whole-blood collections could be processed the next day, during regular working hours. Aluminium plates with 1,4-butanediol were devised as cooling plates. They were refrigerated and were ready for use after 30 min at ambient temperature when these plates had warmed to approximately 10°C. 1,4-Butanediol, with a melting point of 20°C functions as a 'heat sink', and quickly cools the units of whole blood to room temperature when placed under the plates, thereby achieving a uniform 'temperature history'. When whole blood was held overnight for 20 h, the buffy coats had a higher platelet yield and an overall more consistent platelet content than when processed within 3 h [7]. Thus, not only a uniform controlled process was introduced, but also the quality of the platelet concentrates improved.

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## Automated separation of whole blood into components

As articulated by Schiffer [11], the method of buffy coat separation was indeed cumbersome, and needed a high degree of staff training. Hans Loos had foreseen this, and developed an automated separator, of which a first prototype became available in 1976. This separator was equipped with clamping and sealing heads to automatically close or open the tubing leading to satellite containers. It had a scale to determine the weight of the extracted components. To detect the red cell/plasma interface, detectors were used, after which an extendable slide would ensure that the buffy coat layer remained in the upper part of the bag. Two individually moving presses divided the bags in a one-third upper part and two-thirds lower part. The various processing steps could be programmed. After refinements, the first version of what was called the Compomat became available in 1980. It was commercialized in 1984.

This automated separator allowed large-scale, standardized processing of whole blood into cell-'free' plasma, a red cell concentrate and a buffy coat. Importantly, the separator standardized the composition of the buffy coat with respect to volume and haematocrit. This was important, as these parameters determined the composition of the platelet concentrate that was made from that buffy coat.

Automated separators allowed the development of the bottom-and-top blood collection system [13]. In that system, the container in which the blood is collected had an outlet tubing to a satellite container both at the top and the bottom. After centrifugation, the red cells were expressed by the separation device to the bottom container, then plasma was expressed to the top container, and the buffy coat remained in the original collection container [14]. The idea was that, if the buffy coat remained in the original container, leukocytes and platelets sticking to the plastic after centrifugation would not end up in the red cell concentrate, but rather in the buffy coat. A paired comparison of the conventional top-top system with the bottom-and-top system revealed no major differences in the composition of the components, but the red cell concentrates indeed contained a five-fold lower leukocyte contamination than in the conventional system [15].

While previously the conventional system required very skilled staff for careful removal of the buffy coat, now the automated system in combination with the new blood bag design could be implemented easily even if staff was not very familiar with the buffy coat removal process.

One item remained to be solved. The buffy coat now remained in a larger collection container instead of the SAGM container, and the operators found it much harder to centrifuge the units. This resulted in a higher leukocyte contamination of the platelet concentrate as compared to the former procedure. Pooling of buffy coats using a sterile connection device was considered to solve that problem. This sterile connection device, initially developed for peritoneal dialysis, was an invention that revolutionized blood banking. It became available in 1983, and could connect two separate pieces of tubing using a bi-metal wafer that was heated to a temperature of 260°C during cutting and welding to prevent bacterial contamination [16]. It allowed pre-storage pooling of multiple buffy coats without opening the system, but was also instrumental in washing of red cells, or attaching a leukoreduction filter to a red cell or platelet unit. Because the system remained closed, these procedures could already be performed upfront, before receiving a request from the hospital.

Together, these evolutionary adjustments led to the development of a system in which five buffy coats were connected to a piece of tubing with multiple leads, using a sterile connection device [17]. A leukoreduction filter was integrated into the system, and an automated separator was used to express the platelet-containing plasma through the filter to the storage container. These platelets could be stored for 7 days and were introduced in 1996, first without bacterial screening. In 2001, bacterial screening with the BacT/Alert system for all platelet products was introduced in The Netherlands [8].

## International developments

In an academic environment where new developments are openly discussed, and where one can learn from their peers, parallel investigations were started. In the Budapest blood centre, the buffy coat was used for platelet production for immediate transfusion, but with 18–20 h storage of whole blood at 10°C [18]. They also performed studies with pre-storage pooling of buffy coats for immediate transfusion [19]. The Japanese Red Cross blood centre experimented with buffy coat platelets, ultimately ending up in the original red cell storage solution container, inspired by the Amsterdam experience [20]. Shimizu et al. [21] experimented with automated separation of whole blood and buffy coat removal. They simulated the bottom and top system by upside-down centrifugation of the blood collection container, similar to very early experiments of Hans Loos [10]. Other blood centres started picking up the buffy coat technology, and starting from the late 1980s, experiences of other centres, each with their local flavour, were published.

## Current situation and new developments

Overnight hold of whole blood, 1,4-butanediol cooling plates, pooling of buffy coats and storage of the platelets at room temperature are still largely in place in 2021 in The Netherlands. Nonetheless, modifications have taken place. After the merger of all 22 blood banks and the CLB into Sanquin Blood Supply in 1998, steps were taken to standardize the various processes. All blood banks had started using the buffy coat method in the years following its introduction in Amsterdam, but the Rotterdam Blood Center, for example, already used the platelet additive solution (PAS-B at the time), which was replaced with PAS-C in 2012. They supplied a quarter of the country, while the other three blood bank regions still made platelets in plasma. To standardize the processes, all platelets are now routinely prepared in PAS-E for the entire country since 2018. Because of the logistical burden of having to transport 1,4-butanediol plates to and from the mobile sites, we are

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considering temperature-controlled transportation boxes. Also, with the declining demand for red cells, whole-blood collections have dropped almost by half over the last decades (1992, 692,000 donations; 2018, 404,000) and fewer buffy coats are available for platelet preparation, which is becoming problematic for certain blood groups. Occasionally, platelet collection by apheresis is used to replenish shortages, but this takes considerable time from the donor, and is more expensive. We are investigating the possibility of reducing the number of buffy coats per pool to four, or even three, in the context of a platelet dose trial (Dutch trial register NL9204). We are currently also actively investigating fully automated centrifuges that can express the various components while being centrifuged, reducing the manual step to transfer the bags from the centrifuge to the automated separator.

In summary, about 40 years after the buffy coat method was developed for platelet preparation, the technology is still applied in many blood centres around the globe, predominantly, but not only, in Northern Europe, Australia, New Zealand and Canada. Various technological advances have further refined and standardized the technique.

## DEDICATION

This paper is to honour Ruby N.I. Pietersz (1942–2018). She devoted much of her professional career to study and implement the buffy coat method for the routine production of high-quality blood components, as described in this review. Worldwide, the buffy coat method is now applied in many countries, serving patients in need of these precious biological medicines.

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P.M. and H.R. conceived the idea, and P.M. wrote the manuscript; H.R., D.K. and H.L. provided information and reviewed the manuscript; T.K. reviewed the manuscript.

### CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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### SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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ORIGINAL ARTICLE

# Implementation of an Africa-specific donor health questionnaire for human immunodeficiency virus risk screening

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## Abstract

**Background and Objectives:** We had previously developed an Africa-specific donor health questionnaire (ASDHQ) based on local risk factors and designed a scoring scheme. This study assessed the performance of a new donor health questionnaire by comparing the human immunodeficiency virus (HIV) status in accepted versus deferred donors by ASDHQ and comparing the rate of risk deferrals with historical data.

**Materials and Methods:** Data were collected during a cross-sectional study conducted over 15 months at three referral-hospital-based blood services in Cameroon. ASDHQ was administered to blood donors aged 18–65 years in the same screening conditions as the routine questionnaire. The main outcomes of the study were ASDHQ sensitivity and specificity with regard to HIV laboratory testing as well as donor deferral rates for each of the routine screening algorithms and for ASDHQ.

**Results:** Overall, 71/11,120 (0.6%) were confirmed as HIV positive. The mean ASDHQ score was $95.80 \pm 4.4$ in HIV-negative donors and $94.80 \pm 4.4$ in HIV-positive donors ($p = 0.05$). The optimal cut-off provided by the receiver operating characteristic (ROC) curve for the best performance of ASDHQ was 95.04. Using this optimal cut-off, the ASDHQ sensitivity and specificity were 57% and 53%, respectively (area under curve = 0.58 [0.51, 0.64], $p = 0.028$). Using ASDHQ, the HIV prevalence was 0.7% in deferred donors and 0.6% in accepted donors.

**Conclusion:** ASDHQ might be efficient only in specific conditions that maximize truthful donor responses, requiring each blood service to create an environment of trust and transparency to increase donor compliance and improve the accuracy of the questionnaire.

KEYWORDS
Africa, blood safety, donor health questionnaire, HIV

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**Highlights**

- The study found that the Africa-specific donor health questionnaire (ASDHQ) performed poorly in discriminating human immunodeficiency virus (HIV)-negative blood donors from HIV-positive blood donors.
- The study also showed that ASDHQ was associated with a high deferral rate in the context of low HIV prevalence.
- ASDHQ might be efficient only in specific conditions that maximize truthful donor responses, requiring each blood service to create an environment of trust and transparency to increase donor compliance and improve the accuracy of the questionnaire.

# INTRODUCTION

Despite several years of international support, African blood services have some of the poorest blood safety indicators in the world as reported by several multi-centre surveys [1–3]. Recently, some multicentre studies have shown that the risk of human immunodeficiency virus (HIV) transmission by transfusion ranges between 1 in 456 and 1 in 90,200, which is much higher than in high-income countries [4, 5]. To reduce blood-borne HIV transmission, four main strategies need to be considered: more effective identification of blood donors at high risk of HIV infection; better laboratory screening for HIV; pathogen reduction of blood products and reduced blood utilization. Blood donor risk screening comes first in the overall process of collecting safe blood products. The identification and exclusion of donors with high-risk behaviours leads to a significant yet poorly quantified reduction in the risk of infections for the blood recipients and ensures donor safety as well. However, medical selection is inappropriately conducted in several African blood services, and the donor health questionnaire (DHQ) used for it is frequently inefficient [6, 7].

To design an Africa-specific DHQ (ASDHQ) based on local risk factors, we conducted a case–control study in Cameroon in 2017 and gathered risk factor data using audio computer-assisted self-interviews. We identified 16 HIV local risk factors and developed an ASDHQ and designed a scoring scheme to distinguish between HIV-positive and HIV-negative cases using receiver operating characteristic (ROC) curves. Donors who scored over 82.2 on a 100-point HIV risk score were more likely to be HIV negative than those who scored less [8]. However, to validate the new scoring system it needed to be implemented in a real-world setting.

In this study, we assessed the performance of a new DHQ by determining its sensitivity and specificity, comparing the HIV status in accepted versus deferred donors by ASDHQ and comparing the rate of risk deferrals with historical data. We also determined the operational acceptability of the new ASDHQ by collecting qualitative data from the donors. We hypothesized that ASDHQ would reduce the rate of risk deferrals compared to historical data and increase HIV prevalence in deferred donors compared to HIV prevalence in accepted donors.

# METHODS

## Study design and settings

Data used in this study were collected during a cross-sectional study conducted over 15 months at three referral-hospital-based blood services in Cameroon: the Yaounde University Teaching Hospital (YUTH), the Blood Bank of the Yaounde Central Hospital (YCH) and the Bafoussam Regional Hospital blood service (BRH). The sampling was consecutive. All three centres are located in urban areas. They collected between 2000 and 10,000 blood units per year. They have less than 30% of Voluntary Non Renumerated Blood donations (VNRBD). The Nucleic Acid Testing (NAT) is not performed on blood samples in Cameroon.

## Study population and data collection procedures

Following eligibility assessment and informed consent, prospective blood donors were included in the study. Blood donors aged 18–65 years were recruited at the clinics during their routine medical screening, which included a routine donor questionnaire (RQ) at YUTH and an RQ plus pre-donation testing (PDT) at YCH and BRH. ASDHQ was then administered in the same screening conditions as RQ. The donor deferral decision was based only on the routine screening criteria. Investigators collected 5 ml of whole-blood specimens from all the accepted and deferred blood donors for further HIV testing in the laboratory. Investigators also completed a paper-based laboratory tracking form in which they documented PDT and enzyme-linked immunosorbent assay (ELISA) results performed on site by the facilities themselves in addition to other specimens collected.

## Measurements and laboratory analysis

RQ used by the facilities was an empirical questionnaire based on 24 questions with yes/no response possibilities. ASDHQ is a comprehensive questionnaire of 16 questions designed previously by the authors to be administered within 15 min. Both questionnaires are available upon request to the authors. ASDHQ was designed to discriminate HIV-positive donors (score < 82.2) from HIV-negative

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donors (score = 82.2 or more) [8]. Both RQ and ASDHQ are available upon request. Each plasma specimen was first analysed by the facilities themselves using their routine algorithm for HIV diagnosis. It included two rapid determination tests (RDTs) in a serial algorithm as described in Figure 1. Each plasma specimen was tested before donation at YCH and BRH and after donation at YUTH with a rapid test (RDT 1, Alere Determine HIV-1/2, Matsudo, Japan); each specimen was also tested after donation in all three centres with either ELISA Ab (Human Diagnostics Worldwide, Wiesbaden, Germany) or ELISA Ag/Ab (Murex HIV Ag/Ab, Diasorin SpA, Saluggia, Italy). If at least one test was reactive, the donor was considered as at risk of HIV and deferred. If both tests were non-reactive, the donors were considered HIV negative and the blood unit safe for transfusion with regard to HIV. Samples reported discordant (at least one test reactive) or positive (two different assays reactive) were re-tested by the research team with the Oraquick HIV-1/2 (Orasure Technologies, Inc., Bethlehem), Geenius Bio-Rad HIV 1/2 (Bio-Rad, Marnes-la-Coquette, France) and/or the RNA detection for confirmation according to an appropriate algorithm (Figure 2). The confirmatory algorithm was developed following results from a study conducted in the same setting a year before and consistent with the WHO confirmatory approach [9]. Historical data on donor deferral rate and HIV prevalence were collected from blood centre registers

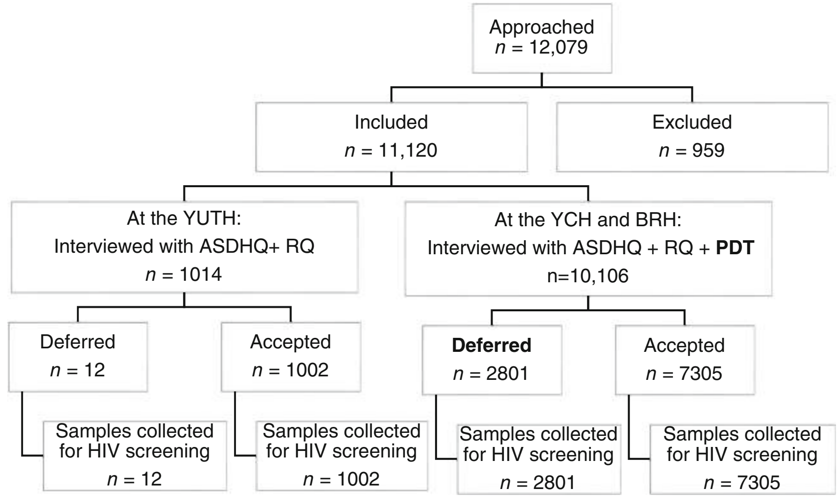


**FIGURE 1** Selection and testing procedures in the three participating facilities. ASDHQ, Africa-specific donor heath questionnaire; BRH, Bafoussam Regional Hospital; PDT, pre-donation testing; RQ, routine questionnaire; YCH, Yaounde Central Hospital; YUTH, Yaounde University Teaching Hospital

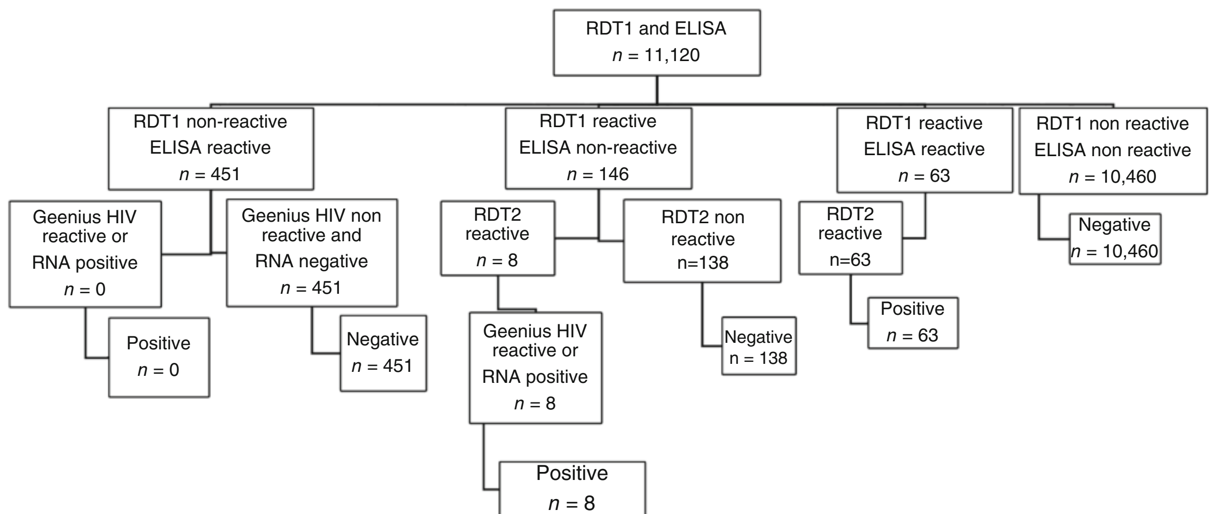


**FIGURE 2** Testing outcomes in the three participating facilities. ASDHQ, African-specific donor heath questionnaire; BRH, Bafoussam Regional Hospital; ELISA, enzyme-linked immunosorbent assay; RDT1, First rapid determination testing (pre or post donation); RDT2, Second rapid determination testing (post donation); RQ, Routine questionnaire; YCH, Yaounde Central Hospital; YUTH, Yaounde University Teaching Hospital

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**TABLE 1** Human immunodeficiency virus (HIV) statutes according to responses to risk factors questions in the study population

| HIV status | Frequently | Occasionally | Rarely | Never |
|---|---|---|---|---|
| C2: Sexual intercourse without condom during the past 12 months ($p = 0.26$) | | | | |
| Negative | 1947 (99.1) | 1514 (99.4) | 1740 (99.1) | 4464 (99.4) |
| Positive | 17 (0.9) | 9 (0.6) | 15 (0.9) | 26 (0.6) |
| Total (%) | 1964 (20.2) | 1523 (15.6) | 1755 (18.0) | 4490 (46.2) |
| C3: Anal sex during the past 12 months ($p = 0.72$) | | | | |
| Negative | 28 (96.6) | 108 (100.0) | 82 (98.8) | 9447 (99.3) |
| Positive | 1 (3.4) | 0 (0.0) | 1 (1.2) | 65 (0.7) |
| Total (%) | 29 (0.3) | 108 (1.1) | 83 (0.9) | 9512 (97.7) |
| C4: Sex with people you are not officially married to during the past 12 months ($p = 0.36$) | | | | |
| Negative | 936 (98.9) | 1157 (99.3) | 1387 (99.4) | 6185 (99.4) |
| Positive | 10 (1.1) | 8 (0.7) | 9 (0.6) | 40 (0.6) |
| Total (%) | 946 (9.7) | 1165 (12.0) | 1396 (14.3) | 6225 (64.0) |
| C6: Sex with sex workers during the past 12 months ($p = 0.29$) | | | | |
| Negative | 38 (100.0) | 77 (100.0) | 178 (99.4) | 9372 (99.3) |
| Positive | 0 (0.0) | 0 (0.0) | 1 (0.6) | 66 (0.7) |
| Total (%) | 38 (0.4) | 77 (0.8) | 179 (1.8) | 9438 (97.0) |
| C7: Sex with drug users during the past 12 months ($p = 0.49$) | | | | |
| Negative | 64 (100.0) | 28 (96.6) | 56 (98.2) | 9517 (99.3) |
| Positive | 0 (0.0) | 1 (3.4) | 1 (1.8) | 65 (0.7) |
| Total (%) | 23 (0.4) | 25 (0.4) | 36 (0.6) | 6264 (98.6) |
| C10: Sex with a man who had sex with another man ($p = 0.000$) | | | | |
| Negative | 10 (100.0) | 59 (100.0) | 38 (100.0) | 9558 (99.3) |
| Positive | 0 (0.0) | 0 (0.0) | 0 (0.0) | 67 (0.7) |
| Total (%) | 7 (0.1) | 17 (0.3) | 33 (0.5) | 6293 (99.1) |
| C12: Sex with somebody who spent at least a night in jail ($p = 0.40$) | | | | |
| Negative | 13 (100.0) | 23 (100.0) | 81 (97.6) | 9548 (99.3) |
| Positive | 0 (0.0) | 0 (0.0) | 2 (2.4) | 65 (0.7) |
| Total (%) | 8 (0.1) | 18 (0.3) | 34 (0.5) | 6291 (99.1) |
| C15: Treated for sexually transmitted infection ($p = 0.15$) | | | | |
| Negative | 62 (98.4) | 285 (98.3) | 734 (99.2) | 8584 (99.4) |
| Positive | 1 (1.6) | 5 (1.7) | 6 (0.8) | 55 (0.6) |
| Total (%) | 38 (0.6) | 190 (3.0) | 537 (8.4) | 5584 (88.0) |
| C17: Use non-injected illegal drugs ($p = 0.000$) | | | | |
| Negative | 26 (100.0) | 64 (100.0) | 97 (100.0) | 9478 (99.3) |
| Positive | 0 (0.0) | 0 (0.0) | 0 (0.0) | 24 (0.7) |
| Total (%) | 21 (0.3) | 47 (0.7) | 55 (0.9) | 6225 (98.1) |
| C18: Use injected illegal drugs ($p = 0.000$) | | | | |
| Negative | 12 (100.0) | 18 (100.0) | 27 (100.0) | 9608 (99.3) |
| Positive | 0 (0.0) | 0 (0.0) | 0 (0.0) | 67 (0.7) |
| Total (%) | 13 (0.2) | 13 (0.2) | 16 (0.3) | 6308 (99.3) |
| C21: Undergone treatment on the street such as pedicure/manicure, tooth care ($p = 0.028$) | | | | |
| Negative | 171 (99.4) | 429 (98.4) | 792 (98.8) | 8273 (99.4) |
| Positive | 1 (0.6) | 7 (1.6) | 10 (1.2) | 49 (0.6) |
| Total (%) | 117 (1.8) | 319 (5.1) | 535 (8.4) | 5380 (84.7) |

(Continues)

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**TABLE 1** (Continued)

| HIV status | Frequently | Occasionally | Rarely | Never |
|---|---|---|---|---|
| C22: Undergone acupuncture ($p = 0.57$) | | | | |
| Negative | 10 (100.0) | 25 (100.0) | 49 (100.0) | 9581 (99.3) |
| Positive | 0 (0.0) | 0 (0.0) | 0 (0.0) | 67 (0.7) |
| Total (%) | 10 (0.2) | 19 (0.3) | 33 (0.5) | 6286 (99.0) |
| C23: Tattoo yourself ($p = 0.23$) | | | | |
| Negative | 11 (100.0) | 42 (97.7) | 155 (98.7) | 9457 (99.3) |
| Positive | 0 (0.0) | 1 (2.3) | 2 (1.3) | 64 (0.7) |
| Total (%) | 9 (0.1) | 30 (0.5) | 112 (1.8) | 6199 (97.6) |
| C24: Piercings on your body ($p = 0.000$) | | | | |
| Negative | 13 (100.0) | 39 (100.0) | 159 (100.0) | 9454 (99.3) |
| Positive | 0 (0.0) | 0 (0.0) | 0 (0.0) | 67 (0.7) |
| Total (%) | 12 (0.2) | 30 (0.5) | 112 (1.8) | 6197 (97.5) |
| C25: Scarifications on your body ($p = 0.65$) | | | | |
| Negative | 13 (100.0) | 43 (97.7) | 268 (99.3) | 9341 (99.3) |
| Positive | 0 (0.0) | 1 (2.3) | 2 (0.7) | 64 (0.7) |
| Total (%) | 15 (0.3) | 45 (0.7) | 238 (3.7) | 6053 (95.3) |
| C30: If you are a woman, are you excised? ($p = 0.45$) | | | | |
| | No | Yes | | |
| Negative | 64 (98.5) | 1472 (99.6) | | |
| Positive | 1 (1.5) | 6 (0.4) | | |
| Total (%) | 6 (0.6) | 17 (1.8) | | |

and published papers [6, 10, 11]. We also interviewed 30 blood donors to measure the mean time spent to fill the questionnaire and their perception and their acceptability of the questionnaire using a set of five open-ended questions.

## Variables, outcomes and analysis

The main outcomes of the study were the ASDHQ sensitivity and the specificity with regard to HIV laboratory testing, donor deferral rates for each of the routine screening algorithms and for the ASDHQ at the recommended cut-off of 82.2 [8], the area under curve (AUC) and the optimal performance cut-off. The internal consistency (reliability) of the questionnaire was tested by calculating Cronbach's alpha. The best threshold of distinguishing between HIV-positive and HIV-negative donors was selected by ROC curves based on the calculated sensitivity and specificity. The cut-off score was adjusted to get the highest sensitivity. We measured and compared deferred donor rate and the HIV prevalence in accepted donors and in deferred donors for the different donor screening approaches (RQ, ASDHQ, RQ + PDT and PDT alone).

The socio-demographic data of the population were based on descriptive statistics: median and interquartile ranges for continuous variables, and counts and proportions for categorical variables. The outcomes described above were determined as proportions measured using EPI info 7.2.6 and Microsoft EXCEL. Sample size calculations were performed using the following baseline parameters: $\alpha = 0.05$, $1 - \beta = 0.80$, an annual study population of 15,000 donors, a baseline deferral rate of 13% and a baseline HIV prevalence of 2% in accepted donors. The proportion of blood donor deferrals and HIV prevalence in different time periods and in the deferred versus accepted blood donors were compared using chi-squared or Fisher's exact tests as appropriate. $p$-Values less than 0.05 were considered statistically significant.

