ISSN 0042-9007 (Print) ISSN 1423-0410 (Online)

Volume 116 Number 9 **OCTOBER 2021**

# VoxSanguinis

The International Journal of Transfusion Medicine

## In this issue



ISBT International Society of Blood Transfusion

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# Vox Sanguinis

International Journal of Blood Transfusion

**Official Journal of the International Society of Blood Transfusion**

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*VOX SANGUINIS*, (Online ISSN: 1423-0410 Print ISSN: 0042-9007) is published monthly except in March, June, September, & December. US mailing agent: Mercury Media Processing, LLC, 1850 Elizabeth Avenue, Suite #C, Rahway, NJ 07065 USA. Periodical postage paid at Rahway, NJ. Postmaster: Send all address changes to *VOX SANGUINIS*, John Wiley & Sons Inc., C/O The Sheridan Press, PO Box 465, Hanover, PA 17331 USA.

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Printed in Singapore by C.O.S. Printers Pte Ltd.

*Vox Sanguinis* (2021) **116**, 933–1020


116/9/2021

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The International Journal of Transfusion Medicine
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Vox Sanguinis (2021)

COMMENTARY


DOI: 10.1111/vox.13091

# COVID-19 convalescent plasma therapy: hit fast, hit hard!

**Daniele Focosi[1] & Massimo Franchini[2]**

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COVID-19 convalescent plasma (CCP) is an investigational treatment for SARS-CoV-2 infection. Several lines of evidence, ranging from expanded access programmes (EAP) to clinical trials employing randomized controls (RCT) (summarized in Table 1) or propensity score-matched (PSM) controls (summarized in Table 2), are now indicating how CCP should be used. Such evidence is supporting the initiation of CCP treatment as early as 44-72 h after hospitalization (or anyway within 7 days from the onset of symptoms) and the use of CCP units with a high neutralizing antibody (nAb) titre. There seems to be no clinical benefit if the CCP units are administered later or with a low nAb titre.

## Why you should treat fast

Timeliness of treatment can be defined in various ways: median duration of symptoms before randomization or transfusion, time between hospital admission and transfusion and time between final diagnosis and transfusion, or can be inferred from the disease stage.

The rationale for administering CCP as early as possible lies in the neutralization stoichiometry itself. The more actively replicating virions there are within the body, the higher the nAb dose needs to be to neutralize them all.

At the very beginning, many historically or internally controlled phase II studies showed clinical benefit from CCP. The largest of them is likely the one by Joyner *et al.*, who showed, in a post hoc analysis from the US open-label EAP (NCT04338360), that 7-day mortality in non-intubated patients younger than 80 years of age and treated within 72 h after diagnosis was 6·3% in those receiving high-titre CCP and 11·3% in those receiving low-titre CCP [1]. Of the 3,082 patients included in a later analysis, death within 30 days after CCP transfusion occurred in 22·3% in the high-titre group, 27·4% in the medium-titre group and 29·6% in the low-titre group; no effect of CCP titre on the risk of death was observed among patients who had received mechanical ventilation [2].

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In a post hoc subgroup analysis of 35,322 transfused patients from the Mayo Clinic (including 52·3% in the intensive care unit and 27·5% receiving mechanical ventilation), the 7-day mortality rate was 8·7% in patients transfused within 3 days of diagnosis but 11·9% in patients transfused ≥ 4 days after diagnosis. Similar findings were observed in 30-day mortality (21·6% vs. 26·7%) in the US EAP [3]. Unfortunately, the main bias of those studies is that controls were neither randomized nor PSM; hence, differences in the treatment outcome between treated and untreated groups may have been caused by a factor that predicted treatment rather than by the treatment itself.

PSM studies balance treatment and control groups on a large number of covariates without losing a large number of observations. In two retrospective PSM studies from two different hospitals in New York, trends for improved outcomes were observed in non-intubated patients and in those treated within 7 days of hospitalization (hazard ratio, 0·33) [4,5]. These findings were later confirmed in a prospective PSM study from Houston [6,7]. Of interest, a retrospective PSM study from Providence in which patients were treated at a median of 7 days after onset of symptoms did no show any benefit [8].