## Ethical considerations

The study protocol was approved by the Cameroonian National Ethical Committee and the University of California San Francisco Committee on Human Research. All the collaborative centres provided an agreement for data and material sharing.

# RESULTS

## Study population

A total of 11,120 blood donors were included in the study, with 1014 (9.1%), 8718 (78.4%) and 1388 (12.5%) from YUTH, YCH and BRH, respectively. In total, 9374 (84.5%) were male and 9560 (86.0%) were family replacement blood donors. The mean age of the study population was 29.94 ± 8.24 (18–64).

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**TABLE 2** Mean score (over 100) of the Africa-specific donor health questionnaire by center and per human immunodeficiency virus (HIV) status

| HIV status | YUTH | YCH | BRH | Total | *p*-Value |
|---|---|---|---|---|---|
| HIV-negative | 95.70 ± 4.6 | 95.93 ± 4.4 | 95.02 ± 3.8 | 95.80 ± 4.4 | |
| HIV positive | 95.72 ± 2.9 | 94.84 ± 4.6 | 92.50 ± 2.88 | 94.80 ± 4.4 | |
| *p*-Value | 0.99 | 0.055 | 0.18 | 0.05 | |
| Total | 95.70 ± 4.6 | 95.93 ± 4.4 | 95.00 ± 3.8 | 95.79 ± 4.4 | 0.000 |

Abbreviations: BRH, Bafoussam Regional Hospital; YUTH, Yaounde University Teaching Hospital; YCH, Yaounde.

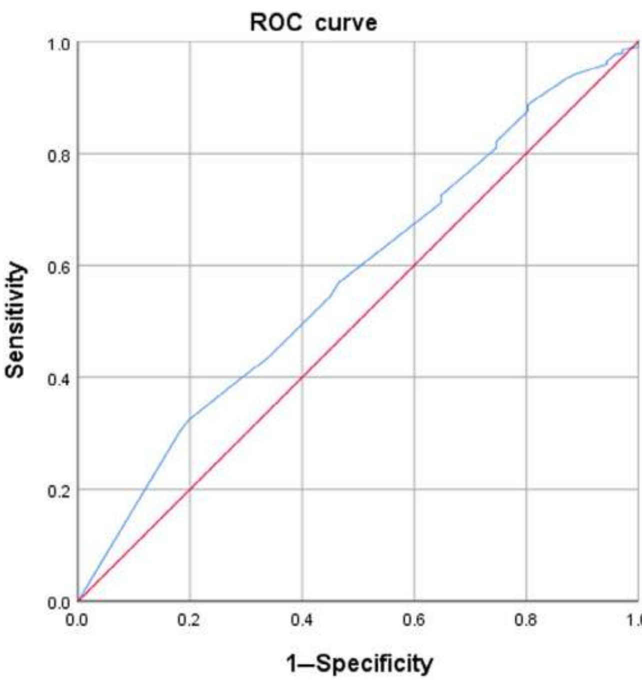


**FIGURE 3** ASDHQ area under the curve using the receiver operating characteristic (ROC)

## Main findings

Overall, 209/11,120 (1.9%) blood donors were reactive to RDT1 and 71/11,120 (0.6%) were confirmed HIV positive. The confirmed HIV prevalence was 0.7% (7/1014), 0.7% (60/8718) and 0.3% (4/1388) in YUTH, YCH and BRH, respectively. The prevalence of HIV was 0.7% (64/9374) in males versus 0.4% (7/1746) in females ($p = 0.11$). Of the 16 HIV risk variables included in ASDHQ, only five were associated with HIV status: sex with a man who had sex with another man, use of illegal drugs, history of treatment on the street such as pedicure/manicure, dental care and body piercings (all $p < 0.05$) (Table 1).

The ASDHQ score ranged from 30 to 100 with an average of 95.79 ± 4.40. The mean ASDHQ score was 95.80 ± 4.4 in HIV-negative donors and 94.80 ± 4.4 in HIV-positive donors ($p = 0.05$) (Table 2). Using the planned cut-off of 82.2, the sensitivity of ASDHQ was 0%. The optimal cut-off provided by the ROC curve for the best performance of ASDHQ was 95.04. Using this optimal cut-off, the ASDHQ sensitivity and specificity were 57% and 53%, respectively (AUC = 0.58 [0.51, 0.64], $p = 0.028$) (Figure 3). A total of 2801 (25.2%) blood donors were deferred in the three sites. The deferral rate for the routine questionnaire alone was 23.9% (2611/10,918) versus 2.0% (202/10,106) for RDT alone and 43.1% (4806/11,120) for ASDHQ at the optimal cut-off of 95.04. Using ASDHQ, the HIV prevalence was 0.7% in deferred donors and 0.6% in accepted donors (Table 3).

The sensitivity and specificity of RQ were, respectively, 40.8% and 76.6%. The sensitivity of RDT1 was 100% (71/71) and the specificity was 98.90% (10,911/11,049). The sensitivity of RDT1 was 100% in PDT in YCH and BRH and 100% in post-donation testing in YUTH. A total of 138/9904 donors were false positive with the PDT and were excluded from donating. The positive predictive value and the negative predictive value of RDT1 were 34% (71/209) and 100% (10,911/10,911), respectively.

The mean time required to fill the questionnaire was 14 min (5–30 min). Concerning the acceptability of the methods, 11/30 blood donors declared they had difficulty understanding all the questions, 28/30 declared the questionnaire to be useful for the safety of the recipient, and 17/30 needed additional explanations during the screening process.

## DISCUSSION

The study revealed that ASDHQ performed no better than RQ; both performed poorly in discriminating HIV-negative blood donors and HIV-positive blood donors. The study also revealed that ASDHQ had a high deferral rate in the context of low HIV prevalence. Finally, rapid testing performed either before or after donation had high sensitivity and specificity.

The sensitivity and specificity of any DHQ is important because they describe the ability of the questionnaire to identify HIV-positive donors while allowing HIV-negative donors to donate blood. The low sensitivity of DHQ increases the risk of collecting blood from at-risk donors, while low specificity would result in the exclusion of too many safe donors and adversely impact the blood supply. Our literature review revealed only a few assessments of DHQ in sub-Saharan Africa, but those that we did find reported that DHQs are inefficient as they were developed without assessment of local HIV risk factors

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**TABLE 3** Deferral rate and human immunodeficiency virus (HIV) status accepted and deferred blood donors

| Donor screening approach | Deferral rate, *n* (%) | HIV in accepted, *n* (%) | HIV in deferred, *n* (%) | Sens./spec. (%) | AUC |
|---|---|---|---|---|---|
| Routine questionnaire alone ($n = 10{,}918$)[a] | 2611 (23.9) | 7 (0.6) | 29 (0.2) | 40.8/76.6 | |
| RDT alone (pre-test) ($n = 10{,}106$)[b] | 202 (2.0) | 0 (0) | 64 (0.6) | 100/98.1 | |
| Routine questionnaire + RDT (pre-test) ($n = 10{,}106$)[b] | 2801 (25.2) | 0 (0) | 64 (0.6) | 100/72.0 | |
| ASDHQ cut-off 82.2[c] ($n = 11{,}120$) | 46 (0.4) | 71 (0.6) | 0 (0) | 0/100 | |
| ASDHQ cut-off 95.04 ($n = 11{,}120$) | 4806 (43.1) | 34 (0.7) | 37 (0.6) | 57/53 | 0.58 [0.51, 0.64] $p = 0.028$ |

Abbreviations: ASDHQ, Africa-specific donor health questionnaire; AUC, area under curve; RDT, rapid determination test.
[a]All sites.
[b]HCY + HRB.
[c]Tagny et al. (2017)[8].

[6, 7]. This study also confirmed the poor performance of RQ used in Cameroon. The poor performance of the RQ was the evidence that supports the development of ASDHQ from locally based risk factors using a rigorous case–control study based on recommended HIV diagnosis algorithm in a quality-assured laboratory [8].

Despite the good performance of ASDHQ in the pilot study and an adjustment to its cut-off, ASDHQ also performed poorly and worse than in the previous study. The ASDHQ scores were higher than expected, with no significant difference in score and HIV prevalence between the HIV-negative group and the HIV-positive group. Several risk factors that were significantly associated with the HIV-positive status in the first study were no longer associated with HIV status in the present study. This may be due to the differences in the screening environment in the two studies, the lower HIV prevalence in this validation phase, and the difference in the study design. Indeed, the pilot study was a case–control study with a large number of HIV cases and was administered using a computer-assisted programme, which was more confidential and conducive to donor attention and thus to more accurate responses.

Audio computed-assisted systems for donor health screening have been shown previously to produce more truthful responses compared to a standard written questionnaire and interview [12–15], and people are more likely to respond truthfully about risk behaviours when the questioning is anonymous [16]. Research on sexual behaviour, smoking, alcohol and drug use has found that use of a computer-assisted questionnaire increased reporting of 'stigmatized' behaviours compared to face-to-face interviews [17, 18]. Previous research has also suggested that the limitations of screening questionnaires in identifying ineligible donors may in part be due to the donors' failure to carefully read the instructions or understand the information [19], particularly donors with low educational status. In our study, a significant number of donors declared that they needed more explanations. Donor screening using ASDHQ may then be more efficient if it is conducted in an environment that maximizes the quality of the responses. Despite an ASDHQ sensitivity of 0.77 and specificity of 0.73 reported by Fonkou et al. (unpublished data), ASDHQ was not reliable in the routine environment in Cameroon because the authors conducted the assessment on a small sample size using RDT as the only test for HIV diagnosis.

Surprisingly, HIV prevalence in Cameroonian blood donors and blood donations was lower compared to that reported in previous studies, confirming its steady decrease in the past 20 years [10, 20]. This is consistent with national and international publications on the trends of HIV in Cameroon [21–23]. The effect of coordinated national and international programmes against HIV/AIDS on the general population, and blood donors in particular, may be the main explanation. The decrease in HIV prevalence may also be explained not only by the increase in the proportion of VNRBD but also by the progressive implementation of good practices during donor deferral in African blood services, and better training programmes and subsequent appointment of trained staff in the facilities under the programmes [1, 24, 25]. Considering the mean HIV residual risk of 1/2028 reported in Cameroon recently [5], 5 blood donors of the study population may have been falsely negative but this will not change significantly the prevalence.

The pre-donation screening (RDT1) had 100% sensitivity. This supports the hypothesis that testing for HIV before donation might be an option in Cameroon. This hypothesis needs to be confirmed by a study appropriately designed to assess laboratory screening methods. Indeed, several studies had previously confirmed the performance of pre-donation screening especially when it is conducted with high quality [26, 27], but other studies demonstrated the limitations of RDT in African blood services [28, 29]. About 140/10,000 donors were false positive and unnecessarily excluded for donation, consistent with previous reports that up to 10% of RDT reactivity are false positive in Cameroon [5, 20, 30].

In summary, despite that good cost effectiveness of PDT is reported in high transfusion-transmissible infection (TTI) prevalence settings in Africa [31], the cost effectiveness in low HIV prevalence settings is still to be assessed. Moreover, stigmatization after HIV donor notification immediately after the donor testing may limit the acceptability of the PDT by benevolent donors and impact on regular blood donation.

We recognize some limitations in this study. The low HIV prevalence may have limited an accurate assessment of ASDHQ. However, the HIV prevalence was still higher than in other countries, suggesting that this assessment outcome is likely to be the same in many settings with lower HIV prevalence. The study was not designed to assess RDT,

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but its outcomes appear to support the need to confirm the cost versus benefit of PDT. For a better assessment of the questionnaire, we could have used implementation science design instead of just observing the use of the questionnaire in the confirmatory algorithm, but the probability of finding a NAT+ sample among HIV-antibody-negative samples was likely very low. This step was important to measure its relevance prior to its full implementation. Finally, donor perception regarding the truthfulness of their responses to ASDHQ was not assessed, although we hypothesized that unadmitted risk factors were the likely explanation for its inability to discriminate HIV positives and negatives.

This study has public health and governmental policy implications regarding blood donor screening strategies to adopt in similar environments. We brought to light the fact that DHQs and the screening processes in Cameroon or in similar environments perform poorly. ASDHQ might be efficient only in specific conditions that maximize truthful donor responses, requiring each blood service to create an environment of trust and transparency to increase donor compliance and improve the accuracy of the questionnaire. Given the deficiencies of the donor history questionnaire, both improved donor recruitment with transition to voluntary donor pool and strengthening of laboratory testing of blood donations for transfusion-transmissible pathogens are needed. The study argues for more attention to RDTs than the DHQs in family replacement blood donations and progressive decrease of HIV prevalence in blood donors and, finally, the donation settings. The study also suggests that blood services may wish to assess the PDT strategies for HIV and other TTIs, taking into account cost effectiveness and donor acceptability.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ORIGINAL ARTICLE

# Blood donor eligibility criteria for medical conditions: A BEST collaborative study

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## Abstract

**Background and Objectives:** Donor eligibility questions and criteria for medical conditions vary between blood centres, suggesting that they are based more on local regulations or experience, rather than on published data, which are limited. As the donor population ages, medical conditions become more common. We assessed donor health assessment criteria at blood centre members of the Biomedical Excellence for Safer Transfusion (BEST) Collaborative. Our aim was to compare eligibility criteria and determine their underlying basis.

**Materials and Methods:** A REDCap survey was sent to blood centre participants, based on medical conditions of greatest interest suggested by the Donor Studies Team of the BEST Collaborative. Participants were asked about current donor health assessment questions, deferral criteria and the basis for their deferral policy (donor risk, recipient risk or both) for 20 medical conditions.

**Results:** Complete responses were received from 26 blood donor centres (24 separate responses) representing a combination of hospital-based centres, large regional centres and community/national blood centres in 14 different countries. Most centres specifically ask about heart and lung conditions, whereas fewer than half inquire about kidney, gastrointestinal or neurological conditions. North American blood centres tended to be less restrictive, while regulatory restrictions are more prevalent in Europe. Most participants felt that the criteria were based on regulatory requirements or experience, rather than on published data.

**Conclusion:** There is considerable variability in criteria by region. Ideally, criteria would be more evidence-based rather than based on regulatory requirements or experience. Deferral criteria must balance donor and recipient safety and maintain an adequate blood supply.

KEYWORDS
blood donation, donor criteria, donor eligibility

## Highlights

- There is considerable variability in eligibility criteria for blood donation, which are based on the geographic region rather than type or size of the blood centre.

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- North American centres tend to be less restrictive, while regulatory restrictions are more common in Europe.
- Most respondents thought that the criteria were mainly based on regulations or experience, rather than published data.

## INTRODUCTION

Blood donor eligibility criteria are established to ensure donor safety, recipient safety, product quality and the adequacy of the blood supply. Some aspects of donor eligibility are based on requirements set by regulatory agencies that are variably amenable to change, depending on the jurisdiction. Although, ideally, the criteria should be evidence-based, in practice, there is often scant evidence available, and it is not obvious how to obtain such evidence in a regulated blood collection environment [1–4]. As a result, donor eligibility rules vary between blood centres, even within similar regions, depending on judgement or experience of the individual blood centre's medical leadership. Similarly, although all blood centres ask donors if they are feeling well on the day of donation, specific questions on the donor health assessment questionnaire about organ systems meant to capture medical illnesses vary.

Previous efforts to develop a standardized health questionnaire and to harmonize donor deferral practices between different jurisdictions/countries (for example, the European TRANSfusion and Transplantation PrOtection and SElection of donor's project, TRANSPOSE) have had difficulty achieving consensus due to a lack of evidence and differing regulations and established practices, as well as risk analysis or perception [4–6].

The high-profile threats to recipient safety in blood transfusion pertain to infectious diseases and teratogenic medications. Government regulations have established stringent rules about donors with such risk factors. However, donor eligibility with respect to underlying medical conditions is more often established at the discretion of medical directors of blood collection organizations and involves both donor and recipient risk considerations. As the donor population ages, the prevalence of medical conditions and the associated pharmaceutical therapy resulting in possible deferral increase.

This study explored donor health assessment questions and deferral criteria for various medical conditions through a survey distributed to blood centre members of the Biomedical Excellence for Safer Transfusion (BEST) Collaborative. The survey was also a forum for the sharing of data and expert opinions that support existing practices. We sought to identify areas of consensus and discrepancy and the rationale behind current criteria in different blood centres. The study highlights where further research would be helpful to provide evidence in support of possible eligibility changes.

## STUDY DESIGN AND METHODS

We developed a REDCap (Research Electronic Data Capture) survey containing questions about the donor health assessment and various medical conditions. Medical conditions were chosen by the BEST Donor Studies subgroup, which is focused on research related to blood and plasma donors. Conditions were chosen because, in many jurisdictions, the condition is not covered by regulations, substantial variability of practice exists and/or the condition arises with relative frequency during donor eligibility screening.

The survey was sent to 32 blood centre members of BEST: 25 large national or regional blood centres, and 7 hospital-based blood collection centres. Each party received a unique invitation link, with the recommendation that the survey be completed by medical staff familiar with the reasons behind eligibility policies.

Study data were collected and managed using REDCap tools hosted at the Clinical and Translational Science Institute at Children's National Hospital (supported by grant UL1TR001876) [7, 8].

Respondents were asked whether their donor questionnaire includes broad questions about organ systems (heart, lung, kidney, endocrine, gastrointestinal tract, neurological), how they address medical conditions not included in their criteria manual and eligibility for new medications. They were asked detailed questions about criteria for blood donors with 20 different medical conditions (see Table 1, and Supplementary material). Centres responded whether they accepted all or some of these donors, or whether they deferred all of them. For neoplastic conditions, the first choice (accept all) was phrased as accept immediately after curative treatment. If a centre accepted certain donors, they were asked about qualifying criteria for acceptance (e.g., disease well-controlled, physician approval necessary). Participants were asked about the reason for the deferral (donor risk, recipient risk or both), the basis for their policy (published data, experience, or established practice, or regulatory and/or standards requirement), and whether they had a specific code for the deferral to enable the tracking of donor deferrals linked to a particular condition or criterion.

The study investigators contacted respondents to clarify individual responses. Responses were tallied for each of the 20 medical conditions. To provide a quick overview of the stringency of criteria for these conditions by region, the overall number of 'accept all', 'accept some', and 'defer all' donor responses were calculated for each region by summing all the answers provided. A percentage was then calculated for each category using the total number of responses for the region as the denominator. For example, for the 11 North American sites answering 20 questions, there were 220 answers; 78 of these (35%) were 'accept all' donors. A similar calculation was performed to determine the basis of deferrals (publications, experience or established practice, or regulations).

Depending on rules in each individual jurisdiction, approval by the institution's Research and Ethics Board was obtained if required.

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**TABLE 1** Medical condition deferrals by blood centre geographic region

| Condition | North America ($n = 11$) | | | Europe ($n = 7$) | | | Other ($n = 6$) | | | Safety rationale donor (D)/ recipient (R) risk[b] |
|---|---|---|---|---|---|---|---|---|---|---|
| | Accept all[a] | Accept some[a] | Defer all | Accept all | Accept some | Defer all | Accept all | Accept some | Defer all | |
| Cardiovascular, metabolic disorders | | | | | | | | | | |
| Hypertension | 1 | 10 | 0 | 0 | 6 | 1 | 0 | 6 | 0 | 25 D, 1 R |
| Type 1 diabetes | 6 | 3 | 2 | 0 | 0 | 7 | 0 | 2 | 4 | 21 D, 4 R |
| Type 2 diabetes | 5 | 6 | 0 | 0 | 7 | 0 | 0 | 6 | 0 | 21 D, 4 R |
| Coronary artery disease | 0 | 11 | 0 | 0 | 1 | 6 | 0 | 1 | 5 | 26 D, 1 R |
| Neoplastic disorders | | | | | | | | | | |
| Cancer[c] | 0 | 11 | 0 | 0 | 0 | 7 | 0 | 3 | 3 | 18 D, 16 R |
| Melanoma | 0 | 8 | 3 | 0 | 2 | 5 | 0 | 2 | 4 | 16 D, 22 R |
| Cervix pre-cancer, in situ[d] | 9 | 2 | 0 | 1 | 5 | 0 | 2 | 4 | 0 | 15 D, 17 R |
| Breast pre-cancer, in situ | 7 | 2 | 2 | 1 | 5 | 0 | 1 | 1 | 4 | 15 D, 17 R |
| Prostate pre-cancer, in situ | 6 | 3 | 2 | 1 | 4 | 1 | 1 | 1 | 4 | 15 D, 17 R |
| GI pre-cancer[e] | 10 | 1 | 0 | 1 | 4 | 1 | 2 | 2 | 2 | 15 D, 17 R |
| MGUS[f] | 4 | 3 | 4 | 1 | 2 | 3 | 0 | 2 | 4 | 14 D, 15 R |
| Benign tumour | 7 | 4 | 0 | 1 | 6 | 0 | 3 | 3 | 0 | 15 D, 5 R |
| Immune disorders | | | | | | | | | | |
| Multiple sclerosis | 2 | 7 | 2 | 0 | 0 | 7 | 0 | 0 | 6 | 21 D, 14 R |
| Inflammatory bowel disease | 1 | 10 | 0 | 0 | 2 | 5 | 0 | 2 | 4 | 19 D, 19 R |
| ITP[g] | 1 | 9 | 1 | 0 | 5 | 2 | 0 | 4 | 2 | 24 D, 7 R |
| Severe allergies[h] | 10 | 1 | 0 | 2 | 3 | 2 | 0 | 4 | 2 | 16 D, 11 R |
| Autoimmune disease[i] | 2 | 9 | 0 | 0 | 6 | 1 | 0 | 4 | 2 | 23 D, 17 R |
| TNFα blocker use[j] | 3 | 5 | 3 | 0 | 2 | 5 | 0 | 1 | 5 | 17 D, 18 R |
| Other disorders | | | | | | | | | | |
| Hypercoagulable condition[k] | 1 | 10 | 0 | 0 | 7 | 0 | 0 | 1 | 5 | 24 D, 13 R |
| Epilepsy | 3 | 8 | 0 | 0 | 4 | 3 | 0 | 5 | 1 | 24 D, 1 R |

Abbreviations: GI, gastrointestinal; ITP, immune thrombocytopenia purpura; MGUS, monoclonal gammopathy of undetermined significance.
[a]For neoplastic conditions, 'accept all' means 'accept immediately after curative therapy' and 'accept some' means 'accept under some circumstances or a specified period of time after curative treatment'.
[b]Some centres reported concerns about both donor and recipient risk for certain medical conditions.
[c]Breast, colon, or prostate.
[d]Abnormal Pap smear, cervical dysplasia, carcinoma in situ.
[e]Barrett's oesophagus, colon adenomas.
[f]Monoclonal gammopathy of undetermined significance.
[g]Idiopathic thrombocytopenic purpura.
[h]Not including allergies to latex or disinfection solutions.
[i]Systemic lupus erythematosus, scleroderma, vasculitis.
[j]Medications such as infliximab, adalimumab, and etanercept, sometimes called biologics and used to treat autoimmune disorders.
[k]Factor V Leiden deficiency, antiphospholipid syndrome, etc.

# RESULTS

## Survey participant geographic distribution

Responses were obtained from 26 centres (81% response rate) in 14 different countries (listed in Table 2). North American participants consisted of nine US centres (six stand-alone blood centres and three hospital-based collection centres) and the two Canadian blood centres. European participants included four national blood centres, one regional blood centre and two hospital-based centres. There were four national blood centres in the Asia/Pacific region and two hospital-based centres in Brazil.

Two American blood centres (New York Blood Centre and Innovative Blood Resources) have merged and use the same donor questionnaire and eligibility criteria. In Europe, the Welsh Blood Service and the English National Health Services Blood and Transplant (NHSBT) use the same eligibility criteria but slightly different donor health questionnaires. Therefore, we analysed 24 separate sets of deferral policies and 25 different health questionnaires. We grouped respondents into three regions: North America, Europe and Other (South America/Asia/Oceania).

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**TABLE 2** BEST study participants

| Country | Organization | Type of institution[a] | Respondent(s) |
|---|---|---|---|
| North America | | | |
| USA | American Red Cross | Large blood centre | Kathleen Grima |
| USA | Vitalant | Large blood centre | Ralph Vassallo, Hany Kamel |
| USA | New York Blood Centre[b] | Large blood centre | Lucette Hall |
| USA | Innovative Blood Resources[b] | Large blood centre | Nancy Van Buren |
| USA | Carter BloodCare | Medium blood centre | Frances Compton |
| USA | OneBlood | Large blood centre | Melissa Lopez, Kelsi Hurt, Rita Reik |
| USA | Stanford Blood Centre | Small blood centre | Suchitra Pandey |
| USA | Children's National Hospital | Hospital-based | Cyril Jacquot |
| USA | Dartmouth-Hitchcock Medical Centre | Hospital-based | Jenna Khan |
| USA | UCLA Blood & Platelet Centre | Hospital-based | Alyssa Ziman |
| Canada | Canadian Blood Services[c] | Large blood centre | Mindy Goldman |
| Canada | Héma-Québec[c] | Medium blood centre | Isabelle Rabusseau |
| South America | | | |
| Brazil | Hospital Israelita Albert Einstein | Hospital-based | Jose Mauro Kutner |
| Brazil | Sírio-Libanês Hospital | Hospital-based | Roberta Maria Fachini, Silvano Wendel |
| Europe | | | |
| Ireland | Irish Blood Transfusion Service[c] | Small blood centre | Ellen McSweeney |
| England, UK | NHS Blood & Transplant[c,d] | Large blood centre | Emanuele Di Angelantonio |
| Wales, UK | Welsh Blood Services[c,d] | Small blood centre | Stewart Blackmore |
| France | Establissement Français du Sang[c] | Large blood centre | Geneviève Woimant, Pierre Tiberghien |
| The Netherlands | Sanquin[c] | Large blood centre | Tanneke Marijt-van der Kreek |
| Germany | German Red Cross | Large blood centre | Torsten Schulze |
| Denmark | Aarhus University Hospital | Hospital-based | Christian Erikstrup |
| Norway | Haukeland University Hospital (Bergen) | Hospital-based | Torunn Oveland Apelseth |
| Asia, Western Pacific | | | |
| Japan | Japanese Red Cross[c] | Large blood centre | Minoko Takanashi |
| Australia | Australian Red Cross Lifeblood[c] | Large blood centre | Robert Harley |
| New Zealand | New Zealand Blood Service[c] | Small blood centre | Anup Chand |
| Singapore | Health Sciences Authority[c] | Small blood centre | Rami Alcantara |

[a]Stand-alone blood centres (not affiliated with a hospital). Large blood centre: >400,000 units collected in 2019. Medium blood centre: 200,000–400,000 units collected in 2019. Small blood centre: <200,000 units collected in 2019.
[b]Merged blood centres with same criteria.
[c]National blood service, or for Canada, two organizations each covering their geographic area.
[d]Very similar criteria.

## Questions about organ systems and being under a physician's care

There was substantial variability about organ-based disease questions. All US participants use the Donor History Questionnaire (DHQ) developed by the Association for the Advancement of Blood and Biotherapeutics (AABB) and recognized by the Food and Drug Administration (FDA). The DHQ includes the general question about heart and lung problems: 'Have you ever had any problems with your heart or lungs' [9, 10]. Three centres also ask a general question about being under a physician's care in the past few months.

Non-US participating centres routinely include questions about heart problems, and most inquire about lung diseases. Questions about medical conditions associated with the renal, gastrointestinal, endocrine, and neurologic systems, such as 'Have you ever had kidney problems', are less common. All non-US centres ask donors if they have been under a physician's care in the last few months to a year. For example, both Canadian blood centres ask donors 'In the last 6 months, have you consulted a doctor for a health problem, had surgery or medical treatment'.

## Deferral policies for medical conditions

Deferral responses associated with medical conditions are listed in Table 1. Variability in criteria was related to geographic location rather

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than the size of the blood centre or whether blood was collected in a stand-alone or hospital-based centre.

In general, for the medical conditions queried, North American blood centres had the least restrictive deferral policies. Of the 20 medical conditions queried, all or some donors are accepted by all North American centres for 12, all European centres for 5, and all other centres for 4. North American blood centres accepted all or some donors with a given condition in 91% of responses (200 out of 220 responses), compared to 59% in Europe (80 out of 135 responses) and 52% in other regions (63 out of 120 responses) (Figure 1).

Table 3 outlines factors used to determine donor eligibility for medical conditions where the blood centre accepts some donors. Again, there was considerable variability in the assessment of these donors; factors influencing eligibility include severity of illness, medication use, time since last exacerbation, time since curative treatment and consultation with the donor's physician and/or centre medical physician. In many cases, North American centres were again less stringent in their determination of donor eligibility. For example, donors with epilepsy were eligible if they had been seizure-free from 1 to 6 months, depending on the blood centre, regardless of anticonvulsant medication use, while European and most other centres required donors to have a 3-year seizure-free period while off anticonvulsant medication.

Criteria for high or low blood pressure (BP) are particularly variable, and may involve measurement of BP, and/or history of hypertension and use of antihypertensive medications.

All US centres measure BP and defer if it is out of range according to FDA regulations. One blood centre allows its physicians to accept donor despite low or high BP after medical evaluation. In Canada, Canadian Blood Services has stopped measuring BP and is monitoring the potential impact on donor reaction rates. Héma-Québec has temporarily paused BP measurements for physical distancing during the COVID-19 pandemic. Ireland, the United Kingdom, Denmark and New Zealand do not measure BP, while Norway-Bergen does on the first donation and annually for donors over 60 years. All other centres measure BP at all visits.

Deferral criteria include BP being out of range (all centres routinely measuring BP), poorly controlled disease (Denmark), recent addition of medications (United Kingdom, Ireland), high number of medications (Singapore) or the presence of hypertensive heart disease or renal disease (Sírio-Libanês Hospital in Brazil). Approval from the blood centre physician is needed under certain circumstances in Japan and Brazil. Since a history of hypertension and/or high BP measured before donation are common in otherwise healthy individuals, variability in policies may result in major differences in deferral rates.

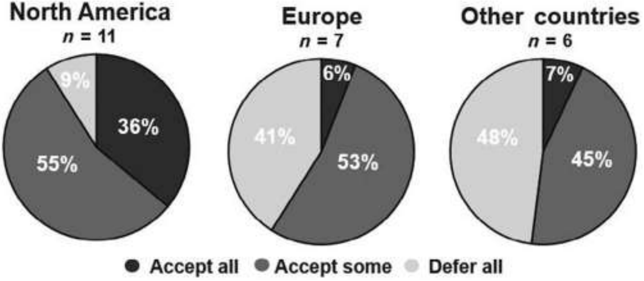


**FIGURE 1** Deferral for medical conditions, broken down by geographic region

## Basis of deferrals

Survey respondents outside of North America reported that regulations played a larger role in their decisions about deferrals associated with medical conditions. Regulations were a factor in 67% of European centre policies, compared to 23% in North America and 46% in other regions (Figure 2).

In the United States, regulatory requirements are important in criteria for hypertension and use of certain medications, but do not cover the other medical conditions in the survey [11]. In Canada, changes to criteria that might theoretically affect the quality of the product or safety of the recipient require a submission to the regulator for approval prior to implementation [12]. Changes to 17 of the 20 criteria (all except hypertension, coronary artery disease and epilepsy) would likely require a regulatory submission.

In Europe, 9 of 20 medical conditions queried in this survey are covered in the Commission Directive of the European Union 2004/33/EC, which dates from 2004 [13, 14]. In particular, the Directive explicitly states that donors with a history of cardiovascular disease (except for surgically corrected or resolved congenital abnormalities), a history of coagulopathy, diabetes being treated with insulin or malignant diseases (except for in situ cancer with complete recovery) should be permanently deferred. Donors with serious active, chronic or relapsing disease of the gastrointestinal and immunological systems are also permanently deferred, leading to deferral of donors with inflammatory bowel disease and other autoimmune conditions such as multiple sclerosis. Criteria are also specific for donors with a history of epilepsy, who must be seizure-free while off medications for at least 3 years to donate. Additionally, the European Directorate for the Quality of Medicines and Healthcare (EDQM) 20th Edition specifies deferral for a documented history of anaphylaxis or an autoimmune disease affecting more than one organ system [14].

For criteria that were not based on a regulatory or standards requirement, respondents indicated that their policies were based largely on experience and current practice. Published data were mentioned as the main basis for the deferral policy in less than 20% of responses.

## Medications new to market

Blood centres usually consult their medical director when a donor reveals that he or she is taking a medication that is new to market and not associated with clearly defined eligibility criteria. At some centres, medical staff are available on site to make this decision about eligibility. At other sites, the donor is temporarily deferred while the case is forwarded to medical staff for review. Decisions

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**TABLE 3** Considerations for determining donor eligibility when some donors are accepted

| Condition | Well controlled disease/ asymptomatic | Time since last event/ flare/ treatment | Donor physician approval | Blood centre physician approval | Medication use | Comments |
|---|---|---|---|---|---|---|
| Hypertension | X | | | X | X | • May include measurement and/or history |
| Type 1 diabetes | X | | | | | • Defer for acute event in last 3 months, complications |
| Type 2 diabetes | X | | | X | X | • Defer for multiple medications, change in medications, insulin use, repeated hypoglycaemia, complications |
| Coronary artery disease | X | X | X | | | • Asymptomatic, no surgery in last in 6 months to 2 years<br>• Ejection fraction >50% |
| Cancer | | X | | X | | • Accepted 1–2 years (USA), 5 years (elsewhere) after curative treatment |
| Melanoma | | X | | | | • Accepted 1–2 years (USA), 5 years (elsewhere) after curative treatment, or in situ disease only |
| Pre-cancerous conditions | | X | | X | | • Accept after cure or 1 year wait<br>• Accept if ongoing routine surveillance |
| MGUS | | | | X | | • Deferral may be a requirement for recovered plasma |
| Benign tumour | | X | | X | | • Location, size of tumour, and recovery from excision |
| Multiple sclerosis | X | X | | | X | |
| Inflammatory bowel disease | X | X | | X | X | • Deferral 3–30 days from flare, 2–3 days from diarrhoea |
| ITP | X | | X | X | | • Accept if cured, no episode in >1 year, less than four bleeding episodes, no splenectomy<br>• Platelet count over 100,000–150,000/μl |
| Severe allergies | X | | | X | | • Symptoms and specific allergen |
| Autoimmune disease | X | X | | X | X | • Severity, time since flare, organs affected, immune-suppressant use |
| TNFα blocker use | | | | | X | • Half-life, teratogenicity of specific medications |
| Hypercoagulable condition | | X | X | X | X | • Asymptomatic period of 1 month to 1 year<br>• History of clotting episode |
| Epilepsy | X | X | | X | | • Seizure-free 1–6 months (North America), 3 years (elsewhere)<br>• Anticonvulsant medication use |

Abbreviations: ITP, immune thrombocytopenic purpura; MGUS: monoclonal gammopathy of undetermined significance.

to accept or defer may be made depending on the time of the last dose of medication, pharmacokinetics, teratogenicity and similarity with other medications.

In the United States, the AABB maintains and updates a medication deferral list in consultation with FDA. This list is part of the donor health assessment process, and donors are asked if they have taken a medication on the list [9, 10].

The two Canadian centres review newly licensed medications quarterly, updating the donor suitability manual to include medications that are a cause for deferral. Donors on licensed new medications are accepted if these are not specifically on the deferral list [12].

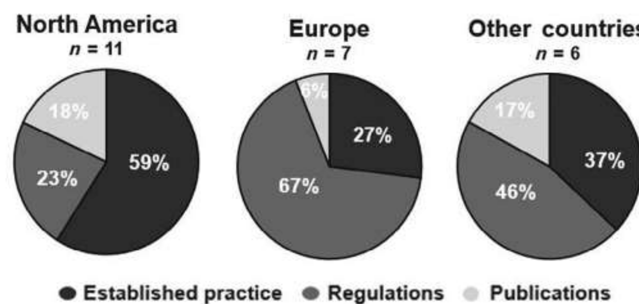


**FIGURE 2** Basis of deferrals: established practices, regulations, or publications

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## Conditions not listed in a criteria manual

If a donor presents with a condition not listed in a criteria manual, most centres attempt to contact a medical director in real time to determine eligibility. Another strategy employed is to defer a donor until a medical director can evaluate risk factors and contact the donor later regarding his or her eligibility.

# DISCUSSION

This survey demonstrated that there is wide variability in how blood donor centres approach donor eligibility for various medical conditions. Organizations within the same geographic region typically demonstrate better agreement, regardless of the size or type of centre.

None of the medical conditions queried in our survey is addressed in US FDA regulations [11]. However, US-based centres tend to have similar criteria. For example, donors who have had a myocardial infarction must wait at least 6 months before they are considered suitable for donation. This symptom-free interval may be in part based on how patients are assessed before being considered for elective surgery [15]. The AABB has taken a leadership role in developing a common donor health assessment questionnaire and unacceptable medication deferral list, and perhaps could do so for various medical conditions [9, 16].

For many medical conditions, European centres have more stringent criteria, based on the 2004 European Directive. Changing the directive requires a change in the law. Since detailed proscriptive criteria are included in the directive, blood centres are not able to make adjustments based on new evidence [4, 6]. A review of the content of the European blood directives, performed by the European Blood Alliance 10 years after their implementation, recommended that an evidence-based approach for donor deferral criteria be promoted [17]. More flexibility may be provided by higher level general directives and laws, which refer to more detailed technical standards that are reassessed and changed on a more frequent basis.

Our study has several limitations. The survey was sent only to BEST members, so our respondents come from a limited geographic distribution. There is no representation from African centres and limited representation from South American and Asian centres. The survey addressed only 20 specific medical conditions, chosen because they were of particular interest to the BEST Donor Studies Team; these are not necessarily the most important donor eligibility concerns regarding the number of donor deferrals. We deliberately chose to focus on medical conditions and did not address deferrals for travel or infectious disease risk, which involve different considerations such as the epidemiology of various infectious agents in different geographic regions. Centres interpreted some questions differently, such as organ system queries on their donor questionnaire. Some centres had different donation criteria for diseases within the same broad category, such as Crohn's disease and ulcerative colitis in the general category of inflammatory bowel disease. Some of these issues were partially addressed by having study investigators follow-up with respondents to clarify answers.

The literature about establishing deferral practices for medical conditions is limited [1, 12, 18]. Our data represent information from a wide range of blood centres and may be helpful for other blood centre medical directors in assessing their eligibility criteria. However, for most medical conditions, respondents noted that the basis for their policies was regulatory requirements or experience and established practice, rather than data or publications.

Although blood centres clearly cannot ethically perform randomized trials to evaluate the safety of various donor eligibility policies, several sources of information are useful in developing more evidence-based criteria. Knowledge about the frequency and natural history of medical conditions such as coronary artery disease, monoclonal gammopathy of undetermined significance, immune thrombocytopenia purpura and epilepsy may be helpful in designing criteria [15, 19–22]. A robust donor haemovigilance system, which includes post-implementation monitoring of the impact of changes on donor reactions, would provide data to assess donor safety criteria [23]. Finally, data linkage studies of donors and recipients assess theoretical transmissibility of various medical conditions, such as cancer and neurodegenerative diseases [24, 25]. Providing a firmer evidence basis for criteria will ensure that they protect donor and recipient health, without unnecessarily deferring people who are eager to contribute to the blood supply.

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## CONFLICT OF INTEREST

All authors declare no conflicts of interest.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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ORIGINAL ARTICLE

# Antioxidants in single methylene-blue-treated plasma units cannot be used to predict pathogen inactivation treatment success

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## Abstract

**Background and Objectives:** Measurement of antioxidant power (AOP) can be useful to validate the execution of the pathogen inactivation (PI) treatment of plasma units. The aim of this study was to evaluate the Theraflex technology for plasma units routinely used in Belgium.

**Materials and Methods:** AOP was tested on plasma units treated by Theraflex with various non-complete treatment scenarios. AOP was quantified electrochemically using disposable devices and was expressed as equivalent ascorbic acid concentration.

**Results:** During a complete PI treatment, AOP rose from $195 \pm 32$ to $230 \pm 42$ μmol/L eq. ascorbic acid after addition of methylene blue (MB), and decreased to $192 \pm 30$ μmol/L eq. ascorbic acid after illumination and finally to $177 \pm 27$ μmol/L eq. ascorbic acid after final filtration. Without MB, the final filtration had no effect on the plasma AOP ($197 \pm 22$ μmol/L eq. ascorbic acid before filtration and $194 \pm 22$ μmol/L eq. ascorbic acid after filtration). With no MB and no illumination, there was no significant difference between the plasma AOP at the beginning ($188 \pm 23$ μmol/L eq. ascorbic acid) and at the end of the process ($179 \pm 21$ μmol/L eq. ascorbic acid).

**Conclusion:** AOP measurement may not indicate the effectiveness of the PI treatment.

KEYWORDS
antioxidant power, plasma units, quality control

## Highlights

- Analysis of the antioxidant power (AOP) measurements does not allow testing the reliability of the pathogen inactivation process of plasmas treated by methylene blue (MB).
- Consequently, an AOP-based approach is unable to properly assess the effectiveness of the MB plasma treatment.
- Evaluation of intermediate measurements is proposed since the use of MB results in a clear increase in AOP.

## INTRODUCTION

The treatment of cellular blood products with pathogen inactivation technologies (PITs) is widely implemented in blood establishments to decrease the risk of bacterial contamination and to face the presence of new, emerging agents in blood components [1–5]. The Theraflex system (MacoPharma, Mouvaux, France) uses methylene blue (MB) and visible light to inactivate pathogens in plasma units [6, 7]. Other technologies exist, such as Mirasol Pathogen Reduction Technology (Terumo BCT, Lakewood, CO) and the Intercept Blood System (Cerus

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Corporation, Concord, CA), which use a combination of ultraviolet (UV) light and photosensitive molecules to inactivate pathogens and white blood cells in blood components used for transfusion [4, 8, 9]. Since 2004, all individual therapeutic plasma units have been treated with the Theraflex system in Belgium.

A quality control test is recommended by the Council of Europe to assess the efficacy of pathogen inactivation (PI) in blood components [10]. Different controls ensure that a unit has been put through instrument control and collaborator training. After illumination, a report is automatically printed to provide proof of completeness. The illuminator may also be connected to a data management system to block the release of non-illuminated or doubly illuminated products [11]. Additional actions can be implemented such as a label applied to the illumination container after treatment to provide visual evidence that the unit received complete treatment in the illuminator and should not be re-illuminated, or a UV indicator label changing from light blue to dark blue after UV-A exposure [12]. These checkpoints are necessary during the treatment process to avoid errors, but they are not a check on the product itself [11]. Finally, even more complex approaches based on the inhibition of mitochondrial DNA replication have been developed, which do not apply to plasma units [13, 14].

A new technology based on measuring the antioxidant power (AOP) can ensure the QC assay on blood products [11, 15–17]. Oxygen metabolism naturally produces reactive oxygen species (ROS) present in all cells in a steady state along with antioxidants [11]. These antioxidants can directly participate in the scavenging of ROS or indirectly by intercepting the chain-carrying radicals during the oxidative process [11, 18]. Oxidative stress appears when the ROS/antioxidant balance is disturbed because of ROS excess, antioxidant depletion, or both [11]. Consequently, with PITs that generate ROS [19] the resulting excess stress leads to a decrease in the AOP in platelet concentrates or plasma units, which has been demonstrated as proof of PI treatment effectiveness [11, 20, 21]. These ROS and the consequent oxidative stress are likely to participate in accelerating storage lesions and oxidative damage [19, 22–27].

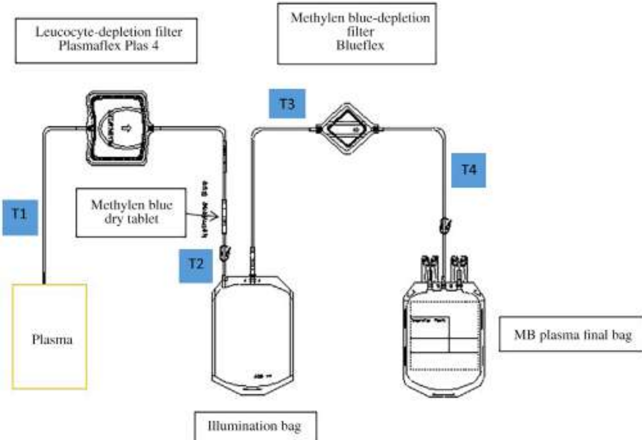


**FIGURE 1** Experimental design and sampling. MB, methylene blue; T1, initial plasma antioxidant power (AOP) level; T2, AOP level with MB before illumination; T3, AOP level after illumination but before MB filtration; T4, AOP level after illumination and after MB filtration

The technology comprises a commercial electrochemical device to measure AOP in a variety of samples, including biological fluids [15, 17]. It is based on an electrical current recording using linear sweep voltammetry. The recorded current corresponds to the ability of the sample to donate electrons for neutralizing free radicals [15].

This study is aimed at quantifying the AOP level in plasma units treated by the Theraflex system. Different protocols were established to understand the role of MB on one hand and the illumination in the other on the AOP values. The aim is to evaluate such a test as a QC assay for documenting the execution of MB pathogen treatment during the preparation of plasma units.

## MATERIALS AND METHODS

### Blood collection

Blood and blood-derived products were collected from healthy volunteer donors who gave their consent for the use of their blood components for research. An ethical committee decision was not required in this study design. The collection day was defined as Day 0. Plasma was obtained from whole-blood donations (CompoSelect, Fresenius Kabi, Bad Homburg, Germany) with a mean volume of 465 ml and was collected in accordance with local standards. Whole blood was cooled to 18–24°C within 18 h of donation and centrifuged at 4500*g* before being separated on MacoPress (MacoPharma, Tourcoing, France) by using a top and bottom kit to collect the plasma units.

### PI treatment

Plasma units were sterile connected to the Theraflex MB plasma disposable set (MacoPharma) and plasma content was filtered through the PLAS 4 membrane plasma filter included in the instrument to remove cellular elements. The plasma passed through the dry MB tablet into the illumination container. The plasma containing MB was illuminated with 180 J/cm$^2$ visible light (B2 Maco-tronic, MacoPharma). The illuminated plasma mixture was passed through the MB reduction filter by gravity into a single storage container to remove MB from the final plasma unit (Figure 1).

### Study design

Four modifications of one PI treatment process were established to understand the role of MB on one hand and the illumination on the other, on the AOP values. A complete MB treatment was executed on 20 plasma units. Four different time-points throughout the PI process were established: T1 corresponded to the plasma unit before any treatment, T2 to a sampling after MB addition but before illumination, T3 to sampling after illumination and T4 to sampling after illumination

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and filtration (Figure 1 and Table 1). Twenty other plasma units received no MB and no illumination, but the timing and final filtration were maintained. Thirty more plasma units underwent incomplete treatment; MB was added but the illumination was not provided for 10 plasma units, and no MB was added but illumination carried out for the remaining 20 plasma units. Finally, we obtained at least 10–60 data points per time-points and conditions (Table 1). These data values were sufficient to give the tendency of the results.

## AOP measurements

AOP measurements were carried out using a potentiostat electrochemical analyser and three electrode-based sensors (Edel-for-life SA, Lausanne, Switzerland) [15]. A 3-μl sample volume was deposited on the electrodes on a single-use microchip. In order to obtain a sample, a segment of the tube was connected through a sterile connection device. The workflow from bag sampling to the AOP readout took less than 5 min (which included the AOP measurement that took 1 min). The measurement is based on a pseudo-titration of a linear sweep voltammogram (recorded from 0–1.2 V with a scan rate of 100 mV/s under ambient conditions) for rapidly measuring the water-soluble AOP in a sample. The mathematical relationship between AOP and the effective number of antioxidant molecules can be found in the literature. This equation considers gaseous exchanges with the sample [15]. AOP is expressed in micromole per litre equivalent ascorbic acid and reflects the redox status of the extracellular low-molecular-weight antioxidants present in the plasma units. The measurement was carried out immediately after the sampling and took only 1 min. The sensitivity of the assay is 19 μmol/L eq. ascorbic acid and the SD is ±6 μmol/L eq. ascorbic acid (data not shown).

## Statistical analyses

The results of the tests are expressed as mean ± SD. Statistical analysis was performed using a computer software (Minitab software, Minitab Inc., State College, PA). Normality and statistical significance were tested for each test variable. Analysis of variance (one-way ANOVA) was used to compare data between T1 and T4. Subsequent statistical analyses were performed with Student's *t*-test between protocol with complete treatment and protocol with no treatment at different times. A *p*-value of <0.05 indicates significant statistical difference.

# RESULTS

## Complete treatment

Twenty plasma samples from male donors were tested, and four measurements were carried out during the process for each plasma unit (Figure 1). The mean results were 195 ± 32 μmol/L eq. ascorbic acid at T1, 230 ± 42 μmol/L eq. ascorbic acid at T2, 192 ± 30 μmol/L eq. ascorbic acid at T3 and 177 ± 27 μmol/L eq. ascorbic acid at T4 (see Table 2 and Figure 2). A clear increase in AOP was observed after the addition of MB, which subsequently dropped down to T1 values after illumination. Filtration slightly decreased the AOP. There was a significant difference over the treatment (ANOVA $p < 0.001$).

## No treatment (no MB, no illumination)

The mean results for 20 plasmas from male donors were 188 ± 23 μmol/L eq. ascorbic acid at T1, 185 ± 23 μmol/L eq. ascorbic acid at T3 and 179 ± 21 μmol/L eq. ascorbic acid at T4 (Table 2). There was no significant difference over the treatment process (ANOVA $p = 0.466$). Differences between MB treatment and no treatment condition at different process steps were not significant (T1 $p = 0.451$, T3 $p = 0.425$ and T4 $p = 0.765$).

## Incomplete treatment

### Plasmas without MB and with illumination

Twenty plasma samples from male donors were tested. The mean results were 217 ± 24 μmol/L eq. ascorbic acid at T1, 204 ± 26 μmol/L

**TABLE 1** Recapitulative table containing the number of samples and modifications compared with the normal process for each incomplete scenario treatments

| PI treatment | *n* | T1 | T2 | T3 | T4 |
|---|---|---|---|---|---|
| Complete treatment | 20 | Sampling before any treatment | Sampling after MB before light | Sampling after light | Sampling after filtration |
| MB− light− | 20 | Sampling before any treatment | — | Sampling after respected process time but no light | Sampling after filtration |
| MB− light+ | 20 | Sampling before any treatment | — | Sampling after light | Sampling after filtration |
| MB+ light− | 10 | Sampling before any treatment | — | Sampling after the required process time but no light | Sampling after filtration |

Abbreviations: MB, methylene blue; PI, pathogen inactivation.

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**TABLE 2** Antioxidant power (AOP) values during the process for complete treatment and no treatment

| Eq. µmol/ml | *n* | T1 | T2 | T3 | T4 |
|---|---|---|---|---|---|
| Complete treatment | 20 | 195 ± 32 | 230 ± 42* | 192 ± 30 | 177 ± 27* |
| MB− light− | 20 | 188 ± 23 | – | 185 ± 23 | 179 ± 21 |

*Note*: Values are expressed in mean ± SD in equivalent micromole per liter. No statistical difference for the incomplete treatment. For the condition 'no MB, no Illumination', no T2 result because no manipulation compared to T1, but T3 was measured to have a result before and after filtration.
Abbreviations: MB, methylene blue; T1, initial plasma AOP level; T2, AOP level with MB before illumination; T3, AOP level after illumination but before MB filtration; T4, AOP level after illumination and after MB filtration.
*Analysis of variance: $p < 0.001$ compared to T1.

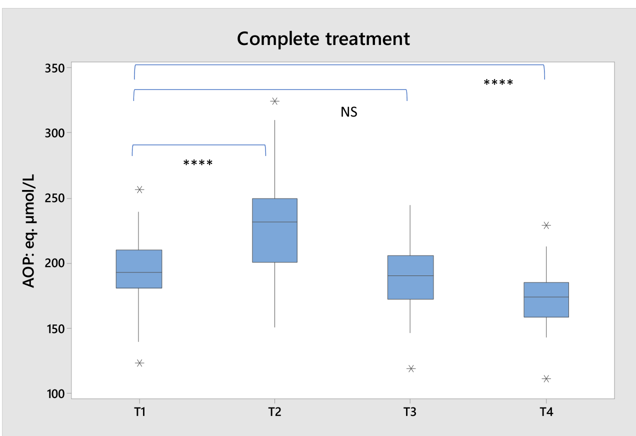


**FIGURE 2** Antioxidant level in illuminated plasmas for complete treatment. The box represents 50% of the data distribution; otherwise, whisker plots represent 25% of data distribution each. Bars represent SE of the mean, and stars represent the outliers. ****Analysis of variance: $p < 0.001$ compared to T1. AOP, antioxidant power; NS, not significant

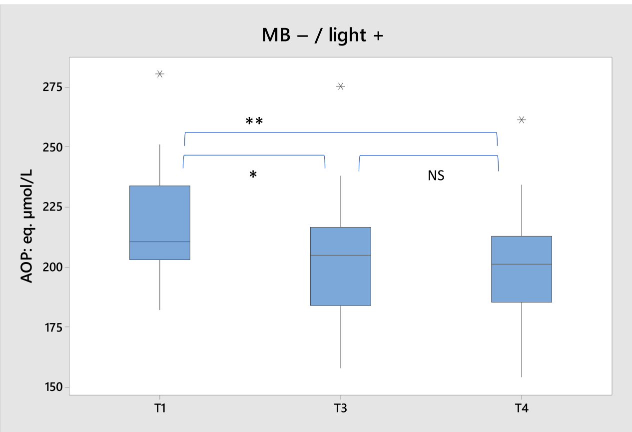


**FIGURE 3** Antioxidant level in non-complete treatment of plasma units: MB−, light+. The box represents 50% of the data distribution; otherwise the whisker plots represent 25% of data distribution each. Bars represent SE of the mean. *$p < 0.05$; **$p < 0.01$. AOP, antioxidant power; MB, methylene blue; NS, not significant

eq. ascorbic acid at T3 and 200 ± 25 µmol/L eq. ascorbic acid at T4 (see Figure 3). In this configuration, only a decrease was reported as a result of the illumination but not following the filtration. There was a significant difference between T1 and T3 and between T1 and T4, but no statistical difference between T3 and T4.

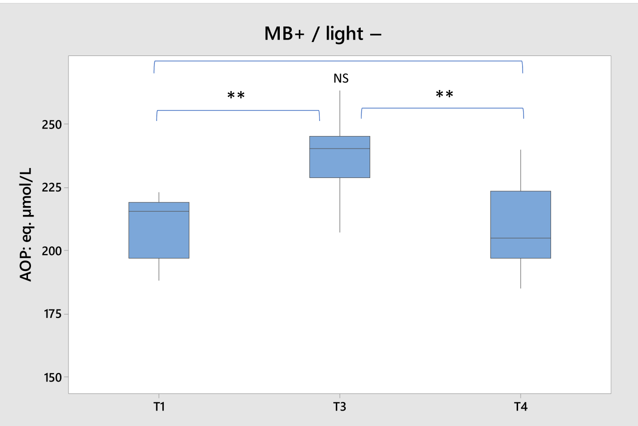


**FIGURE 4** Antioxidant level in incomplete treatment of plasma units: methylene blue+, light−. The box represents 50% of the data distribution, otherwise whisker blots represent 25% of data distribution each. Bars represent SE of the mean. **$p < 0.01$. AOP, antioxidant power; MB, methylene blue; NS, not significant

### Plasmas with MB and without illumination

This group included 10 plasmas from male donors. Mean results were 210 ± 13 µmol/L eq. ascorbic acid at T1, 238 ± 16 µmol/L eq. ascorbic acid at T3 and 209 ± 17 µmol/L eq. ascorbic acid at T4 (Figure 4). As for the complete treatment, the addition of MB increased the AOP, which decreased after filtration. There was a significant difference between T1 and T3 and between T3 and T4 but it was not statistically significant between T1 and T4.

## DISCUSSION

Analysis of the results regarding the complete treatment of plasma units shows an increase between T1 and T2. It was reported in the scientific literature that MB has antioxidant properties [28, 29] and it is responsible for the AOP increase after the addition of this molecule. The result decreases after illumination, probably as a result of the transformation of MB into its photoproducts by the illumination.

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A decrease is also observed between T3 and T4, which can be explained by the action of the filter that retains MB and its photoproducts (Figures 2 and 4) [30]. With no MB and no illumination, no significant difference is found before and after the treatment of the plasma units (process duration maintained and filtration completed) (Table 2). When the process is carried out without illumination, an increase is observed between T1 and T3 through the action of MB, and a decrease is seen between T3 and T4, again through the action of the MB filter. The result at T1 is similar to that at T4 (Figure 4). And finally, when the process is initiated without MB, the decrease between T1 and T3 can be explained by the effect of illumination, but the non-significant difference between T3 and T4 may demonstrate that MB filtration alone has no effect on the plasma AOP (Figure 3).

As AOP can reliably be measured in plasma units that contain various antioxidants such as urate or ascorbic acid [31], different conditions involving the plasma treatment by the Theraflex system were investigated. ANOVA showed that the plasmas' AOP mean values at T1 in each series do not show any significant difference. We observed an increase after the addition of MB and before illumination. This is confirmed by the literature showing that MB has antioxidant properties [28, 29, 32]. After illumination, the result decreases to less than 50%, which is to be expected because of the degradation of MB due to its photoproducts during illumination [30]. In the tests including illuminated plasmas and plasmas without MB but with illumination, the illumination seems to have an effect by itself, with or without MB, which was also reported in the case of riboflavin/UV treatment of platelets concentrates [20, 21]. The final filtration is aimed at eliminating the residual MB and its photoproducts. After this step, the values decreased as expected. However, in the absence of MB, the results at T1 and T4 were not statistically different. Finally, the filtration decreases the AOP rate, except when there is no MB.

To be used as a QC assay, an analysis has to be fast, straightforward, robust, reliable, and of affordable cost. The electrochemical-based technology meets these requirements, as it only requires a portable device that is plugged into a computer and single-use sensors. There is no sample preparation, as only a few microlitres of plasma is to be dispensed into the sensor. Nevertheless, significant differences must be quantified before and after the treatment to prove its execution.

The AOP level of a plasma unit significantly decreases between the beginning of the PI treatment process and the end of the process. The objective was to establish an AOP threshold ensuring that the PI treatment was completed. But the box plot indicates that it is not possible to determine a threshold between treated and untreated plasmas with MB (Figure 2), which is contrary to results of Abonnenc et al. [11], who found a threshold for the plasma units treated by INTERCEPT technology. Indeed, in INTERCEPT technology, amotosalen is an ROS generator and is responsible for the decrease in AOP value, whereas MB has an antioxidant effect and avoids the drop in AOP. Considering the light sources, UV light without amotosalen in INTERCEPT treatment induces a moderate decrease in AOP [11], while visible light without MB in Theraflex treatment has no effect on the AOP value.

In conclusion, the analysis of AOP measurements does not permit testing the reliability of the PI process of plasmas treated by MB. Consequently, an AOP-based approach cannot properly assess the effectiveness of plasma treatment by MB. Intermediate measurements could be evaluated since MB induces a clear increase in AOP.

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M.P. reviewed the manuscript; A.L. collected and analysed the data and wrote the paper, and carried out the analyses and reviewed and edited the manuscript; A.R. designed and supervised the research and reviewed the manuscript.

## CONFLICT OF INTEREST

The authors declare no conflict of interests.

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ORIGINAL ARTICLE

# Weak D type 42: Antigen density and risk of alloimmunization in the province of Québec

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## Abstract

**Background and Objectives:** A high proportion of suspected weak D patients referred to Héma-Québec were genotyped as weak D type 42 (368/2105, 17.5%). These patients are currently considered D with regard to RhD immunoprophylaxis in pregnancy and transfusion. The goal of this study was to retrospectively evaluate the risk of alloimmunization in weak D type 42 patients and to characterize their RhD surface molecule expression on red blood cells (RBCs) in comparison to other weak D types (1, 2 and 3).

**Materials and Methods:** A retrospective analysis using the weak D type 42 patients' medical data to verify potential anti-D alloimmunization events was conducted. Quantitative analyses using flow cytometry were also performed on RBCs to quantify the cell surface density of the D antigen.

**Results:** Data on 215 subjects with weak D type 42 were reviewed. None developed immune allo-anti-D; three had definite exposure to D+ red cells and 41 had possible exposure through pregnancy. Flow cytometry analysis showed that weak D types 1, 2, 3 and 42 had relative antigen densities of 2.7%, 2.2%, 8.1% and 3.6%, respectively, with R1R2 red cells referencing 100% density. The estimated antigen density range of weak D type 42 was 819–1104 sites per RBC.

**Conclusion:** Our retrospective alloimmunization data analysis and antigen density study establish a basis for the consideration of a weak D type 42 individual as D+. This consideration would allow for a targeted reduction of RhD immunoprophylaxis in pregnancy and the unjustified use of D– units for transfusion.

KEYWORDS
alloimmunization, anti-D, flow cytometry, weak D

## Highlights

- No anti-D was found among 215 studied subjects, of whom 44 were potentially exposed to a situation with risk of immunization.
- Weak D type 42 red blood cells have an antigen density between those of weak D types 1 and 3.
- National guidelines should consider recommending the clinical management of weak D type 42 as D+.

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## INTRODUCTION

RhD variants are commonly classified as weak D or partial D. Weak D, previously called D$^{u}$, has been serologically defined as the D antigens on red cells that are agglutinated not by IgM anti-D but by IgG anti-D in an antiglobulin test. While originally defined as a D antigen that can lead to the formation of anti-D [1], clarification of the molecular basis of partial D has led it to be defined as a D antigen that lacks epitopes, with a qualitative defect [2]. This dichotomy is, however, challenged, as alloimmunization has not been demonstrated in all D variants classified as partial, and some classified as weak D have been associated with the development of an anti-D alloantibody, such as weak D type 4.2, 15 and others [3, 4].

While data are sufficient to consider weak D types 1, 2 and 3 as not associated with alloimmunization [5, 6], this is not the case with most other weak D variants, and it is recommended that women with another serologically weak D type be managed as RhD negative, with Rh immunoglobulin (RhIg) administration as appropriate and transfusion of RhD negative blood products [7, 8]. The same authors also recommended that alloimmunization outcomes of transfusion and pregnancies be published. Haemolytic disease of the foetus and newborn (HDFN) occurs in approximately 1 in 21,000 births [9]; and while exact data are not available in Quebec, alloimmunization during pregnancy remains a relevant clinical problem in Canada [10, 11].

While weak D types 1, 2 and 3 are the most common in individuals of European descent, this is not homogeneous in all Caucasian populations. In the province of Québec, Canada, where a majority of the population is French Canadian, weak D type 42 (*RHD*01W.42*) has been previously identified as the most common *RHD* variant. Among 2105 suspected weak D samples tested between 2016 and 2020, 368 (17.5%) were found to be weak D type 42, while 323 (15.3%) were found to be weak D type 1, 69 (3.3%) type 2, and 180 (8.6%) type 3 [8]. This is, to our knowledge, the largest weak D type 42 cohort identified yet. The potential for alloimmunization of weak D type 42 remains unknown; therefore, the current recommendation is to provide RhIg prophylaxis as necessary and to transfuse D− blood products for these individuals.

In accordance with recommendations from Flegel and coworkers in 2015 and Robitaille et al. in 2016 [7, 12], Héma-Québec has been offering *RHD* genotyping for women of childbearing potential (≤45 years old) with evidence of a serological weak D since June 2016, identifying further individuals with type 42 variant through time and confirming its status as the most prevalent variant in the Québec population. Using this larger cohort, we undertook a two-part study aimed at evaluating the risk of alloimmunization in women with weak D type 42 variant. The first part is a retrospective study looking at the rates of exposure to RhD-positive red cells and anti-D alloimmunization. The second part is a comparative characterization of D antigen density in weak D type 42 individuals using flow cytometry.

## MATERIALS AND METHODS

Both parts of this study were conducted following approval from Héma-Québec research ethics board in December 2018, as well as from the directors of professional services from the concerned hospital centres in accordance with Québec laws for the first part of the study. For the second part, all subjects provided informed consent to participate in the study.

### Retrospective study of the incidence of anti-D alloimmunization

#### Study design

We conducted a retrospective study using the medical records of all subjects identified with a weak D type 42 *RHD* variant at Héma-Québec's Immunohematology Reference Laboratory (IRL), the only laboratory performing red cell genotyping in Québec, between June 2016 and July 2018. Weak D genotypes were determined as previously described [13, 14], and subjects of this study were identified through Héma-Québec's database. All subjects were women with childbearing potential (defined as age ≤45 years) because genotyping is currently offered only for this population. The aim of the study was to evaluate the risk of anti-D alloimmunization in individuals exposed to D+ red cells through either transfusion or pregnancy.

TraceLine (MAK-SYSTEM, Paris, France) is the software used by all blood banks in the province, and it houses information on serology results and transfusion of labile and stable blood products. Hospital data were obtained from the central Traceline database at the Direction de la Biovigilance et de la Biologie Médicale from Québec's Ministry of Health.

#### Data collection

Data were reviewed for demographic variables including sex, age and ethnicity; results of serological investigations and antibody identification history of blood product transfusion, including labile products and RhIg; history of pregnancy; and RhD type of neonates (if available).

#### Definitions and statistical analysis

Anti-D alloimmunization was defined as presence of an immune anti-D antibody in any serological investigation. Auto-anti-D was recorded as such and not considered as alloimmunization. If antibody identification was unavailable, a positive antibody screen was considered likely to be due to passive anti-D if this screen was within 12 weeks of RhIg administration and/or a later screen became negative.

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Exposure to D+ red cells could occur through transfusion or pregnancy. Definite exposure was defined as either transfusion of one or more D+ red cell units or a recorded history of a pregnancy with a D+ neonate, without exposure to RhIg. Possible exposure was defined as a recorded history of pregnancy for which the RhD type of the neonate was unknown, without exposure to RhIg. Because of missing data on pregnancies in a large number of subjects, an 'unknown exposure' category was defined for subjects with isolated serial antibody investigations performed during a 7 to 9-month period without associated transfusion and without exposure to RhIg; by definition, the RhD status of newborns was unknown. The studied population being comprised of only women of childbearing potential, in whom serological investigations are infrequent outside of pregnancy (and especially without evidence of transfusion), this pattern was considered as having a high likelihood of being associated with pregnancy. Subjects with no transfusion history or transfusion of D– blood products, and with no evidence of pregnancy or only pregnancies with administration of RhIg, were considered as unexposed because of the low failure rate of RhIg (0.24%–0.31%) in standard RHD, although the efficacy in weak D type 42 is unknown [15, 16]. Among this population, based on a prevalence of 85% of D+ status in fathers given the majority is Caucasian, of which 45% are homozygous and 55% are heterozygous, 60% of these possible pregnancies were considered as likely to be D+. Descriptive statistics were used to report the rates of RhD alloimmunization and exposure to D+ red cells.

## Flow cytometric analysis of weak D type 42

### Samples and phenotyping

RBCs were obtained by venipuncture and collected into EDTA-anticoagulated tubes for each weak D types and controls. RHC, c, E and e phenotypes of RBCs were determined using commercial reagents as per the manufacturer and IRL standard operating procedures.

**TABLE 1** RhD alloimmunization results

| Antibody screen results | Number (%) |
|---|---|
| All | 215 (100) |
| Negative | 142 (66) |
| RhIg-treated | 3 (1) |
| Positive | 73 (34) |
| Investigation results available | 18 (8) |
| Non-anti-D alloantibody[a] | 2 (1) |
| Non-specific autoantibody | 1 (1) |
| Both allo and autoantibody[b] | 1 (1) |
| Passive anti-D | 14 (7) |
| Results unavailable | 55 (26) |
| Likely passive anti-D | 52 (24) |

Abbreviation: RhIg, Rh immunoglobulin.
[a]Anti-M (1) and anti-Kpa, anti-Bg and anti-Wra (1).
[b]Anti-E and non-specific auto-antibody.

### Flow cytometry

Surface antigen density was determined by flow cytometry (Accuri C6, BD Biosciences, Mississauga, Canada). Briefly, 250,000 washed red blood cells were incubated with 30 μl of anti-D-R-PE (clone NaTH109-IG2, D epitope 5.2) coupled to phycoerythrin (PE) human anti-D (IQ Products, Groningen, The Netherlands) in stain buffer (BD Biosciences) for 30 min in the dark at room temperature. After incubation and washing, 10,000 positive events per sample were acquired by flow cytometry. The PE mean surface equivalent (MSE) was established with a standard curve using SPHERO Rainbow Calibration Particles (8 peaks, Spherotech, Lake Forest, IL). Different RBCs were tested: $R_1R_2$, weak D type 1, weak D type 2, weak D type 3, weak D type 42 and rr. The NaTH109-IG2 clone was used because it provided a reaction force similar to that of weak D type 42 compared to the Gamma Clone (Immucor, GA) antibody validated and used in our IRL. The BRAD-3 anti-D clone, which recognizes the D epitope 6.2, was also initially tested and provided similar results (data not shown).

# RESULTS

## Retrospective study of the incidence of anti-D alloimmunization

Two-hundred and fifteen subjects with weak D type 42 were included in our study. All subjects were female; 213 were of childbearing age and two were children. The median age was 29 (range: 0–49). Self-declared ethnicity was known for 91 subjects (42%); of these, 89 (98%) were Caucasian, 1 (1%) was Hispanic and 1 (1%) was Black.

### RhD alloimmunization

Results of serological analyses are presented in Table 1. No immune anti-D was documented. Negative antibody screens were found only in 142 subjects (66%). Sixty-six (31%) had passive anti-D and four had other antibodies. In three cases, an unexplained positive antibody was identified; all three patients had been exposed to RhIg 4–7 months prior to the last available antibody screen, and the last available screen was positive. Allo-anti-D was excluded.

### Exposure to D+ red cells

Two subjects were transfused two units of D+ red cells each. Three others received only D– red cells units. Both had negative antibody screens months to years after exposure.

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Only one definite exposure due to pregnancy was identified in a 46-year-old woman who delivered a D+ newborn without exposure to RhIg during pregnancy (Table 2). A prior pregnancy, also without exposure to RhIg, was suspected. Antibody screens were negative. A total of 58 pregnancies in 41 women without exposure to RhIg were suspected based on serial antibody screens, but without definitive documentation. All subjects had at least one negative antibody screen months to years after their suspected exposure(s). As pointed out above, 60% of these pregnancies are likely to be associated with a D+ foetus, for a total of 25 women with a possible but unknown exposure. RhIg administration was documented in all other pregnancies.

## Flow cytometric analysis of weak D type 42

### D antigen density determination

Flow cytometry analyses were carried out using fluorescence calibration beads to establish a PE standard curve, and MSE quantitative analyses were performed as described. Table 2 shows the MSE results for weak D type 1, 2, 3 and 42, as well as $R_1R_2$ and rr used as maximum (100%) and minimum (0%) D expression controls. Weak D types 1, 2 and 3 showed MSE values of 2.7%, 2.2% and 8.1%, respectively, of that of $R_1R_2$, with weak D type 42 at 3.6% (Table 3). The estimated antigen density was calculated using the published density range of 23,000–31,000 of $R_1R_2$ as reference, multiplied by its MSE value and divided by the sample MSE value, estimating antigen density of weak D type 42 in the range 819–1104 sites per RBC. The order of antigen density was rr < type 2 < type 1 < type 42 < type 3 < $R_1R_2$. It should be noted that all five weak D type 42 patients were DcE/ce (R2r) (Table 3). RhC, c, E and e phenotypes of other variants are also described in Table 3, and each weak D type had, unexpectedly, the same RhC/c/E/e phenotype.

**TABLE 2** Weak D type 42 women with pregnancies

| Exposure to RhIg and D+ foetus | Number (%) |
|---|---|
| All | 213 (100) |
| Exposed to RhIg | 172 (81) |
| Not exposed to RhIg | 41 (19) |
| Possible exposure to D+ foetus | 25 (12) |
| Confirmed exposure to D+ foetus | 1 (1) |

Abbreviation: RhIg, Rh immunoglobulin.

## DISCUSSION

The risk of alloimmunization with most RhD variants, whether they are classified as weak or partial, is unknown. In this retrospective study, among 215 mostly French–Canadian women of childbearing potential with weak D type 42, none of the 44 susceptible subjects had an anti-D documented in their health records.

Three patients had unequivocal documented exposure to D+ red cells, two through transfusion and one through pregnancy; none had an anti-D documented in their health record. While not confirmed, our data also suggest that an additional 41 subjects may have been exposed through pregnancy, without any evidence of alloimmunization. In the original study reporting this variant, one subject received D+ red cells, without alloimmunization [19]. In the previously published series of 17 subjects, 6 had a reported anti-D; however, 3 had an auto-anti-D, 1 had a doubtful anti-D with an anti-C, and the 2 others also had an anti-C but anti-G was not ruled out, meaning none of them had a demonstrated immune anti-D [14]. Seven were stated as having been transfused, although the RhD status of the received red cells was not recorded; of these, three had no anti-D, two had an auto-anti-D, and the two others were among the subjects not fully investigated. Another report described two cases of weak D type 42, including one presenting an auto-anti-D [20]. Therefore, although exposure to D+ red cells is difficult to evaluate, there has been no report of an allo-anti-D in a subject with known weak D type 42.

The polymorphism responsible for weak D type 42 is a 1226A>T substitution, causing a change from a lysine to methionine at position 409. This position is close to the C-terminal end of the protein, which contains 416 amino acids, and is in the intracellular section of the protein [21]. This localization is consistent with the observed quantitative change in the expression of the molecule, which in turn explains the absence of any detectable alloimmunization. Furthermore, weak D type 42 was shown in our study to have an estimated antigen density

**TABLE 3** D antigen density quantification

| RBC | N | MSE | As % of $R_1R_2$ | Reported Ag density[a] | Estimated Ag density[b] | RHCE phenotypes |
|---|---|---|---|---|---|---|
| rr | 5 | 12.2 ± 10.6 | <0.4 | 0 | – | ce/ce |
| Weak D type 2 | 4 | 69.3 ± 15.4 | 2.2 | 466–818 | 507–684 | cE/ce |
| Weak D type 1 | 5 | 85.0 ± 11.2 | 2.7 | 533–1283 | 623–839 | Ce/ce |
| Weak D type 42 | 5 | 111.8 ± 23.6 | 3.6 | Unknown | 819–1104 | cE/ce |
| Weak D type 3 | 5 | 253.2 ± 29.4 | 8.1 | 1333–2650 | 1855–2500 | Ce/ce |
| $R_1R_2$ | 5 | 2929 ± 500 | 100 | 23,000-31,000 | – | Ce/cE |

Abbreviations: MSE, mean surface equivalent; RBC, red blood cells.
[a]References [17, 18].
[b]Antigen density was estimated using the median $R_1R_2$ MSE-PE result and the published mean antigen density range of 23,000–31,000.

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of 819–1104 molecules per RBC, which is similar to that of weak type 1 and slightly less than that of type 3. This is consistent with a previous study showing that weak D type 42 reaction strength against commercial anti-D in both tube (immediate spin) and gel was similar to that of weak D type 1 but weaker than that of type 3 [13].

There are significant limitations to this study. Regarding the retrospective chart review, data were unavailable concerning antibody screen and identification results in a large number of subjects. Results of positive serological investigations are permanently recorded in TraceLine only in the case of clinically significant antibodies; non-significant antibodies such as passive anti-D may be initially recorded but will become unavailable at a later date, leading to missing results for a large number of positive antibody screens of the study population. However, as the database used combines results from all serological testing across the province of Quebec, the likelihood of an allo-anti-D being detected in a subject but absent in the database is low because of the strong clinical significance of such an antibody. Unequivocal documentation of D+ red cell exposure due to pregnancy was also unavailable in most subjects, as pregnancies are not recorded in this database, and RhD status of neonates was unavailable as it is recorded only in the neonates chart, leading to the assumption of exposure in some of these cases. Potential exposure was also made less likely by the current practice of administering RhIg prophylaxis during pregnancy to weak D type 42 patients. However, considering that all but two included subjects are women of childbearing age with a median age of 29, there is a high likelihood that some of them have been exposed to D+ blood through pregnancy without exposure to RhIg, suggesting that weak D type 42 may not be associated with alloimmunization.

These limitations also underline the importance of better documentation and accessibility of data regarding pregnancies and alloimmunization, as these are essential to study and improve care in this population.

Nonetheless, our study is of significant importance given the large number of weak D type 42 cases studied. Such a large cohort has not been reported anywhere else in the world, as Québec appears to be the location where weak D type 42 is the most prevalent [13]. Our study is also the first to measure the antigen density of weak D type 42 on the RBC surface, allowing comparison with weak D types 1, 2 and 3 and finding a similar density.

For the prevention of alloimmunization and HDFN, adequate management with transfusion of D– red cells and RhIg prophylaxis are essential. However, inadequate use of these measures is not without consequences. Unnecessary use of RhIg is associated with significant costs and may also be associated with side effects [22, 23]. Blood supply for D– products, especially O–, is also limited. As RhIg is obtained through alloimmunized donors, which is now uncommon because of prophylactic measures, RhIg supply may also become limited. Therefore, care should be taken to limit needless use of these blood products.

Overall, our data suggest that the likelihood of RhD alloimmunization in subjects with weak D type 42 is low, thereby challenging the need for RhIg prophylaxis during pregnancy and transfusion with D– RBC units. While the results of more, ideally prospective, studies in patients with weak D type 42 would allow us to confirm this hypothesis, such studies would be challenging to perform and could be unethical (such as voluntarily not exposing pregnant women to RhIg without the certainty that there is no risk of alloimmunization). Therefore, our data may be used by policy makers to evaluate whether a change in practice is advocated and whether weak D type 42 subjects should be considered as D+, as is the current practice with weak D types 1, 2 and 3.

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## CONFLICT OF INTEREST

The authors declare no conflict of interests.

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ORIGINAL ARTICLE

# *RHD* alleles contributing to serologically weak D phenotypes in China: A single-centre study over 10 years

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## Abstract

**Background and Objectives:** In cases of serologically weak D phenotypes, *RHD* genotyping may identify discrepant serotyping results and protect the patient against allogeneic immunization. This study aimed to conduct a comprehensive analysis of weak D alleles in China.

**Materials and Methods:** We collected samples carrying weak D antigen during a 10-year period from 2005 to 2014. The intensity and epitopes of D were analysed serologically. Genomic DNA was extracted and used for *RHD* sequencing and heterozygote analysis. In particular, an in vitro expression method for functional verification of the rare and novel in-frame deletion mutation was developed and then combined with homologous modelling results for analysis.

**Results:** We studied a total of 283 weak D samples from volunteer blood donors and identified 45 *RHD* alleles among them, 11 of which were reported for the first time. Ten (3.5%) samples surprisingly carried DEL allelic variants and as many as 40 (14.1%) carried the wild-type *RHD* genotype. Combination of the results of functional experiments and in silico analysis suggested that the rare in-frame deletion mutation may reduce the expression of D antigen by affecting the RhD protein structure.

**Conclusions:** This study provides an enhanced overview of the distribution characteristics of *RHD* alleles in Chinese subjects with serologically weak D. An in vitro method to predict the biological significance of variant *RHD* alleles was also provided. We found inconsistent genotyping and phenotypic results in some samples, indicating the existence of additional regulatory mechanisms.

**KEYWORDS**
Chinese, lentiviral overexpression, *RHD* allele, weak D

## Highlights

- A total of 45 *RHD* alleles were identified in Chinese blood donors, and 11 of them were novel.
- An available functional research method of *RHD* mutants was developed and introduced.

## INTRODUCTION

The D antigen is generally recognized as the most immunogenic and significant in the Rhesus blood group system. In China, only the ABO system and D antigen of the Rh system are routinely tested in clinical blood typing. Weak D, formerly known as $D^u$, reacts with IgM anti-D as impaired or undetectable agglutination (≤2+) and with IgG anti-D as moderate to strong agglutination [1]. The accuracy of weak

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D serological detection depends on anti-D reagents and test methods. In recent years, studies have shown that *RHD* genotyping is a better method to resolve the problems of discordant serological D typing results, especially for patients undergoing chronic transfusion and for women of childbearing age [1, 2].

A comprehensive understanding of the *RHD* alleles that leads to specific phenotypes is obviously essential for accurate genetic diagnosis of D-antigen expression, especially in specific regions and populations. Initially, the weak D phenotype was defined as one or more missense mutations that resulted in transmembrane or intracellular amino acid substitutions in the RhD protein. More than 150 *RHD* alleles with this property have been described in the Human RhesusBase [3]. In addition, recent studies have revealed more diversified genetic mutation types of weak D, such as silent mutations, intronic mutations, in-frame variations and large duplication or deletion [3].

Although weak D is the main D variant in China, previous studies of the allelic composition of weak D phenotype in the Chinese population consisted mainly of sporadic case reports or genetic analysis of dozens of samples that lacked large data. Thus, proposing effective and targeted genetic testing approaches is difficult. In the Shanghai Blood Center, over 10 years of research was conducted on the *RHD* alleles that cause the weak D phenotype of blood donors, so as to develop more targeted detection strategies based on population data. Besides, an efficient method to express recombinant RhD protein in vitro was developed to verify the biological functions of rare *RHD* variants. An in silico tool was also used to complement the experimental results at the molecular level.

## METHODS

### Blood samples and routine tests

We collected peripheral whole-blood samples from random voluntary blood donors at the Shanghai Blood Center. Informed consent was obtained from all blood donors prior to blood collection. Then we carried out routine laboratory tests, such as blood typing and testing for transfusion-transmitted infection. Blood samples that showed weak or no agglutination with anti-D antibodies during the initial screening were sent to the Immunohematology Reference Lab for a weak D test. After testing by one monoclonal IgM anti-D and three IgG anti-Ds, the samples were divided into D− and D variants. RhCE typing was carried out simultaneously. The methods and reagents were as described in the literature [4].

### D epitope analysis

We further tested blood samples reported as D variants from 2005 to 2014 with a commercial panel of anti-D reagents (D-screen, Diagast) for D epitope analysis [4]. When the results of the anti-D panel showed DVI and other specific partial D types, the samples were excluded from this survey.

**TABLE 1** New *RHD* alleles

| *RHD* allele | Nucleotide change(s) | Exon/intron | Type of mutation | Amino acid substitution(s) (localization) | GenBank accession no. | rs# | Sample no. |
|---|---|---|---|---|---|---|---|
| *RHD*254A* | c.254C>A | Exon 2 | Missense | p.Ala85Glu (TM) | MZ592918 | NA | 1, 2 |
| *RHD*526A* | c.526G>A | Exon 4 | Missense | p.Ala176Thr(TM) | MZ592917 | rs141615605 | 3, 4, 5 |
| *RHD*577A, 594T, 602G* | c.577G>A; c.594A>T; c.602C>G | Exon 4 | Missense | p.Glu193Lys; p.Lys198Asn; p.Thr201Arg (IC) | MZ592909 | rs1053352; rs569974439; rs1053355 | 6 |
| *RHD*612G* | c.612A>G | Exon 4 | Missense | p.Ile204Met (IC) | MZ592910 | NA | 7 |
| *RHD*781G* | c.781C>G | Exon 5 | Missense | p.Pro261Ala (IC) | MZ592919 | NA | 8 |
| *RHD*947A* | c.947G>A | Exon 7 | Missense | p.Cys316Tyr (TM) | MZ592911 | rs1431008299 | 9 |
| *RHD*1005A* | c.1005G>A | Exon 7 | Synonymous | p.Leu335Leu (TM) | MZ592912 | rs1400980567 | 10 |
| *RHD*1102_1104del* | c.1102_1104delGGG | Exon 8 | In-Frame Deletion | p.Gly368del (TM) | MZ592913 | NA | 11 |
| *RHD*1142C* | c.1142G>C | Exon 8 | Missense | p.Gly381Ala (TM) | MZ592914 | NA | 12 |
| *RHD*1183A* | c.1183G>A | Exon 9 | Missense | p.Ala395Thr (IC) | MZ592915 | NA | 13 |
| *RHD*1227+4G* | c.1227+4A>G | Intron 9 | Splice Site | NA | MZ592916 | NA | 14 |

Abbreviations: IC, intracellular; NA, not available; TM, transmembrane.

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**TABLE 2** Serotyping results and possible *RH* haplotypes of weak D samples carrying new alleles

| Sample no. | | | | | D-screen | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Clone Isotype | Rum-1 IgM | MS-26 IgG | Polyclonal anti-D serum A IgG | Polyclonal anti-D serum B IgG | HM10 IgM | HM16 IgG | P3*61 IgG | P3*35 IgG | P3*212 11 F1 IgM | P3*212 23 B10 IgG | P3*241 IgM | P3*249 IgG | P3*290 IgM | CE type | *RHD* zygosity | Most likely *RH* haplotype |
| 1 | 1+s | 2+ | 2+ | 1+ | Neg | 2+ | Neg | 2+ | Neg | Neg | 1+w | 3+ | 1+w | ccEe | *D*/− | *DcE*, *ce* |
| 2 | 1+ | 3+ | 2+ | 2+s | ± | 3+ | 1+ | ± | Neg | Neg | Neg | 4+ | 3+s | NA | *D*/− | NA |
| 3 | 2+ | 4+ | 2+ | 3+ | 1+ | Neg | 2+ | Neg | Neg | 1+ | Neg | 2+ | 2+ | NA | *D*/− | NA |
| 4 | 1+ | 3+ | 4+ | 2+ | 1+w | 2+ | 1+ | 2+ | 1+w | 1+ | 2+s | 4+w | 3+ | CCee | *D*/− | *DCe*, *Ce* |
| 5 | 2+ | 3+ | 3+ | 3+ | 1+w | 1+ | 2+ | 1+ | Neg | Neg | 2+ | 2+ | 3+ | CCee | *D*/− | *DCe*, *Ce* |
| 6 | NA | NA | NA | NA | Neg | 3+ | 1+ | 2+ | Neg | ± | 3+ | 3+ | 2+ | ccee | *D*/− | *Dce*, *ce* |
| 7 | 1+ | 1+ | ± | 2+ | NA | NA | NA | NA | NA | NA | NA | NA | NA | Ccee | *D*/− | *DCe*, *ce* |
| 8 | 2+s | 3+ | 1+s | 2+ | 1+w | 2+s | 1+ | 2+ | ± | ± | 3+ | 3+s | 2+s | CCee | *D*/− | *DCe*, *Ce* |
| 9 | 2+w | 3+ | 2+ | 3+ | 1+s | 2+s | Neg | 3+ | 1+s | 1+ | 3+s | 4+ | 3+ | CCee | *D*/− | *DCe*, *Ce* |
| 10 | 1+ | 3+ | NA | 2+s | 1+w | 3+ | 1+w | 3+ | ±w | ±w | 3+s | 4+w | 4+w | Ccee | *D*/− | *DCe*, *ce* |
| 11 | ± | 1+ | Neg | Neg | Neg | ± | Neg | Neg | Neg | Neg | Neg | ± | Neg | Ccee | *D*/− | *DCe*, *ce* |
| 12 | 1+ | 2+ | 1+w | ± | 1+ | Neg | 2+ | Neg | 1+w | Neg | Neg | 1+ | 2+ | Ccee | *D*/− | *DCe*, *ce* |
| 13 | 2+w | 2+s | 2+ | 2+w | ± | 3+ | 2+ | 3+ | 1+w | Neg | 3+ | 4+ | 2+s | NA | *D*/− | NA |
| 14 | 1+ | 4+w | 1+ | 2+ | ± | 2+s | 1+ | 3+ | Neg | ± | 2+ | 4+ | 2+ | Ccee | *D*/− | *DCe*, *ce* |

*Note*: Neg means negative reaction; ± means a very weak positive reaction; 1+ to 4+ mean strength of positive reaction; s means strong, w means weak.
Abbreviation: NA, not available.

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## *RHD* sequencing

Genomic DNA was extracted from 300 whole-blood samples by commercial DNA extraction kits (QIAamp DNA Blood Mini Kit, Qiagen) in accordance with the manufacturer's instructions for further genotyping. All 10 exons and intron–exon junctions of *RHD* were specifically amplified from the genomic DNA and fully sequenced for 295 samples as described previously [5, 6]. Five samples failed to be fully sequenced because of poor DNA quality. Seventeen samples with mutations in the extracellular domain of RhD were further excluded from the analysis of this study.

## *RHD* zygosity genotyping

Droplet digital polymerase chain reaction (ddPCR) was used for *RHD* zygosity genotyping. Briefly, a 20-μl mixture was used for ddPCR reaction, consisting of approximately 50 ng genomic DNA, 1× ddPCR Supermix for Probes (No dUTP, Bio-Rad), 900 nM specific exon (either of *RHD* exon 5 and *RHD* exon 7) and internal control (IC) primers, and 250 nM of the corresponding FAM or HEX-labelled probes. The sequences of each primer and probe are given in the literature [7, 8]. PCRs were carried out in a Veriti 96-well thermal cycler (Thermo Fisher Scientific) with the following steps: 95°C for 10 min; 40 cycles of 94°C for 30 s; 60°C for 1 min and, finally, 98°C for 10 min. After PCR, the samples were analysed using fluorescence signals from the QX200 droplet readers. Bio-Rad QuantaSoft v1.0 software was used for data analysis.

## In vitro overexpression assays and in silico analysis

Full-length wild-type *RHD* and *RHAG* cDNA fragments were amplified and cloned into T-vector pUC57 (Sangon Biotech, Shanghai, China). The mutated *RHD* plasmid was prepared by site-directed mutagenesis. Subsequently, all expression fragments were sub-cloned into commercial lentivirus vectors (Shanghai Genechem Co., Shanghai, China). The GV326 vector was used for wild-type and mutated *RHD* fusion and the GV341 vector was used for *RHAG* fusion. For lentivirus package, 293T cells were co-transfected with each lentiviral vector and two helper vectors, pHelper1.0 and pHelper2.0 (Shanghai Genechem Co.). Virus supernatant was collected after 48–72 h, and the virus titre was evaluated by fluorescence microscopy and quantitative PCR. The wild-type or mutated *RHD* lentivirus was co-infected with *RHAG* lentivirus by using 293T cells. The uninfected 293T cells served as the blank control group. The negative control group was co-infected with empty lentivirus vectors. Flow cytometry (Accuri C6, BD) was performed to detect D antigen expression on the infected 293T cells and the controls by using an IgG anti-D (Clone MS-26) as the primary antibody and an FITC-conjugated secondary antibody (709-095-149, Jackson ImmunoResearch). By using *GAPDH* as an IC, real-time reverse transcription PCR (qRT-PCR) was used to detect the expression of *RHD* and *RHAG* transcripts in each group, as described in the literature [9]. Delta–delta Ct method was used for qRT-PCR data analysis. Homology modelling was carried out using the SWISS-MODEL server [10].

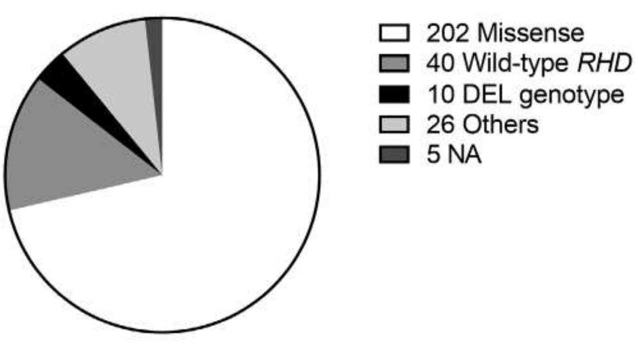
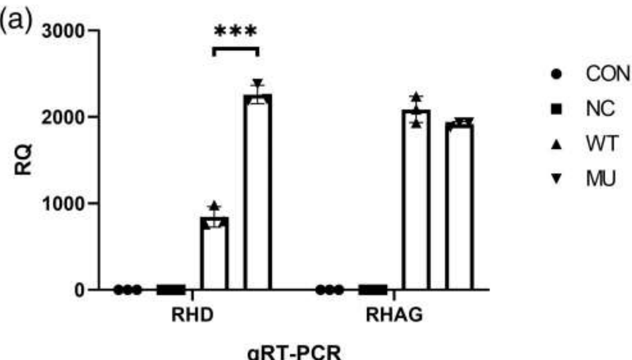



**FIGURE 1** Distribution of different types of *RHD* mutations. Others include synonymous, intronic and in-frame mutations. The corresponding sample size of each type is listed



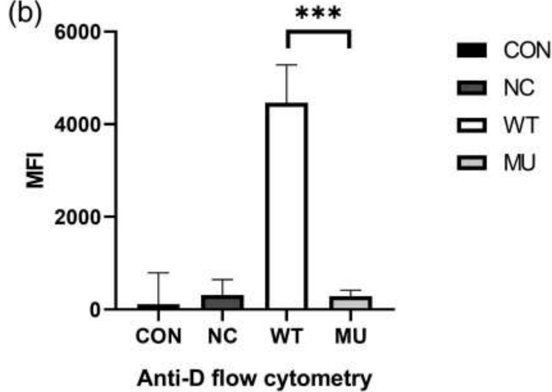




**FIGURE 2** Levels of *RHD* transcripts (a) and D antigen (b) in overexpression assays. All MFI values were normalized by subtracting the MFI of cells stained with the secondary antibody only for each group. Mean ± SD, $n = 3$. ***$p < 0.001$, unpaired two-tailed Student's *t*-test. CON, uninfected control; MFI, mean fluorescence intensity; MU, mutated *RHD* co-transfected with wild-type *RHAG*; NC, negative control infected with empty lentivirus vector; qRT-PCR, real-time reverse transcription PCR; RQ, relative quantity value; WT, wild-type *RHD* co-transfected with wild-type *RHAG*

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## RESULTS

### Weak D samples

We studied a total of 283 samples with weak D phenotype from approximately 3 million blood donors. In China, as confirmed by serological screening and gene sequencing, the prevalence of serologically weak D phenotype was estimated to be 0.01%, which is similar to previous reports [11–13]. Details on 6 of 283 samples have been published in advance [4].

### *RHD* alleles, serotypes and genotypes

Table 3 shows that a total of 45 *RHD* alleles were identified, mainly *RHD*15*, and 11 alleles in 14 samples showed attenuated expression of D antigen for the first time. Table 1 summarizes the detailed molecular characteristics of the novel alleles, and Table 2 summarizes their serological properties and speculative *RH* haplotypes. Another notable detail is that the allele *RHD*526A* has been detected in a compound heterozygous form in trans to wild-type *RHD* without serological profiles in a survey conducted by another group [14]. The sequencing results of the new alleles are shown in Figure S1. Table 3 shows the serological and genotyping results from the remaining 269 samples. The distributions of all the subsets of genotypes are shown in Figure 1. Amino acid substitutions in the transmembrane or intracellular regions in RhD protein remain the most common reason for serologically weak D phenotypes, which are caused by single or multiple base substitutions. No mutation showed relative dominance over other mutations, except for weak D type 15.

### Functional verification of novel in-frame deletion

A novel in-frame deletion mutation of *RHD* was identified, that is, *RHD* c.1102delGGG, p.368delGly (Sample 11). All the recombinant plasmids were successfully constructed. After transfection into 293T cells, the lentivirus was collected, purified and subsequently used for infection. As shown by flow cytometry, the D antigen expression in the mutated *RHD* group co-transfected with wild-type *RHAG* significantly decreased compared with that in the wild-type infection group, consistent with the serological findings (Neg to 1+), although a relatively high level of *RHD* transcripts could be found in the mutated group (Figure 2). The results of homology modelling suggested that structural variation of the mutated RhD protein occurred in the last transmembrane helix (Figure 3).

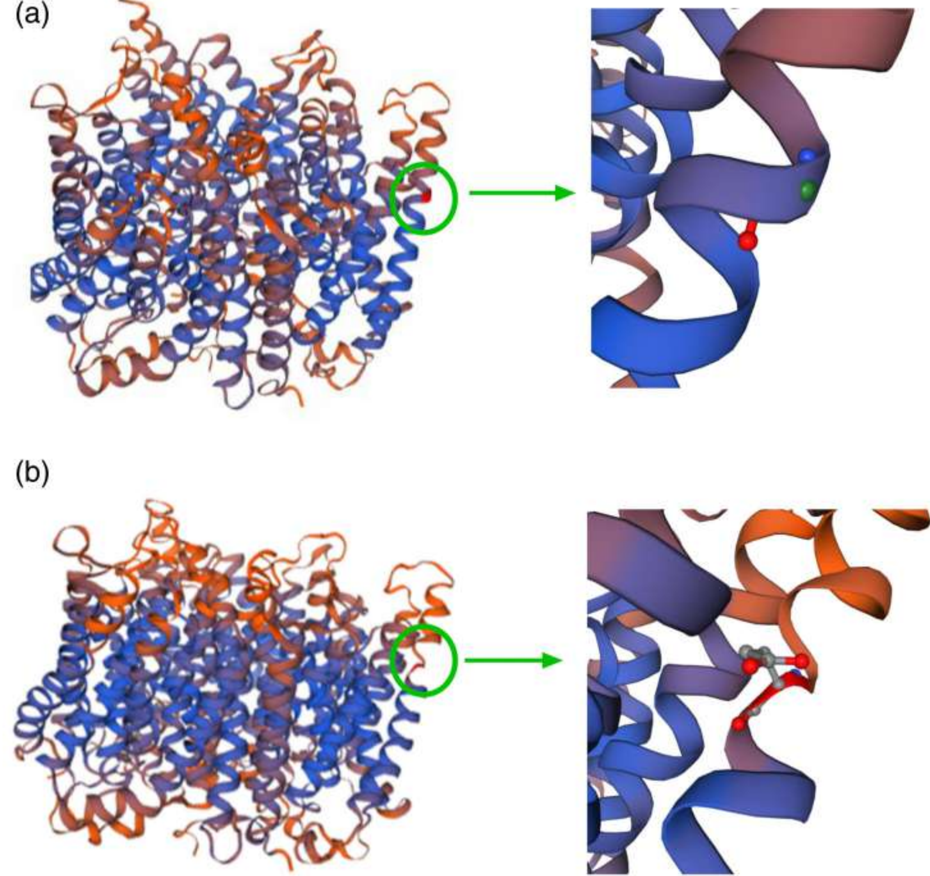


**FIGURE 3** Wild-type (a) and mutated (b) RhD protein structures based on homology modelling. Left, complete structure; right, local structure. The circles and the arrows indicate the positions of the mutated and the corresponding wild-type amino acids

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**TABLE 3** Molecular and serological results of weak D samples with previously reported alleles

| Weak D type or designation | ISBT allele | Nucleotide change(s) | Amino acid change(s) | *RHD* zygosity | Agglutination with anti-Ds: Direct | Agglutination with anti-Ds: AGT | CE type (number) | Number | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Type 15 | *RHD*15* | c.845G>A | p.Gly282Asp | *D/–* | Neg to 1+ | Neg to 3+ | ccEe(59), CcEe(19), CCee(1), ccee(1), ccEE(4), NA(20) | 115 | [3] |
| | *RHD*15* | c.845G>A | p.Gly282Asp | *D/D* | Neg to 1+ | Neg to 3+ | CcEe(3), ccEE(3) | | |
| | *RHD*15, RHD*01EL.01* | c.845G>A, c.1227G>A | p.Gly282Asp | *D/D* | Neg | Neg to 3+ | CcEe(5) | | |
| Type 1 | *RHD*01W.1* | c.809T>G | p.Val270Gly | *D/–* | ± to 1+ | 1+ to 3+ | Ccee(2) | 2 | [3] |
| Type 2 | *RHD*01W.2* | c.1154G>C | p.Gly385Ala | *D/–* | Neg to 1+ | 1+ to 4+ | ccEe(1), ccEE(1) | 2 | [3] |
| Type 6 | *RHD*01W.6* | c.29G>A | p.Arg10Gln | *D/–* | Neg to 1+ | ± to 3+ | Ccee(2), CCee(1) | 3 | [3] |
| Type 18 | *RHD*01W.18* | c.19C>T | p.Arg7Trp | *D/–* | 1+ to 2+ | 1+ to 3+ | Ccee(3) | 3 | [3] |
| Type 25 | *RHD*01W.25* | c.341G>A | p.Arg114Gln | *D/–* | Neg to 2+ | 1+ to 4+ | CcEe(3), Ccee(2), ccEE(1), ccEe(4), | 12 | [3] |
| | *RHD*01W.25, RHD*01EL.01* | c.341G>A, c.1227G>A | p.Arg114Gln | *D/D* | Neg to ± | Neg to 2+ | CCee(1), CcEe(1) | | |
| Type 31 | *RHD*01W.31* | c.17C>T | p.Pro6Leu | *D/–* | ± to 1+ | 1+ to 3+ | Ccee | 1 | [3] |
| Type 33 | *RHD*01W.33* | c.520G>A | p.Val174Met | *D/–* | 1+ to 3+ | 1+ to 4+ | CCee(3), Ccee(2), ccEe(1), CcEe(1), NA(2) | 9 | [3] |
| Type 54 | *RHD*01W.54* | c.365C>T | p.Ser122Leu | *D/–* | 1+ to 2+ | 1+ to 3+ | Ccee(3), NA(1) | 5 | [3] |
| | *RHD*01W.54, RHD*01EL.01* | c.365C>T, c.1227G>A | p.Ser122Leu | *D/–* | Neg to 2+ | 1+ to 4+ | CCee(1) | | |
| Type 71 | *RHD*01W.71* | c.29G>C | p.Arg10Pro | *D/–* | Neg to 2+ | ± to 3+ | Ccee(2), NA(1) | 3 | [3] |
| Type 72 | *RHD*01W.72* | c.1212C>A | p.Asp404Glu | *D/–* | Neg to 2+ | Neg to 4+ | Ccee(3), CcEe(1), CCee(1) | 5 | [3] |
| Type 73 | *RHD*01W.73* | c.1241C>T | p.Ala414Val | *D/–* | ± to 2+ | 2+ to 4+ | Ccee | 1 | [3] |
| Type 100 | *RHD*01W.100* | c.787G>A | p.Glu263Arg | *D/–* | 1+ to 2+ | 1+ to 4+ | ccEe(5), NA(1) | 8 | [3] |
| | *RHD*01W.100, RHD*01EL.01* | c.787G>A, c.1227G>A | p.Glu263Arg | *D/D* | 1+ to 2+ | 1+ to 4+ | CcEe(2) | | |
| Type 105 | *RHD*01W.105* | c.200C>G | p.Ser67Trp | *D/–* | Neg to 3+ | Neg to 3+ | Ccee | 1 | [3] |
| Type 119 | *RHD*01W.119* | c.818C>T | p.Ala273Val | *D/–* | 1+ to 3+ | 1+ to 4+ | ccEe | 1 | [3] |
| Type 122 | *RHD*01W.122* | c.208C>T | p.Arg70Trp | *D/–* | ± to 2+ | 1+ to 4+ | CcEe(1), Ccee(1), ccEe(1) | 7 | [3] |
| | *RHD*01W.122, RHD*01EL.01* | c.208C>T, c.1227G>A | p.Arg70Trp | *D/D* | Neg to 2+ | ± to 3+ | CcEe(4) | | |
| Type 129 | *RHD*01W.129* | c.1208A>T | p.Asp403Val | *D/–* | Neg to ± | Neg to 2+ | ccEE | 1 | [3] |
| Type 132 | *RHD*01W.132* | c.394G>A | p.Gly132Arg | *D/–* | Neg to 1+ | ± to 3+ | CCee(2) | 2 | [3] |
| Type 136 | *RHD*01W.136* | c.41C>T | p.Pro14Leu | *D/–* | Neg to 1+ | 1+ to 3+ | CcEe | 1 | [3] |
| RHD(T32N) | *RHD*95A* | c.95C>A | p.Thr32Asn | *D/–* | Neg to 2+ | NA | Ccee | 1 | [3, 15] |
| RHD(H166D) | *RHD*496G* | c.496C>G | p.His166Asp | *D/–* | 1+ | 1+ to 2 | Ccee | 1 | [3, 16] |
| RHD(G255R) | *RHD*763A, RHD*01EL.01* | c.763G>A, c.1227G>A | p.Gly255Arg | *D/D* | 1+ | ± to 1+ | CCee | 1 | [3, 17] |
| RHD(G307E) | *RHD*920A, RHD*01EL.01* | c.920G>A, c.1227G>A | p.Gly307Glu | *D/D* | Neg to 1+ | 1+ to 4+ | CcEe | 1 | [3, 18] |

(Continues)

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**TABLE 3** (Continued)

| Weak D type or designation | ISBT allele | Nucleotide change(s) | Amino acid change(s) | *RHD* zygosity | Agglutination with anti-Ds: Direct | Agglutination with anti-Ds: AGT | CE type (number) | Number | Reference |
|---|---|---|---|---|---|---|---|---|---|
| weak RHD(960G>A) | *RHD*960A* | c.960G>A | p.Leu320Leu | *D/−* | Neg to 2+ | ± to 4+ | Ccee(9), NA(1) | 11 | [3, 19] |
| | *RHD*960A*, *RHD*01EL.01* | c.960G>A, c.1227G>A | p.Leu320Leu | *D/D* | Neg to 1+ | Neg to 2+ | CCee(1) | | |
| RHD(I341N) | *RHD*1022A* | c.1022T>A | p.Ile341Asn | *D/−* | Neg to ± | Neg to 3+ | CCee(1), Ccee(3), NA(1) | 5 | [3, 20] |
| RHD(IVS3+3G>C) | *RHD*486+3C* | c.486+3G>C | NA (not applicable) | *D/−* | Neg to 3+ | Neg to 3+ | ccEe(4) | 4 | [3, 4] |
| RHD(IVS4+5G>A) | *RHD*634+5A* | c.634+5A | NA | *D/−* | Neg to 1+ | Neg to 2+ | ccEe | 1 | [3, 4] |
| RHD(IVS4+5G>T) | *RHD*634+5T* | c.634+5T | NA | *D/−* | Neg to 2+ | Neg to 2+ | CCee(1), NA (1) | 2 | [3, 4] |
| RHD(IVS6-14delTAA) | *RHD*940-16_14delTAA* | c. 940-16_14delTAA | NA | *D/−* | ± to 2+ | 1+ to 3+ | Ccee(1), ccEe(1) | 2 | [3, 4] |
| RHD(1227G>A) | *RHD*01EL.01* | c.1227G>A | NA | *D/−* | Neg to ± | Neg to 2+ | CCee(3), Ccee(4), NA(2) | 9 | [3] |
| RHD(M1I) | *RHD*01EL.02* | c.3G>A | p.Met1Ile | *D/−* | Neg | Neg to 2+ | Ccee | 1 | [3] |
| RHD(165C>T) | *RHD*165T* | c.165C>T | NA | *D/−* | ± to 1+ | 1+ to 2+ | NA(1) | 2 | [3, 21] |
| | *RHD*165T*, *RHD*01EL.01* | c.165C>T, c.1227G>A | NA | *D/D* | Neg to 1+ | 1+ to 4+ | CcEe(1) | | |
| RHD(357T>C) | *RHD*357C* | c.357T>C | NA | *D/−* | Neg to 1+ | 1+ to 4+ | ccEe | 1 | [3, 12] |
| standard RHD | *RHD*01* | NA | NA | *D/−* | ± to 2+ | Neg to 3+ | CCee(11), Ccee(18), ccEe(3), NA(2) | 40 | [3] |
| | | | | *D/D* | 1+ to 2+ | 2+ to 4+ | CCee(3) | | |
| | *RHD*01*, *RHD*01EL.01* | c.1227G>A | NA | *D/D* | ± to 2+ | ± to 3+ | CCee(1) | | |
| | *RHD*01*, *RHD*01N.16* | c.711del C | NA | *D/D* | Neg to 1+ | 1+ to 2+ | CCEe(1) | | |
| | *RHD*01*, *RHD*357C* | c.357T>C | NA | *D/D* | 1+ to 3+ | 2+ to 4+ | CcEe(1) | | |
| NA | NT | NA | NA | NA | Neg to 2+ | ± to 3+ | CCee(2), Ccee(1), ccEe(2), | 5 | |
| Total | | | | | | | | 269 | |

*Note*: Neg means negative reaction; ± means a very weak positive reaction; 1+ to 4+ mean strength of positive reaction.
Abbreviations: AGT, antiglobulin test; NA, not available; NT, not tested.

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## DISCUSSION

In recent years, *RHD* genotyping has been increasingly involved in the identification of serologically weak D to avoid ambiguity. Given that only limited investigations on the genetic characteristics of weak D have been conducted in a relatively large group in China, a single-centre study took more than 10 years to provide an overall analysis of the genotypes of serologically weak D phenotypes. Overall, the diversity of *RHD* genotypes suggested the complexity of D antigen expression, as discussed below.

As expected, weak D type 15, which is currently renamed as weak partial type 15, is the most common weakened D expression phenotype in China, consistent with several previously published findings in small groups from other areas of the country [11–13]. Differentiating this type from normal D+ or D– blood is essential and particularly important for patients undergoing transfusion who should receive D– units to avoid the risk of isoimmunization. In the present study, the most common haplotype in weak D type 15 was *DcE*, as could be inferred from the CE types. In addition, a rare ccee phenotype was found in one sample, suggesting the existence of the *Dce* haplotype in weak D type 15.

In particular, a novel in-frame deletion variant *RHD*1102_1104del* was found. We developed an efficient method for the functional interpretation of the correlation between *RHD* mutation and D antigen expression by in vitro recombinant RhD protein expression combined with in silico analysis. Small in-frame insertions/deletions are relatively uncommon *RHD* variant types, with only a few cases reported [3, 22–24]. In the first report of in-frame deletions in *RHD* coding regions, the authors hypothesized that amino acid deletions could be triggered by nucleotide repeats at nine positions [22]. Interestingly, although some publications were consistent with this conjecture [22, 24], mutations failed to be located in the speculative regions in some cases [6, 15], as in the present study. This finding suggested other mechanisms, which led to unexpected in-frame deletions. Although in-frame mutations could lead to distinct phenotypic states, such as D–, weak D and partial D, none has studied the molecular connection between the genotypes and the phenotypes caused by this type of mutation. Here, an in vitro model was used as an efficient tool for functional interpretation of novel *RHD* variants. Combining the results at the RNA and protein levels and the homology modelling analysis showed that the new in-frame deletion may significantly lower the expression of D antigen through altered protein structure in the last transmembrane helix instead of reducing RhD protein production. This suggests impaired membrane integration of the RhD protein or the interruption of RhD–RhAG complex formation. Therefore, the development of effective functional study methods will help improve the understanding of rare mutants and shed light on the complex genotype–phenotype correlations.

Twenty-five samples showed silent or intronic mutations, which have long been underestimated in weak D study. In recent years, increasing evidence has shown the importance of such mutations in various contexts. Silent mutations, also called synonymous variants, may affect the protein expression levels and function models by changing *cis*-acting elements, altering mRNA structures, and influencing protein synthesis [25]. Intronic mutations could affect pre-mRNA splicing by impairing core or auxiliary *cis*-splicing elements. Interestingly, 12 out of the 15 silent mutation samples showed leucine mutations (*RHD*960A* and *RHD*1005A*).

Interestingly, 14.1% of the samples (40/283, including 3 compound heterozygous samples and 3 samples with homozygous wild-type genotype) had no apparent mutations in the coding regions or near exon–intron junctions. Although uncommon, normal *RHD* alleles leading to weakened or even extremely low D antigen expression have been reported recently in other populations [26–28]. The mechanisms leading to unexpectedly low expression of D antigen remain poorly understood. Some likely reasons include down-regulation at the transcriptional level by unknown *cis*-acting elements or trans-acting factors (e.g., deep intronic mutations that could hardly be detected by conventional gene sequencing) and structural variations that do not alter the sequences of bases in exons and adjacent introns. A notable report has shown that a kind of structural variation (duplication of *RHD* exon 3) is the predominant variant *RHD* allele in the Indian population but not in others [26]. For solving such structural variation problems, detecting the copy numbers of each *RHD* exon or in-depth high-throughput sequencing may be a possible solution.

In addition, the same mutant allele surprisingly led to different phenotypic traits on several occasions. Remarkably, 10 samples serologically typed as weak D initially were confirmed as DEL genotype. However, serologically distinct weak D patterns were found in these nine samples, suggesting as-yet-undefined modifiers of D antigen expression. In addition, three samples harboured controversial silent mutations of *RHD*. Two of them were *RHD*165T* [21] and third was *RHD*357C* [12]. However, one homozygous *RHD*357C/RHD*357C* sample presenting normal D+ phenotype and D antigen density was previously identified in a Chinese $Rh_{mod}$ pedigree [29]. As another example, although the allele *RHD*634+5T* has the ISBT destination *RHD*DEL14*, the two samples encountered in our study showed distinct weak D patterns. Even though the serological phenotypes differ considerably between individuals having the same mutated variants, the underlying mechanisms remain unclear. Such peculiarities may complicate the serological interpretation of genotyping results for some variants.

In summary, we found distinct characteristics of *RHD* allele distribution in Chinese persons with serologically weak D when compared with reports from other populations. Our effective method to assess the possible function of rare variants of *RHD* may contribute to a better understanding of D antigen expression. One flaw in this single-centre study is that the true frequency of serologically weak D in China may be underestimated (i.e., some examples may be missed because of a limited number of primary screening anti-Ds). With the widespread application of blood group genotyping, solving the problem of inconsistency between genotyping and serotyping is a new challenge (e.g., inconsistent antigen expression caused by the same allele). Meanwhile, we also need clinical data on the risk of anti-D production by people with different types of weak D.

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Y.Q. contributed to genomic DNA extraction; Z.Z. designed the study and revised the manuscript. All authors reviewed the data, provided comments and approved the final manuscript.

## CONFLICT OF INTEREST

The authors declare no conflict of interests.

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## SUPPORTING INFORMATION

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ORIGINAL ARTICLE

# A new high-prevalence LW antigen detected by an antibody in an Indigenous Australian homozygous for *LW*A* c.309C>A variant

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## Abstract

**Background and Objectives:** The *LW* gene encodes the LW glycoprotein that carries the antigens of the LW blood group system. LW antigens are distinct from D antigen, however, they are phenotypically related and anti-LW antibodies are often mistaken as anti-D. An antibody was detected in an Australian patient of Aboriginal descent who consistently typed as LW(a+b−). This study aimed to describe the antibody recognizing a high-prevalence antigen on the LW glycoprotein.

**Study Design and Methods:** Samples from the patient and her four siblings were investigated. DNA was genotyped by single nucleotide polymorphism (SNP)-microarray and massively parallel sequencing (MPS) platforms. Red blood cells (RBCs) were phenotyped using standard haemagglutination techniques. Antibody investigations were performed using a panel of phenotyped RBCs from adults and cord blood cells.

**Results:** SNP-microarray and MPS genotyped all family members as *LW*A/A*, (c.299A), predicting LW(a+b−). In addition, a novel *LW*A* c.309C>A single nucleotide variant was detected in all family members. The patient and one of her siblings (M4) were *LW* c.309C>A homozygous. Antibody from the patient reacted positive to all reagent panel RBCs and cord blood cells but negative with RBCs from LW(a−b−), $Rh_{null}$ and sibling M4. Antibody failed to react with RBCs treated with dithiothreitol.

**Conclusion:** Antibody detected in the patient recognized a novel high-prevalence antigen, LWEM, in the LW blood group system. LWEM-negative patients who developed anti-LWEM can be safely transfused with D+ RBCs, however, D− is preferred. Accurate antibody identification can help better manage allocation of blood products especially when D− RBCs are in short supply.

KEYWORDS
GenBank MN412704, Indigenous Australian, LW blood group system, LW high-prevalence antigen, *LW*A* c.309C>A polymorphism, LW8, LWEM

## Highlights

- LWEM is a new high-prevalence antigen in the LW blood group system.

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- Individuals homozygous for *LW* c.309C>A are LWEM-negative and are at risk of alloimmunization when exposed to LWEM-positive red blood cells (RBCs).
- LWEM-negative individuals with anti-LWEM can safely receive D-positive/D-negative LW-positive RBCs.

## INTRODUCTION

The LW blood group system (ISBT 016) was named after Landsteiner and Wiener who first reported the production of LW antiserum derived from guinea pigs and rabbits [1, 2]. LW antigens are carried on the LW glycoprotein—$LW^a$ and $LW^{ab}$ are high-prevalence antigens and $LW^b$ is a low-prevalence antigen [3, 4]. $LW^b$ is found in Europeans (4.0%–5.9% in Estonia, Finland, Lithuania and Latvia) but absent in other population groups [5–7]. The two main alleles of the *LW* gene are *LW*A* and *LW*B*, which encode antithetical antigens $LW^a$ (c.299A) and $LW^b$ (c.299G), respectively (Table 1) [9].

The *LW* gene, also known as the intracellular adhesion molecule 4 (*ICAM4*) gene, is located in chromosome 19p13.3 and encodes LW glycoprotein [8, 10]. The three exons of the *LW* gene produce a mRNA transcript (isoform 1) encoding a 271 polypeptide chain that includes a signal peptide of 30 amino acids [8, 9]. The LW glycoprotein, together with Rh proteins, forms the Rh macromolecule associated with the band 3 complex in the red blood cell (RBC) membrane. Although LW and D are distinct antigens, they share a strong phenotypic association and anti-LW antibodies are often mistaken as anti-D.

Short insertion–deletion (indels) and single nucleotide polymorphism (SNP) in *LW* gene give rise to LW(a−b−) phenotype, (Table 1). Indels cause a frameshift forming a stop codon in the *LW* coding region (Figure S1) [10, 11]. Individuals that are phenotypically and genotypically LW(a−b−) can produce anti-LW antibodies [13]. A review of 1960s and 1970s case reports in individuals with anti-LW was summarized in an article by Giles [13]. Case reports from the 1980s and onwards are summarized in Table 2.

Anti-LW in patients who are phenotypically LW(a−b−) but carry a wild-type *LW* gene have also been observed [18]. This LW(a−b−) phenotype is due to the temporary loss of LW observed in pregnancy and blood transfusion, and in certain disease conditions such as lymphoma and leukaemia [19]. In patients with the transitory LW(a−b−) phenotype, the disappearance of anti-LW antibodies is simultaneous with the emergence of LW antigens [19]. More background information on the LW blood group system is found in these review articles [13, 19, 20].

## CASE PRESENTATION

A female Indigenous Australian patient of Aboriginal descent presented at a community health clinic four times for antenatal screening from 1975 up to 1982 (Table 3). Irregular red cell antibodies were first detected in her sample during her third pregnancy in 1980 and again detected in 1982. She is Group O, D+C+E−c−e+, LW(a+b−) and her serum agglutinated all reagent RBCs. Stronger reactions were observed with D+ than D− reagent cells. Laboratory investigations reported that the patient's serum contains anti-LW, active in saline at 37°C, and auto anti-pdl (partially-deleted) antibodies, active in indirect antiglobulin test (IAT) and papain at 37°C. Patient's serum failed to react with two examples of $Rh_{null}$ and one example of LW(a−b−) RBCs. Patient's RBCs were direct antiglobulin test (DAT) negative and were typed as LW+ against a panel of six anti-LW reagents.

In July 1996, the patient's plasma was negative in the antibody screen and was transfused with four crossmatch-compatible units of Group O, D+ packed RBCs (PRBCs). This is the first blood transfusion event for the patient. Blood samples taken from the patient dated

**TABLE 1** LW blood group alleles

| Phenotype | Allele name | Nucleotide change | Exon | Amino acid change | rs number | Reference | Accession number |
|---|---|---|---|---|---|---|---|
| LW(a+) | *LW*A* or *LW*05* | c.299A | 1 | p.Gln100 | | Bailly et al. [8]<br>Hermand et al. [9] | AH004780.1<br>NG_007728.1<br>NM_001544.4 |
| LW(b+) | *LW*B* or *LW*07* | c.299A>G | 1 | p.Gln100Arg | rs77493670 | Bailly et al. [8]<br>Hermand et al. [9] | AH004780.1 |
| Null phenotypes | | | | | | | |
| LW(a−b−) | *LW*05N.01* | c.346_355del | 1 | p.Thr116Glufs*19 | rs778248852 | Hermand et al. [10] | X93093.1 |
| LW(a−b−) | | c.137delT[a,b] | 1 | p.Val46Glyfs*7 | | Gauthier et al. [11] | |
| LW(a−b−) | | c.2T>A[b] | 1 | p.Met1Lys | | Gauthier et al. [11] | |

*Note*: *LW*A* is the reference allele. The nucleotide numbering in this table includes the 90 nucleotides that encode for the 30 amino acid signal peptide. *LW* gene rs numbers were accessed from dbSNP [7, 12].
[a]The *LW* c.137delT variant was originally reported as +47delT [11].
[b]The original report did not associate *LW* c.137delT and c.2T>A variants to either *LW*A* or *LW*B* alleles.

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December 1996 and January 1997 were antibody screen positive. Antibody activity was still present after autoadsorption suggesting an alloantibody rather than autoantibody. Investigations detected anti-LW and an autoantibody weakly reactive by papain at 37°C, which is similar to the previous report. In 2010, the patient was hospitalized and received six units of Group O, D− PRBCs. In June 2020, the patient was again admitted to hospital due to end-stage renal failure and received one unit of Group O, D− and two units of Group O, D+ PRBCs. The patient consistently typed as LW(a+b−).

Based on the patient's laboratory records, there was nothing to indicate that the patient was investigated for haemolytic transfusion reaction or that one of her pregnancies was associated with haemolytic disease of the foetus and newborn (HDFN).

**TABLE 2** Selected reports of LW(a−b−) individuals with anti-$LW^{ab}$

| Genetic basis | Year | Description of report |
|---|---|---|
| Not determined | 1985 | Group A, D+ white male individual with no history of transfusion. Phenotyped as LW(a−b−) consistently in an 8-month period [14]. It is unclear whether the anti-$LW^{ab}$ detected was auto or alloantibody. |
| Not determined | 1986 | D+ female with no history of transfusion. She had been pregnant twice. Safely received D− PRBCs (first event) and thereafter. Persistently typed as LW(a−b−) [15]. |
| Not determined | 1989 | Mrs MMJ—a Caucasian Canadian patient. She is Group A, D+, had two pregnancies and had been previously transfused. Her RBCs failed to react with Mrs Bigelow's serum. The patient remained LW(a−b−) for 2 years eliminating the possibility that this phenotype is transitory [16]. |
| Not determined | 1992 | A previously transfused male Papua New Guinean individual, AK, and his sister. RBCs from AK were consistently typed as LW(a−b−) for 16 months. Anti-$LW^{ab}$ was detected in patient AK [17]. |
| None found | 1996 | D+, 92-year-old male individual (Nic). No deletion or polymorphism was detected in the *LW* gene to explain LW(a−b−) phenotype [10]. |
| *LW* c.137delT | 2012 | A female patient with a D−C−E−c+e+ phenotype [11]. |
| *LW* c.2T>A | 2012 | A female patient and her sister. Both have the Rh phenotype D−C−E+c+e+. Anti-$LW^{ab}$ was detected in the patient but not with her sister [11]. |

Abbreviation: PRBCs, packed RBCs.

# MATERIALS AND METHODS

## Sample collection and preparation

Ethylenediaminetetraacetic acid (EDTA)-whole blood samples from the patient (propositus; M2) and her four siblings (M1, M3, M4 and M5) were collected and referred to Australian Red Cross Lifeblood for serological and molecular investigations. Plasma was separated from whole blood samples for antibody identification investigation. RBCs were washed and suspended in phosphate-buffered saline (PBS) at 3%–5% concentration for haemagglutination tests. DNA was isolated using EZ1 DNA blood extraction kit (QIAGEN) and BioRobot EZ1 Workstation (QIAGEN) according to manufacturer's instructions. DNA was quantitated and quality checked on two instruments: NanoDrop 2000c spectrometer (Thermo Fisher Scientific) and Qubit 4 fluorometer.

## Molecular typing

Blood group genotype was determined by massively parallel sequencing (MPS, TruSight One Sequencing Panel, Illumina) as previously described [21]. SNP-microarray (PreciseType HEA Test, BeadChip, Immucor) was performed according to manufacturer's instructions. MPS was performed on a MiSeq sequencing platform generating a 150-bp paired-end reads [21]. MiSeq-generated FASTQ files were

**TABLE 3** Clinical history for patient M2

| Date | Clinical notes | Antibody screen | Transfused? |
|---|---|---|---|
| October 1975 | Antenatal screening | Negative | No |
| May 1977 | Antenatal screening | Negative | No |
| December 1980 | Antenatal screening | Positive | No |
| December 1982 | Antenatal screening | Positive | No |
| July 1996 | Anaemia, menorrhagia | Negative | Yes. 4× Group O, D+ PRBCs |
| December 1996 | Anaemia | Positive | Unable to confirm |
| January 1997 | Anaemia | Positive | Unable to confirm |
| April 2010 | Anaemia, renal failure | Positive | Yes. 6× Group O, D− PRBCs |
| December 2018/January 2019 | Anaemia, renal failure | Positive | No |
| June 2020 | End-stage renal failure | Positive | Yes. 1× Group O, D− and 2× Group O, D+ PRBCs |

Abbreviation: PRBCs, packed RBCs.

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**TABLE 4** LW phenotyping for M2

| | Reaction with RBCs | |
|---|---|---|
| | M2 | Control |
| CTT method | | |
| Anti-LW$^{ab}$ (Big) | +++ | +++ |
| Anti-LW (Woj) | ++ | ++++ |
| Anti-LW$^{ab}$ (Fra) | ++ | ++ |
| Anti-LW (Mik) | ++ | ++ |
| Anti-LW$^{a}$ (VW) | ++ | ++ |
| Anti-LW$^{a}$ (25262) | ++ | ++ |
| Anti-LW$^{a}$ (Kri) | ++ | (+) |
| CAT method | | |
| Anti-LW$^{ab}$ (Big) | +++ | +++ |
| Anti-LW$^{a}$ (SG) | ++ | ++ |
| Anti-LW$^{a}$ (Lai) | ++ | ++ |

*Note*: Commercial reagent RBCs (Grifols) and Panocell were used as positive control cells. In-house LW antigen-negative RBCs were used as negative control cells. All control cells gave expected results. Data not shown for negative control RBCs. (+) = weak positive; +, positive.
Abbreviations: CAT, column agglutination technology; CTT, conventional tube technique; RBCs, red blood cells.

imported into a bioinformatics software, CLC Genomics Workbench Software 20 (QIAGEN). Trimmed sequence reads were mapped to the human reference genome hg19/GRCh37 to detect variants. Variant Call Format files from CLC Genomics Workbench were imported into a Microsoft Excel file to generate a list of variants [21].

## RBC phenotyping

Standard haemagglutination techniques, conventional tube technique (CTT) and column agglutination technology (CAT) were used to phenotype RBCs. A panel of nine antisera targeting epitopes on LW glycoprotein was used to characterize RBCs from the propositus (M2) (Table 4).

## Antibody identification

A panel of phenotyped cells (RBCs from adults and cord blood cells) were used to determine the specificity of red cell antibody. In addition, three commercial reagent RBCs were used as control RBCs in this study: Grifols Data-Cyte 3% (Grifols), Phenocell (Immulab, ParagonCare), Abtectcell III (Immulab) and Panocell-20 (Immucor). All haemagglutination reactions were performed in test tubes except where stated. Gel-based agglutination technique was performed on ID Card LISS/Coombs (BioRad) or DG Gel Cards (Grifols) according to manufacturer's recommendation. RBCs were treated with enzymes or chemicals to confirm antibody specificity.

## Antibody elution (acid glycine–EDTA)

Antibody-sensitized RBCs or cord blood cells were washed in PBS six times. PBS from the last wash was collected and used as a negative control solution. Four parts of 0.1 M glycine–hydrochloric acid (pH 1.8) were combined with one part 10% EDTA to make glycine–HCl EDTA solution. Two parts of glycine–HCl EDTA solution is combined with one part washed, PRBC and mixed by gentle inversion for 2 min. One drop of 1 M Tris–NaCl was added into the suspension, then mixed and centrifuged immediately. Supernatant (eluate containing anti-LW antibodies) was transferred into a clean tube. One drop of 30% bovine serum albumin was added into the tube. Eluate was used for IAT.

## Anti-A adsorption from patient's plasma

M2, being group O, has anti-A and anti-B antibodies present in the plasma in addition to anti-LW. To prevent anti-A from interfering with haemagglutination reactions against Group A panel and sibling test cells, anti-A from the patient's plasma was removed using dithiothreitol (DTT)-treated Group A D− RBCs. LW antigens are sensitive to DTT. Briefly, Group A D− RBCs were added with 0.2 M DTT in a test tube. Tubes were incubated at 37°C for 30 min and then washed in PBS. DTT-treated RBCs are then added to patient's plasma to adsorb out anti-A. The adsorbed plasma was then used against papain-treated RBCs with known ABO, D and LW phenotype. CAT, in lieu of CTT, was used in this part of this investigation to conserve the remaining M2 plasma sample.

## Haemagglutination assessment

All tube haemagglutination reactions were assessed microscopically and were scored from 0 (negative) to ++++ (strong positive reaction). Gel-based haemagglutination reactions were assessed as per manufacturer's instructions.

# RESULTS

## *LW* genotyping by massively parallel sequencing

The propositus (M2) was homozygous for c.299A (p.Gln100), *LW*A/A*, predicting LW(a+b−). In addition, M2 was homozygous for a novel c.309C>A (p.Asp103Glu) variant in Exon 1 (Figure 1a). The p.Asp103Glu residue is only three amino acids away from p.Gln100 encoding LW$^{a}$ (Figure 1b). Accession numbers were assigned for this novel allele: GenBank MN412704, and ClinVar SCV001761173 and VCV001185005.1. Due to the phenotypic relationship between LW and D antigens, the *RHD* gene was analysed. No variants were detected in the *RHD* gene.

Family members M1, M3, M4 and M5 were all *LW*A/A* (c.299A) predicting LW(a+b−). M1, M3 and M5 were heterozygous, and M4 was homozygous for the *LW*A* c.309C>A variant (Figure 2).

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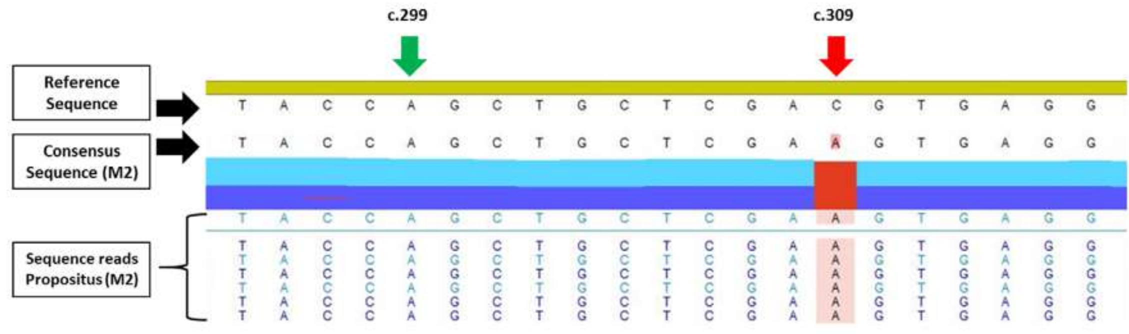


**FIGURE 1** Comparison of *LW* sequence between the reference and propositus (M2). (a) Screenshot of massively parallel sequencing (MPS) sequence reads from CLC Genomics Workbench software. The green arrow at c.299 indicates the $LW^a/LW^b$ polymorphic site. Red arrow indicates the site for the novel *LW* c.309C>A polymorphism. Nucleotide sequencing showed the *LW* c.309C>A variant *in cis* with *LW*A* allele (c.299A). (b) Comparison of amino acid sequences between the reference and the propositus (M2)

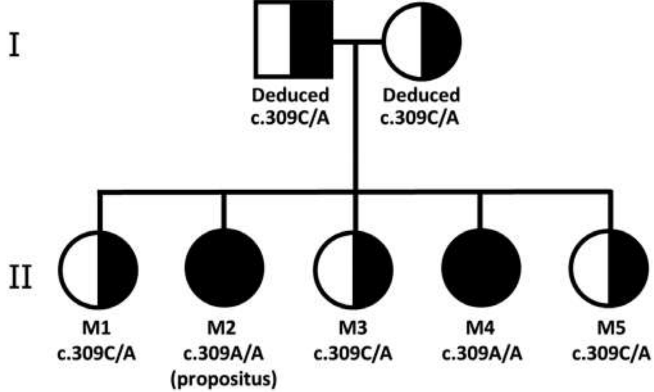


**FIGURE 2** Pedigree chart for the family carrying the *LW*A* c.309C>A variant. No blood samples were received from the parents. The *LW*A* c.309C/A genotype was assigned for the mother and father to support the inheritance of a homozygous *LW*A* c.309C>A for the propositus (M2) and M4. However, the probability that one parent is heterozygous and the other is homozygous for *LW*A* c.309C>A (*LW*A* c.309A/A) cannot be ruled out. Consanguinity between the parents is not known. Black shade indicates the presence of the *LW*A* c.309C>A variant. Male and female members of the family are represented as square and circles, respectively

## *LW* genotyping by SNP-microarray

All family members were genotyped as *LW*A/A*, predicting LW(a+b−) consistent with the MPS data. BeadChip SNP-microarray does not target the nucleotide at position *LW* c.309. SNP-microarray genotyping data are provided in Table S1.

## ABO and Rh phenotyping

M2 was Group O, D+C+E−c−e+, K−k+, Fy(a+b−) Jk(a+b+), M+N+S−s+. ABO and Rh phenotype for all family members were shown in Table S1. The C, E, c and e phenotype is consistent with the predicted phenotype based on SNP-microarray data.

## LW phenotyping

M2 RBCs reacted positive with all antisera in the CTT and CAT methods (Table 4). The LW(a+) and LW(ab+) phenotype were consistent with phenotype predictions based on SNP-microarray and MPS data. The reactivity profile for M2 RBCs against seven typing reagents were comparable to control RBCs. M2 cells reacted weaker with anti-LW (Woj) and stronger with anti-$LW^a$ (Kri) than control cells.

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**TABLE 5** Reactivity profile of M2 plasma

| a. Neat plasma (IAT CTT) | Untreated RBC (PEG) | Papain-treated | DTT-treated |
|---|---|---|---|
| Versus cell panel (Abtectcell III) | | | |
| Adult D+ ($R_1R_1$) | ++ | +++ | 0 |
| Adult D+ ($R_2R_2$) | ++ | +++ | 0 |
| Adult D− (rr) | + | +++ | 0 |
| Auto cells | w | +++ | NT |
| Cord cells D+ | +++ | +++ | NT |
| Cord cells D− | +++ | +++ | NT |

| b. Eluate (IAT CAT) | Eluate | PBS (last wash) |
|---|---|---|
| Versus control RBC panel (papain-treated) | | |
| D+ cord cells | ++++ | 0 |
| D+ ($R_ZR_1$) adult cells | +++ | 0 |
| D− (rr) adult cells | w | 0 |
| Versus RBC from siblings (papain-treated) | | |
| M1 D+ ($R_1R_1$) LW(a+b−) (*LW* c.309C/A) | ++ | 0 |
| M5 D+ ($R_ZR_1$) LW(a+b−) (*LW* c.309C/A) | +++ | 0 |
| M4 D+ ($R_ZR_1$) LW(a+b−) (*LW* c.309A/A) | 0 | 0 |
| M2 D+ ($R_1R_1$) LW(a+b−) (*LW* c.309A/A) Auto | 0 | 0 |

| c. Anti-A adsorbed plasma (IAT CAT) | Plasma |
|---|---|
| Versus RBC panel (papain-treated) | |
| RC1744 Group A, D+ LW(a−b−) | 0 |
| RC0483 Group A, D+ LW(a−b−) | 0 |
| $Rh_{null}$ | 0 |
| Group O, D+ cord cells | +++ |
| Group O, D− cord cells | +++ |
| Group O, D+ ($R_2R_2$) | + |
| Group O, D− (rr) | w |
| Group A, D+ | w |
| Group A, D− | w |
| RC0821 Group A, D+ LW(a−b+) | w |

*Note*: In 5B, D+ $R_ZR_1$ adult cells were chosen to match the Rh phenotype of M2's siblings. In 5C, RC1744, RC0483 and RC0821 are in-house reagent RBCs.
Abbreviations: CAT, column agglutination technology; CTT, conventional tube technique; DTT, dithiothreitol; IAT, indirect antiglobulin test; NT, not tested; PBS, phosphate-buffered saline; PEG, polyethylene glycol; RBC, red blood cells; w, weak positive; 0, negative; +, positive.

## Reactivity profile of plasma

M2 plasma gave a positive reaction to all untreated RBC panel cells (polyethylene glycol, PEG medium) (Table 5a). The pattern of positive reactions varied widely between cells. The weakest was observed with adult D− RBCs and the strongest (3+) with D+ and D− cord cells. Auto RBCs showed a weak positive reaction indicating the presence of an autoantibody. All papain-treated RBCs gave a consistent 3+ positive reaction. A stronger positive reaction was observed in papain-treated adult RBCs compared to untreated adult RBCs. All DTT-treated adult RBCs gave a negative reaction suggesting that the epitope recognized by the antibody is DTT-sensitive. This reactivity pattern is consistent with anti-LW.

## Reactivity profile of eluate

Pooled D+ and D− cord cells were used to adsorb and elute antibody from patient's plasma. The eluate gave a stronger positive reaction with D+ than D− cells (Table 5b).

Eluate reacted positive with RBCs from siblings M1 and M5. Both M1 and M5 were heterozygous for *LW*A* c.309C>A. Eluate failed to react with M4 RBCs. This was expected as M4 was also homozygous for the *LW*A* c.309C>A (p.Asp103Glu) variant. This reactivity profile suggests that the antibody recognized an epitope on normal LW glycoprotein (p.Asp103) and not carried on variant LW glycoprotein (p.Glu103).

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## Reactivity profile of anti-A adsorbed plasma

Adsorbed patient's plasma failed to react with two examples of LW(a−b−) cells and one $Rh_{null}$ RBCs (Table 5c). The remaining seven panel cells were positive. All were assumed to express, at least, the $LW^a$ antigen except panel cell RC0821, which is LW(a−b+). This indicates that the antibody recognizes an antigen on LW glycoprotein common to LW(a+) and LW(b+) RBCs.

# DISCUSSION

Serological and molecular evidence were provided for an antibody recognizing a high-prevalence antigen on LW glycoprotein. We propose the name LWEM for this antigen. LWEM, *LW* c.309C (p.Asp103), has been provisionally designated as LW8 (016008) by the ISBT Working Party on Red Cell Immunogenetics and Blood Group Terminology.

*LW* c.309C>A variant encodes p.Asp103Glu, which is only three residues from p.Gln100 ($LW^a$). Due to the proximity of p.Asp103Glu to $LW^a$, change in the expression of LW glycoprotein on M2 RBCs was anticipated, however, this was not observed in this study (Table 4). The patient is homozygous for *LW*A* c.309C>A (p.Asp103Glu) and is therefore LW(a+b−) LWEM-negative. LWEM-negative individuals when exposed to normal LW are at risk of alloimmunization and can produce anti-LWEM. It is anticipated that this study could lead to the characterization of a novel low-prevalence antigen antithetical to LWEM.

Historical data and data from this current investigation consistently typed the patient as LW(a+). The patient developed anti-LWEM in response to pregnancy and not due to transfusion. The anti-LWEM in this case is not an autoantibody but an alloantibody and is not the transient type. In comparison, most anti-LW made by phenotypically LW(a−b−) individuals but who carry wild-type *LW* are of the transient type [19]. In this report, the patient with anti-LWEM safely received D− and D+ RBCs.

Few cases of anti-LW production stimulated by pregnancy have been reported [13, 15]. In one report, autoanti-LW caused a mild HDFN [22]. RBCs from the baby were DAT positive. The baby had an elevated bilirubin level and received phototherapy [22]. Anti-LW antibodies, although rarely clinically significant, needs to be distinguished from the clinically significant anti-D [23]. Anti-LW that is incorrectly identified as an anti-D could result in withdrawal of anti-D immunoglobulin prophylaxis in D− pregnant women [24]. When 'anti-D' is detected in D+ individual with a normal *RHD* gene, anti-LW should be considered. Accurate antibody identification can help better manage allocation of blood products especially when D− RBCs are in short supply.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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SHORT REPORT

# Reported non-compliance with pre-donation screening among blood donors in Québec, Canada: A focus on the 3-month deferral for men who have sex with men

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## Abstract

**Background and Objectives:** In Québec (Canada), the donation deferral for men who have sex with men (MSM) has recently been shortened to 3 months. Whether this change impacted compliance with pre-donation screening is unknown. We assessed compliance with the disclosure of male-to-male sex and other behavioural risk factors for HIV amid this change.

**Materials and Methods:** Québec residents who donated from 14 July 2020 to 30 November 2020 were invited to participate in an online survey. Donors were informed that the survey was optional and anonymous. Survey questions were those used for routine pre-donation screening. Rates of reported non-compliance were weighted based on several characteristics.

**Results:** Of 21,918 contacted donors, 7113 (32.45%) participated. Among male participants ($N = 3347$), six (0.27% [95% confidence interval (CI) = 0.09%–0.44%]) were not compliant with a 3-month MSM deferral. Among female participants ($N = 3766$), two (0.06% [95% CI = 0.00%–0.13%]) were not compliant with a 3-month deferral for sex with a man who had male-to-male sex ≤12 months. Other risk factors exhibited similar or lower rates of reported non-compliance.

**Conclusion:** Reported non-compliance with a 3-month MSM deferral and the disclosure of other HIV behavioural risk factors was low. These results warrant the investigation of behavioural donor risk assessment approaches to further improve the inclusiveness of blood donation.

KEYWORDS
blood collection, blood safety, donors, men who have sex with men, transfusion-transmissible infections

## Highlights

- Reported non-compliance with a 3-month deferral for male-to-male sex and other HIV risk factors was extremely low among blood donors in Québec, Canada.
- Other risk factors for HIV were associated with similar or lower reported non-compliance rates, suggesting an overall high level of compliance.
- These reassuring results pave the way to the evaluation of an individualized donor risk assessment approach to further improve the inclusiveness of blood donation.

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# INTRODUCTION

The 1980s HIV epidemic prompted regulators to implement a lifetime deferral for all male blood donors who had ≥1 male-to-male sexual contact since 1977. Since then, improvements in the sensitivity of diagnostic tests have shortened the window period for HIV and other infections [1], which justified the relaxation of this criterion in most developed countries [2]. In Canada, the deferral for men who have sex with men (MSM) has been progressively shortened from a lifetime deferral to a 5-year (2013), 1-year (2016) and 3-month (2019) deferral, with virtually no impact on incident HIV cases [3, 4].

Regardless of their duration, deferrals for HIV risk factors can only be effective if donors disclose this information pre-donation. Non-compliance with MSM deferrals has been observed in 0.67%–2.61% of male donors for a lifetime deferral [3, 5, 6], 0.44% for a 5-year deferral [3] and 0.23%–0.73% for a 1-year deferral [4, 7–9]. Studies in Australia and France showed that most male donors found positive for HIV did not comply with deferral policies [10, 11].

In Canada (outside Québec), shortening the MSM deferral to 1 year did not impact donor compliance [3, 4]. However, no studies have evaluated the effect of a 3-month deferral on donor compliance, which is crucial to guiding future policies to improve the inclusiveness of blood donation. Therefore, we assessed donor compliance under the 3-month MSM deferral policy in Québec.

# METHODS

## Data source and study design

On 15 October 2020, donors were invited by e-mail to participate in an online survey. New invitations were sent monthly up until ~4000 males and ~4000 females (margin of error = 0.19%, assumed non-compliance rate = 0.40%) participated. New invitations were only sent to donors who gave blood ≤3 months. Sex and donor type were the only characteristics that influenced which donors were contacted.

Donors were informed that the survey aimed to ‘better understand the history of donors [through a] study on the criteria for qualifying for blood donation’ (see Appendix S1 for survey questions). Only donors who confirmed understanding the voluntary and anonymous nature of the survey — and their responses would not be linked to their donor record — were allowed to participate.

Survey questions were those in the pre-donation questionnaire for routine blood donation. Because certain questions were sex specific, a male- and female-specific questionnaire was developed. Participants were assigned the sex-specific questionnaire consistent with their sex in Héma-Québec's database (rather than their response to the survey's question on sex). Participants were not required to answer all questions; unanswered questions were categorized as ‘No response’. This study was approved by Héma-Québec's ethics review board.

## Study population

Donors were included if they (1) had a known e-mail address, (2) were aged ≥18 years, (3) donated a blood-derived product for an allogeneic medical use ≤3 months, (4) had no active deferral; (5) lived in Québec and (6) were not recently solicited by Héma-Québec for another survey.

## Study outcome

This report focuses on HIV behavioural risk factors, including substance use and other at-risk sexual behaviours that disqualify for blood donation. Reported non-compliance was defined as the proportion of donors whose responses to survey questions differed from those given at the time of their last donation. Reported non-compliance rates were weighted based on age, sex and donor type (first-time vs. repeat donor) to better represent the population of

**TABLE 1** Participant characteristics

| | Included donors ($N$ = 7113) $N$ (%) |
|---|---|
| Age | |
| 18–19 years | 200 (2.8%) |
| 20–24 years | 430 (6.0%) |
| 25–29 years | 483 (6.8%) |
| 30–39 years | 853 (12.0%) |
| 40–49 years | 1089 (15.3%) |
| 50–59 years | 1426 (20.0%) |
| ≥60 years | 2399 (33.7%) |
| No response | 233 (3.3%) |
| Sex[a] | |
| Male | 3181 (44.7%) |
| Female | 3624 (50.9%) |
| No response | 308 (4.3%) |
| Highest level of education | |
| Elementary school | 31 (0.4%) |
| High school or equivalent | 1613 (22.7%) |
| College (i.e. CÉGEP) | 2654 (37.3%) |
| Bachelor's degree | 1902 (26.7%) |
| Masters or doctorate degree | 844 (11.9%) |
| No response | 69 (1.0%) |
| Donor type | |
| Repeat donor | 6710 (94.3%) |
| First-time donor | 390 (5.5%) |
| No response | 13 (0.2%) |

Abbreviation: CEGEP, Collège d'enseignement général et professionnel.
[a]Self-reported by participants.

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**TABLE 2** Weighted[a] rates of HIV behavioural risk factors reported by participants

| Risk factor | Ever | | ≤12 months | | ≤6 months | | ≤4 months | | ≤3 months | | No response | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *N* | %[a] (95% CI) | *N* | %[a] (95% CI) | *N* | %[a] (95% CI) | *N* | %[a] (95% CI) | *N* | %[a] (95% CI) | *N* | %[a] (95% CI) |
| Among males (*N* = 3347)[b] | | | | | | | | | | | | |
| Male-to-male sexual contact[c] | 67 | 2.42 (1.89–2.94) | 18 | 0.84 (0.53–1.15) | 10 | 0.43 (0.21–0.66) | - | | **6** | **0.27 (0.09–0.44)** | 10 | 0.25 (0.08–0.42) |
| Among females (*N* = 3766)[b] | | | | | | | | | | | | |
| Sex with a man who had male-to-male sex ≤12 months[d] | 27 | 0.81 (0.53–1.09) | 7 | 0.23 (0.08–0.38) | 5 | 0.16 (0.03–0.29) | - | | **2** | **0.06 (0.00–0.13)** | 19 | 0.46 (0.25–0.67) |
| Overall sample (*N* = 7113) | | | | | | | | | | | | |
| Sex with a prostitute[e] | 146 | 2.01 (1.68–2.33) | **4** | **0.08 (0.01–0.14)** | 3 | 0.05 (0.00–0.10) | - | | 2 | 0.03 (0.00–0.07) | 57 | 0.65 (0.46–0.84) |
| Sex with HIV-positive partner[f] | 5 | 0.07 (0.01–0.14) | **1** | **0.02 (0.00–0.05)** | 1 | 0.02 (0.00–0.10) | - | | 1 | 0.02 (0.00–0.05) | 73 | 0.88 (0.66–1.09) |
| Received cash or drugs for sex[g] | 10 | 0.17 (0.07–0.26) | **0** | - | 0 | - | - | | 0 | - | 41 | 0.53 (0.36–0.69) |
| Imprisoned for >48 h[h] | 26 | 0.32 (0.19–0.45) | **0** | - | 0 | - | - | | 0 | - | 29 | 0.36 (0.22–0.49) |
| Sex with injection drug user[i] | 39 | 0.59 (0.41–0.61) | **3** | **0.05 (0.00–0.11)** | 0 | - | - | | 0 | - | 79 | 0.95 (0.73–1.18) |
| Use of non-prescribed injection drugs[j] | **2** | **0.03 (0.00–0.06)** | 0 | - | 0 | - | - | | 0 | - | 31 | 0.41 (0.26–0.57) |
| Received PrEP or PEP for HIV[k] | | - | | - | | - | **0** | 0.00% | | - | 54 | 0.77 (0.57–0.98) |
| STD diagnosis[l,m,n] | 761 | 10.99 (10.27–11.7) | 34 | 0.55 (0.38–0.72) | 20 | 0.30 (0.17–0.43) | - | | 13 | 0.20 (0.10–0.31) | 35 | 0.42 (0.27–0.57) |

*Note*: Bold values indicate non-compliance rates with current donor selection criteria.

Abbreviations: CI, confidence interval; HIV, human immunodeficiency virus; PEP, post-exposure prophylaxis; PrEP, pre-exposure prophylaxis; STD, sexually transmitted disease.

[a]Rates were weighted based on age, sex and donor type (first-time vs. repeat).

[b]Participants were assigned the sex-specific questionnaire consistent with their sex reported in Héma-Québec's database (rather than their response to the survey's question on sex).

[c]Male participants were asked the following question: 'Have you ever had sex with another man, even once?'

[d]Female participants were asked the following question: 'Have you ever had a sexual contact with a man who, in the last 12 months, has had a sexual contact with a man?'

[e]All participants were asked the following question: 'Have you ever had a sexual contact with a prostitute, even once?'

[f]All participants were asked the following question: 'Have you ever had sex with an HIV-positive partner?'

[g]All participants were asked the following question: 'Have you ever received money or drugs in exchange for sex?'

[h]All participants were asked the following question: 'Have you ever been in prison for more than 48 consecutive hours?'

[i]All participants were asked the following question: 'To your knowledge, have you ever had a sexual contact with someone who has used intravenous street drugs, even once?'

[j]All participants were asked the following question: 'Have you ever taken illegal drugs by injection (e.g. heroin, crack, heroin, methamphetamine)?'

[k]All participants were asked the following question: 'In the past 4 months, have you taken medications to prevent HIV infection such as pre-exposure prophylaxis (PrEP) or post-exposure prophylaxis (PEP)?'

[l]All participants were asked the following question: 'Have you ever had a sexually transmitted and blood-borne infection (STBBI) (e.g. gonorrhoea, syphilis, genital herpes, chlamydia, human papillomavirus)?'

[m]Since deferrals pertaining to STDs vary based on the underlying infection (see note 'd' for details), no figures are bolded for this indicator.

[n]Donors who tested positive for HIV or syphilis are permanently deferred. Those who tested positive for genital herpes are deferred until symptoms resolve. Those who tested positive for chlamydia are deferred for 7 days after the end of antibiotic treatment. Those who tested positive for gonorrhoea are deferred for 12 months. Those with human papilloma virus are not subject to any exclusion criteria.

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whole blood donors who donated between 14 July 2020 and 30 November 2020. Data and code are available upon request.

## RESULTS

Overall, 21,918 donors were invited to participate ≤3 months after their last donation. Of these, 7261 (33.13%) responded to the invitation, and 7113 (32.45%) completed the questionnaire.

Most participants were aged ≥50 years (53.77%; no response = 3.28%). A slight majority were female (50.95%; no response = 4.33%; Table 1). Most had at least some post-secondary education (75.92%; no response = 0.97%). Furthermore, 94.33% of them were repeat donors (no response = 0.18%). On a scale of 1–5 (5 = comfortable), 92.49% rated their level of comfort at 4 or 5 (no response = 3.57%).

Among male participants, six (0.27% [95% CI = 0.09%–0.44%]) were reportedly not compliant with the 3-month MSM deferral (no response = 0.25%; Table 2). Among female participants, two (0.06% [95% CI = 0.00%–0.13%]) were reportedly not compliant with a 3-month deferral for sexual contact with a man who had an MSM contact ≤12 months (no response = 0.46%).

In the overall sample, reported non-compliance with other HIV behavioural risk factors was rare: The highest non-compliance rate (excluding that for the 3-month MSM deferral) was for sexual contact with a prostitute ≤12 months, which was reported by four (0.05% [95% CI = 0.00%–0.11%]) participants (no response = 0.65%). Only 34 (0.55% [95% CI = 0.38%–0.72%]) participants reported receiving ≥1 diagnosis of a sexually transmitted disease ≤12 months (no response = 0.42%).

## DISCUSSION

Because current screening tests may fail to detect recently acquired infections (i.e. owing to the window period), donor compliance with pre-donation questions is paramount to ensure blood safety. In this study, only 0.27% of male donors reported not complying with a 3-month MSM deferral. Similarly, only 0.06% of female donors reported not complying with a 3-month deferral for sexual contact with another man who had an MSM relationship ≤12 months. Other risk factors for HIV exhibited similar or lower non-compliance rates, suggesting an overall high level of compliance. Taken together, these data build on the existing literature suggesting that a 3-month MSM deferral period bears minimal or even no additional risk for donor compliance.

To the best of our knowledge, this study is the first to report compliance with a 3-month deferral for MSM donors. The 0.27% reported non-compliance rate is largely similar to that observed in Canada (outside Québec) for a lifetime deferral (0.67%), a 5-year deferral (0.44%) and a 1-year deferral (0.26%) [3, 4]. This rate is also at the lower end of those reported outside of Canada for a 1-year deferral (range: 0.23% [Australia] to 0.73% [France]) [7–9] and a lifetime deferral (range: 0.7%–1.4% [Netherlands] to 2.61% [United States]) [5, 6]. Of note, studies that assessed non-compliance over longer deferral periods (e.g. >1 year) are likely prone to a more significant recall bias [12], so that the difference between the current MSM non-compliance rate and those previously reported may be underestimated.

Although not assessed in our study, many factors can explain the low non-compliance rate. Donors' perception of MSM deferral as unfair [13, 14] may be alleviated with less restrictive exclusion criteria and hence improve compliance. Furthermore, donors may be more inclined to disclose recent at-risk behaviours if selection criteria are consistent with their self-assessment of their blood safety. Indeed, donor responses are often framed by their self-assessment of their blood safety rather than the individual questions [15]. Lastly, we cannot exclude that some undocumented regional factors may also be at play given that this is the first study on donor compliance in Québec.

The low rate of non-compliance complements evidence from a recent modelling study, which suggests that the risk of HIV remains low under a 3-month MSM deferral [16]. Together, this body of evidence suggests new measures may be implemented to further improve the inclusiveness of blood donation. One such measure is to individualize donor risk assessment using gender-neutral, behavioural questions that are not based on sexual orientation. The United Kingdom and Netherlands have recently become the first countries to adopt evidence-based, individualized frameworks to assess donor eligibility. In the United Kingdom, all individuals who have had only one sexual partner ≤3 months are eligible to donate, regardless of their gender and sexual orientation [17]; in the Netherlands, MSM are allowed to donate if they have been in a committed, monogamous relationship for ≥1 year [18]. Although the potential impact of these changes on blood safety remains unknown, several blood banks are evaluating the possibility to adopt a similar policy.

The current study is subject to some limitations. First, selection bias is a limitation inherent to all survey studies. However, the response rate was relatively good (i.e. 32.35%) and falls within the range of those of previous, similar surveys (range: 11.5%–49.7%) [3–5, 7–9]. Second, results may be affected by a social desirability bias, especially given the sensitive nature of many questions. However, online surveys are less prone to social desirability bias than other types of survey [19]. In addition, the first question of the survey consisted in a consent statement which clarified that responses were anonymous and could not impact subsequent donation eligibility, which likely further mitigated the risk of social desirability bias. Third, despite all measures to preserve survey anonymity, some donors might have felt compelled to provide the same responses as those given at their previous donation, in which case non-compliance would be underestimated. Lastly, some questions had relatively high non-response rates. While some donors may have intentionally skipped certain (sensitive) questions, the vast majority of participants reported feeling comfortable answering these questions. Further, non-response rates did not appear to be related to the sensitivity of questions. For example, the highest non-response rates were observed for questions related to donor characteristics (i.e. age and sex).

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In this study of blood donors in Québec, non-compliance with a 3-month MSM deferral and with the disclosure of other HIV behavioural risk factors was low. These reassuring results support the investigation of behavioural donor risk assessment approaches to further improve the inclusiveness of blood donation.

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A.L., Y.G., G.D., E.P., C.V. and C.R. designed the study; A.L. and Y.G. collected the data; A.L. and Y.G. analysed the data, with input from G. D. and E.P. C.T., C.M. and C.R. helped interpret the results; A.L. drafted the manuscript; Y.G., G.D., C.T., C.M., E.P. and C.R. critically revised it for important intellectual content. All authors approved the final version to be published. Medical writing assistance was provided by Samuel Rochette, an employee of Héma-Québec.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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SHORT REPORT

# Reduced neutralizing antibody potency of COVID-19 convalescent vaccinated plasma against SARS-CoV-2 Omicron variant

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## Abstract

**Background and Objective:** The SARS-CoV-2 Omicron variant displays increased infectiveness as well as mutations resulting in reduced neutralizing activity of antibodies acquired after vaccination or infection involving earlier strains. To assess the ability of vaccinated COVID-19 convalescent plasma (CCP-V) collected before November 2021 to seroneutralize Omicron, we compared neutralizing antibody (nAb) titres of 63 samples against Omicron and earlier B.1 (D614G) strains.

**Methods and Findings:** Relationship between anti-Omicron titres and IgG anti-S1 levels (binding arbitrary unit: BAU/ml) was studied. Although correlated, anti-Omicron titres were significantly lower than anti-B.1 titres (median = 80 [10–1280] vs. 1280 [160–10,240], $p < 0.0001$). Omicron nAb titres and IgG anti-S1 levels were correlated (Spearman's rank correlation coefficient = 0.67). Anti-S1 IgG threshold at 7000 BAU/ml may allow to discard CCP-V without anti-Omicron activity (nAb titre <40). Conversely, only those with highest titres (≥160) had systematically anti-S1 IgG levels >7000 BAU/ml.

**Conclusion:** A fraction of CCP-V collected before November 2021 retains anti-Omicron seroneutralizing activity that may be selected by quantitative anti-IgG assays, but such assays do not easily allow the identification of 'high-titre' CCP-V. However, collecting plasma from vaccinated donors recently infected with Omicron may be the best option to provide optimal CCP-V for immunocompromised patients infected with this variant.

**KEYWORDS**
convalescent plasma, neutralizing antibodies, Omicron, SARS-CoV-2, vaccination

## Highlights

- Omicron neutralizing antibody titres were significantly lower than anti-B.1 titres in vaccinated COVID-19 convalescent plasma (CCP-V) collected before November 2021.
- A fraction of CCP-V collected before November 2021 retains anti-Omicron seroneutralizing activity that may be selected by quantitative anti-IgG assays.
- Providing CCP with potent anti-Omicron activity may require collecting CCP from vaccinated donors who have recovered from an Omicron infection.


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## INTRODUCTION

The emergence and swift spreading of the Omicron variant (B.1.1.529) of the SARS-CoV-2 virus in November 2021 have raised concern due to the high number of mutations (>30) deletions and insertions in its genome when compared to the D614G strain [1]. Most of these mutations are concentrated on functional epitopes of the spike receptor binding domain (RBD), resulting in a significant risk of immune evasion [2]. Furthermore, the infectivity of the Omicron has been estimated to be 13 and 2.8 times higher than that of the original and delta strains, respectively [3]. Accordingly, the number of Omicron infections has skyrocketed worldwide since the end of 2021. In France, at the end of January 2022, more than 95% of new infections were caused by Omicron [4]. COVID-19 convalescent plasma (CCP), notably high-titre CCP may improve clinical outcomes, in particular when administered to high-risk patients early after symptoms onset [5], as well as to immunosuppressed patients throughout their disease [6, 7]. Assessing the Ab-mediated neutralizing activity in CCP collected prior to the Omicron wave is critical when considering the use of such CCP to treat patients infected with the Omicron variant.

## MATERIALS AND METHODS

### CCP donors

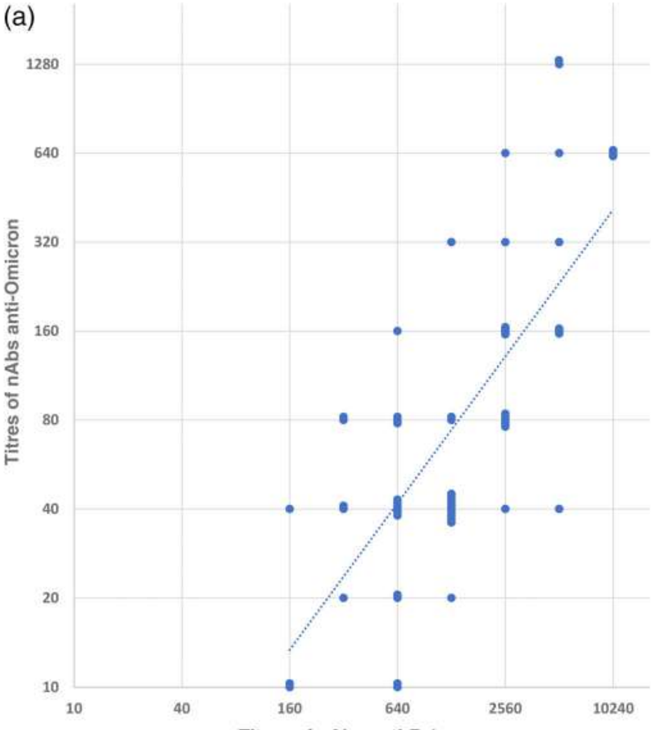
The strategy for selecting CCP donors has been described recently [8]. The sample panel was drawn from 63 plasmas collected between 10 June and 21 September 2021 from donors (sex ratio M/F = 2.15; mean age = 41.2 years old [19–65]) who had been infected with SARS-CoV-2 and subsequently vaccinated (CCP-V). All had a complete vaccination schedule (at the time, one dose of vaccine for individuals with a history of SARS-CoV-2 infection) and none had received a booster dose. The mean time from clinical onset to donation was 325 days ($n$ = 53, median = 321 days [45–554]). The mean time from vaccination to donation was 76.6 days ($n$ = 63, median = 70 days [13–215]).

### Anti-SARS-CoV-2 IgG testing

Samples were tested for specific anti-SARS-CoV-2 antibodies using three ELISA assays according to the manufacturer's instructions. Anti-S1 IgG screening was performed using the 'ELISA SARS-CoV-2 IgG' Euroimmun test [8], anti-S1 IgG were quantified with the Euroimmun Anti-SARS-CoV-2 QuantiVac ELISA (IgG) assay and IgG antibodies targeting the nucleocapsid (anti-N) were tested by the Euroimmun SARS-CoV-2-NCP (IgG) ELISA.

### Seroneutralization testing

Neutralizing antibodies (nAbs) were detected using a virus neutralization test (VNT) as previously described [9]. We used VeroE6 cells expressing the human transmembrane serine protease 2 (TMPRSS2) cultured in 96-well microplates, 100 TCID50 of SARS-CoV-2 and serial dilutions of plasma (1/10 to 1/20,480). Virus strains included the ancestral D614G BavPat1 European strain (B.1 lineage) and a French clinical strain of Omicron. Specimens with a VNT titre ≥40 were considered positive for both strains.



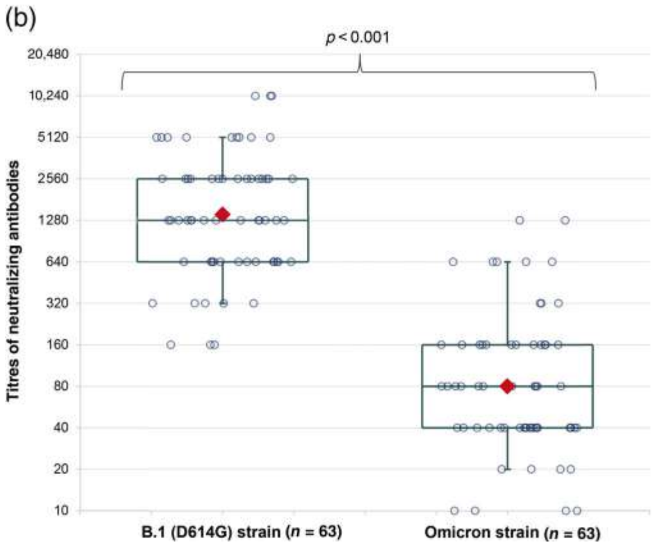




**FIGURE 1** Neutralizing activity of vaccinated COVID-19 convalescent plasma (CCP-V) antibodies against B.1 and Omicron strains. (a) Titres correlation between both strains. Titres are expressed in log2 scale, and the power trend line is provided ($y = 0.2029x^{0.8248}$, $R^2 = 0.54$). (b) Distribution of titres (in log2 scale) against B.1 and Omicron strain. The boxes represent the median and interquartile range. The whiskers represent the 10th and 90th percentiles. The red diamonds indicate the geometric mean

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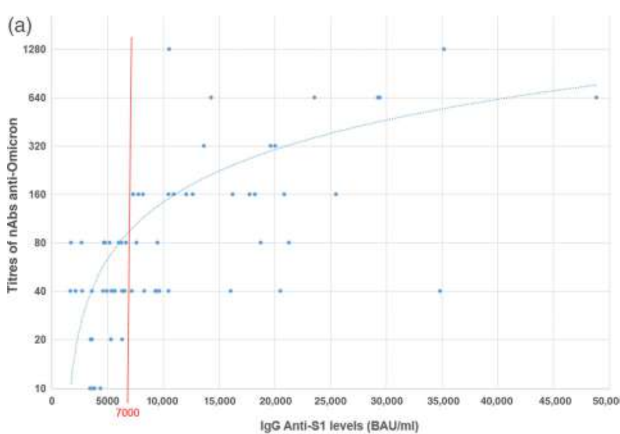
**TABLE 1** Titres in CCP-V assessed by VNT for B.1 and Omicron strains and quantitative levels of anti-S-1 antibodies: geometric mean, median and range

| | | Minimum | Maximum | Geometric mean | Median | |
|---|---|---|---|---|---|---|
| All samples ($n = 63$) | B.1 VNT titre | 160 | 10,240 | 1413 | 1280 | |
| | Omicron VNT titre | 10 | 1280 | 80 | 80 | |
| | IgG level (BAU/ml) | 1699 | 48,854 | 8548 | 7782 | |
| IgG anti-N Abs positive ($n = 20$) | B.1 VNT titre | 160 | 10,240 | 2009 | 2560 | |
| | Omicron VNT titre | 10 | 1280 | 149 | 160 | |
| | IgG level (BAU/ml) | 2657 | 34,851 | 12,070 | 13,157 | |
| IgG anti-N Abs negative or indeterminate ($n = 43$) | B.1 VNT titre | 160 | 10,240 | 1200 | 1280 | $p = 0.004$ |
| | Omicron VNT titre | 10 | 1280 | 60 | 40 | |
| | IgG level (BAU/ml) | 1699 | 48,854 | 7281 | 6557 | $p = 0.015$ |
| Vaccination <60 days ($n = 27$) | B.1 VNT titre | 320 | 10,240 | 1930 | 2560 | |
| Median = 44 days (13–57) | Omicron VNT titre | 20 | 1280 | 124 | 80 | |
| | IgG level (BAU/ml) | 2124 | 48,854 | 11,872 | 10,540 | $p = 0.012$ |
| Vaccination >60 days ($n = 36$) | B.1 VNT titre | 160 | 5120 | 1119 | 1280 | |
| Median = 99 days (61–215) | Omicron VNT titre | 10 | 1280 | 58 | 40 | |
| | IgG level (BAU/ml) | 1699 | 35,209 | 6682 | 6312 | $p = 0.004$ |

*Note*: Median values in subpopulations were compared using the Wilcoxon test.
Abbreviations: Abs, antibodies; CCP-V, vaccinated COVID-19 convalescent plasma; VNT, virus neutralization test.



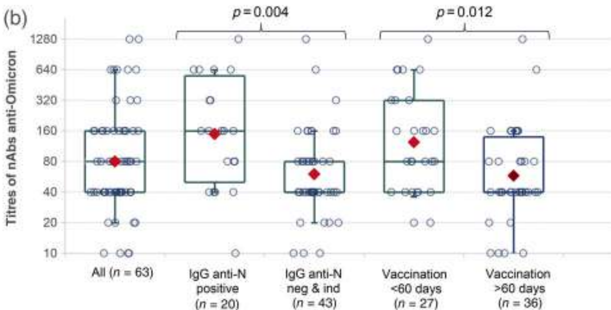




**FIGURE 2** Distribution of SARS-CoV-2 anti-Omicron neutralizing antibody (nAb) titres (log2 scale). (a) According to the IgG anti-S1 levels (BAU/ml), trend curve is provided ($y = 0.016x - 16.563$, $R^2 = 0.3364$); (b) according to the presence of IgG anti-N and a delay < or ≥60 days between vaccination and plasma donation. The boxes represent the median and interquartile range. The whiskers represent the 10th and 90th percentiles. The red diamonds indicate the geometric mean

## Statistical analysis

Paired data (VNT titres against B.1 and Omicron strains) were compared using the Wilcoxon signed-rank test, and Spearman's rank correlation coefficients were calculated between titres against Omicron and titres against B.1, anti-SARS-CoV-2 IgG anti-S1 and time since vaccination. Other comparisons were made using the Wilcoxon test. Statistical analyses were conducted in SAS, version 9.4 (SAS Institute, Cary, NC). The cut-off for significant difference was $p < 0.05$.

# RESULTS

## Neutralizing activity of anti-SARS-CoV-2 Abs

The nAb titres against Omicron and against B1 were correlated (Spearman test = 0.73, $p > 0.0001$, Figure 1a). However, serum neutralization titres of CCP-V were significantly ($p < 0.0001$) lower against the Omicron strain (median = 80 [10–1280]) than against the B.1 strain (median = 1280 [160–10,240]) (Table 1, Figure 1b), corresponding to a mean 4 log2 reduction. Notably, 8 (12.7%) of the 63 tested samples had no anti-Omicron neutralizing activity (titre <40), although they neutralized B.1 strain at titres ranging from 160 to 1280. Among the 55 samples with anti-Omicron neutralizing activity (titre ≥40), 22 (40%) had titres ≥160, the lowest anti-B.1 titre observed in the studied panel. The highest nAb titres were 1280 and 10,240 for Omicron and B.1 strains, respectively.

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## Association between anti-Omicron titres and anti-SARS-CoV-2 IgG

Anti-S1 IgG levels of CCP-V averaged 11,480 BAU/ml [1699–48,-854 BAU/ml]. Anti-Omicron titres and quantitative anti-S1 IgG were correlated as depicted in Figure 2a (Spearman's rank correlation coefficient = 0.67, $p < 0.001$). The eight samples with no anti-Omicron seroneutralizing activity (titre <40) ranged from 3447 to 6290 BAU/ml. A receiver operating characteristic (ROC) plot analysis conducted to predict anti-Omicron titres ≥40 from anti-S1 IgG levels revealed a ROC plot area under curve at 0.8636. Using the Youden index, the best threshold was at ≥6300 BAU/ml (sensitivity, 39/55 = 0.71%; specificity, 8/8 = 100%). Considering a 7000 BAU/ml threshold, we observed that all samples ($n = 28$) with more than 7000 BAU/ml exhibited anti-Omicron nAb titres ≥40, but above this threshold, the distribution of titres was heterogeneous with a poor correlation between BAU levels and nAb titres. Only samples with anti-Omicron titres ≥160 ($n = 22$) (160: $n = 12$; 320: $n = 3$; 640: $n = 5$ and 1280: $n = 2$) had systematically IgG anti-S1 levels >7000 BAU/ml. Below 7000 BAU/ml, 20 samples had anti-Omicron nAb titres between 40 and 80.

In addition, median anti-Omicron nAb titre was significantly higher ($p = 0.004$) in anti-N positive samples (median = 160, $n = 20$) than in anti-N negatives or indeterminates (median = 40, $n = 43$) (Table 1, Figure 2b).

## Association between anti-Omicron titres and time between vaccination and donation

We observed a weak negative correlation between anti-Omicron titres and the number of days between vaccination and plasma donation (Spearman's rank correlation coefficient = −0.38, $p = 0.002$). Anti-Omicron nAb titres were significantly ($p = 0.012$) higher among donors who reported a vaccination <60 days prior to donation (median titre = 80) versus those with a vaccination delay >60 days prior to donation (median titre = 40) (Table 1, Figure 2b). The anti-S1 IgG levels of donors vaccinated less than 60 days prior to donation were significantly higher ($p = 0.004$) than those vaccinated more than 60 days prior to donation (Table 1).

## DISCUSSION

The impact of the mutations observed in the genome of the SARS-CoV-2 Omicron variant, especially those described in the RBD of the spike viral protein, and its increased contagiousness compared to the previous circulating strains required a re-evaluation of the anti-SARS-CoV-2 potency of available vaccines and therapeutic antibodies to treat patients with COVID-19. It has been reported that most available therapeutic antibodies lose their activity against the Omicron variant [10, 11]. Moreover, there was a substantial decrease in neutralizing titres after primary course vaccination with some vaccinated exhibiting undetectable neutralization activity against Omicron [12]. Similar findings were reported in convalescent patients infected before the Omicron wave [10, 13]. However, individuals who had received a booster vaccination [10, 13–15] or who have been vaccinated after being previously infected [13, 16, 17] exhibited lesser decreases of anti-Omicron titres when compared to titres in individuals after two vaccines doses or in non-vaccinated convalescent patients. As therapeutic resources are limited to treat patients infected with Omicron, availability of CCP active against this variant may provide a therapeutic solution in immunocompromised patients, notably B-cell depleted patients [6, 7, 18, 19].

Anti-Omicron neutralizing activity of CCP-V collected prior to the circulation of the Omicron variant was found significantly diminished (by approximately a factor of 10) compared to the wild-type strain. This is in agreement with recently reported 8.4- to 53-fold decrease of anti-Omicron neutralizing activity when compared to the D614G strain, including in vaccinated convalescent individuals [13, 20, 21].

Therefore, the selection criteria for CCP should be revised. Our data suggest that an anti-S1 IgG threshold set at 7000 BAU/ml may help to discard some samples without anti-Omicron activity (all samples with titres <40 had less than 7000 BAU/ml). Conversely, among samples with anti-S1 IgG levels >7000 BAU/ml, anti-Omicron activity was present with nAb titres ranging from 40 to 1280. In our experience, only those with titre ≥160 had systematically anti-S1 IgG levels >7000 BAU/ml. Overall, despite a correlation between anti-Omicron nAb titres and anti-S1 IgG levels (Figure 2a), an anti-S1 IgG threshold with a strong predictive value for selecting plasmas with high anti-Omicron nAb titres remain difficult to identify. In addition, although anti-Omicron titres were overall higher in anti-N positive individuals or in those who have been vaccinated within the 60 days prior to donation, these parameters could not be used to complement biological selection in order to discriminate CCP-V with or without anti-Omicron activity. Our study had some limitations. First, the number of tested CCP-V was limited and they were likely not fully representative of all collected CCP-V. Secondly, we have considered a seroneutralization titre of ≥40 as reflecting significant anti-Omicron activity in vitro, but this may not be the case in vivo after transfusion. Careful assessment of potential relationships between in vitro assessment of CCP-V potency, volume of plasma transfused, patient status and clinical outcome is of paramount importance. CCP-V serological screening using quantitative assays calibrated to international standards should be implemented to allow for comparability between studies. While detection of nAbs to Omicron remains the golden standard to quantify Ab-mediated activity, it remains tedious and unsuitable for large series. Therefore, the development of alternative methods such as anti-RBD serological tests specific to the Omicron variant should be encouraged. Neutralizing activity of CCP-V must be verified upon emergence of SARS-CoV-2 variants with high propensity to escape the immune response, while taking into account booster(s) vaccine that may enhance CCP-V potency.

In conclusion, despite the increased seroneutralization titres and widened immune spectrum provided by the vaccination of convalescent donors [22], the activity of the nAbs contained in currently available CCP-V is considerably diminished against the Omicron variant. Only the fraction CCP-V with the highest anti-B.1 nAb titres or

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anti-S1 levels (BAU/ml) retain anti-Omicron seroneutralization activity. It is therefore necessary to evolve the biological and clinical selection criteria to ensure the most appropriate production of CCP for evaluating the treatment of immunocompromised patients infected with the Omicron variant. Providing CCP with potent anti-Omicron activity may require collecting CCP from vaccinated donors having recovered from an Omicron infection.

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S.L.C., C.I., N.B., B.P., A.A. and E.N. performed the research; X.L. and P.G. wrote the first draft of the manuscript; S.L., P.R. and P.M. designed the research study; P.G. and L.M. acquired and analysed the data; S.L., X.L., P.M. and P.T. supervised the research and reviewed the manuscript. The authors thank the blood donors for their motivation to contribute to the CCP collection, and the EFS medical staff involved in this project from blood collection to plasma supply management.

## CONFLICT OF INTEREST

C.I., P.G., S.L., S.L.C., N.B., L.M., P.R., P.M. and P.T. are employed by the French transfusion public service (Etablissement Français du Sang) in charge of the manufacturing and issuing of blood components in France.

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## CORRIGENDUM

We would like to draw the reader's attention to an error in the following publication:

Abstracts of the 37th International Congress of the ISBT, Virtual meeting, 4–8 June 2022. VoxSang. 2022.;117(Suppl 1):6–271. https://onlinelibrary.wiley.com/doi/full/10.1111/vox.13285

- The word cobas® in the abstract on page 151 with title "P118 | COBAS® MPX performance equivalency between the COBAS® 5800 system and COBAS® 6800/8800 systems" has been changed to lowercase.

- The word cobas® in the abstract on page 152 with title "P119 | Performance comparison of COBAS e 801, Alinity s, and Alinity i serological screening parameters" has been changed to lowercase.

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DOI: 10.1111/vox.13148

# DIARY OF EVENTS

| See also https://www.isbtweb.org/events/hvwebinars.html | |
|---|---|
| 18.08.2022 | Haemovigilance Webinars: Sharing international haemovigilance experiences. Session 1: International Programs |
| 25.08.2022 | Haemovigilance Webinars: Sharing international haemovigilance experiences. Session 2: Consumer Engagement |
| 01.09.2022 | Haemovigilance Webinars: Sharing international haemovigilance experiences. Session 3: Data |
| 06.09.2022 | Haemovigilance Webinars: Sharing international haemovigilance experiences. Session 4: Donors |


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