Since PSM only accounts for observed (and observable) covariates and not latent characteristics, RCTs remain the gold standard for highest level evidence (Table 1). In the PlasmAr RCT, the primary and secondary outcomes in the small number of early arrivals (within 72 h) were better in the CCP arm (n = 28) than in the placebo arm (n = 11), but the minimal contribution of this group to the overall cohort (228 CCP and 105 placebo) made the advantage disappear in the final outcomes at day 30 [9]. In another Argentinean RCT on 160 patients older than 65 years of age with mild COVID-19 who were treated with CCP within 72 h, progression to severe COVID-19 was halved at day 30 [10]. In another RCT from India, patients younger than 67 years of age treated at a median of 4 days after hospital admission showed superior mitigation of hypoxia and survival in the CCP arm [11]. Another RCT in Spain enrolling patients at less than 7 days of hospitalization showed benefit [12]. Many more RCTs are ongoing.

**Table 1** Randomized controlled trials of COVID-19 convalescent plasma reported to date

| RCT identifier | Country | Recruitment (out of expected) (randomization strategy) | Control arm components | Median days from symptoms or hospital admission | Baseline recipient WHO score* | Median nAb in CCP units | Median nAb in recipient | Transfused CCP volume (ml) | Outcome | Refs |
|---|---|---|---|---|---|---|---|---|---|---|
| ChiCTR2000029757 | China | 103 (out of 200) (1:1) | BSC | 30 (from symptoms) | 5–6 | not assessed | not assessed | 200 | Reduced mortality at day 28 only in WHO score 5 patients (HR 2·5) | [21] |
| NCT04342182 (ConCOVID) | Netherlands | 86 (out of 426) (1:1) | BSC | 10 (from symptoms) | 5–6 | 1:160 | 1:160 in 79% of recipients | 300 | No benefit at day 15 | [14] |
| CTRI/2020/04/024775 (PLACID) | India | 464 (1:1) | BSC | 6 (from symptoms) | 4–5 | 1:40 | 1:90 | 200 + 200 | No benefit at day 28 | [13] |
| NCT04345523 (ConPlas-19) | Spain | 81 (out of 278, still recruiting) (1:1) | BSC | 8 (from symptoms) | 3 (25%)<br>4 (75%) | 1:292 | not assessed | 250–300 | Reduced mechanical ventilation or death (0% vs. 14%). Mortality rates were 0% vs. 9·3% at days 15 and 29 for the active and control groups, respectively. | [12] |
| NCT04375098 | Chile | 58 (1:1) | late CCP | 6 (from symptoms) | 3-4 | ≥1:160 | 59% <1:160 (16% of patients enrolled before day 5 were ≥1:160 vs. 60% of those enrolled after day 6 | 200 + 200 | NO benefit at day 30 in death, mechanical ventilation or prolonged hospitalization compared to CCP administration only in case of clinical worsening or >7 days after enrolment | [22] |
| NCT04479163 | Argentina | 160 (out of 210) (1:1) | normal saline | ≤3 (from symptoms; and > 65 yrs) | 3 | Not assessed | Not assessed | 250 | Progression to severe COVID-19 halved at day 30 | [10] |
| NCT04383535 (PlasmAr) | Argentina | 333 (2:1) | normal saline | 8 (from symptoms) | 5 | 1:300 $IC_{80}$ | Not assessed | 500 | No benefit at day 30 (16·2% vs. 31·2%) | [9] |
| CTRI/2020/05/025209 | India | 80 (1:1) | BSC | 4·2 (from hospital admission) | 5 | Not assessed | Not assessed | 200 + 200 | Immediate mitigation of hypoxia, reduction in hospital stay as well as survival benefit was recorded in severe COVID-19 patients with ARDS aged less than 67 years | [11] |
| NCT04356534 | Bahrain | 40 (1:1) | BSC | n.a. | 4 (95%)<br>5 (5%) | Not assessed | Not assessed | 200 + 200 | No difference in requirement for ventilation, white blood cell count, LDH, CRP, troponin, ferritin, D-dimer, procalcitonin, mortality rate at 28 days | [23] |
| NCT04346446 | India | 29 (1:1) | FFP | <3 (from symptoms) | 4-5 | not assessed | not assessed | 250 + 250 | Better median improvement in $PaO_2/FiO_2$ at 48-h [42 vs. 231] and at day 7 | [24] |

**Table 1** (Continued)

| RCT identifier | Country | Recruitment (out of expected) (randomization strategy) | Control arm components | Median days from symptoms or hospital admission | Baseline recipient WHO score* | Median nAb in CCP units | Median nAb in recipient | Transfused CCP volume (ml) | Outcome | Refs |
|---|---|---|---|---|---|---|---|---|---|---|
| BKH-CT-012 | Iraq | 49 (1:1) | BSC | <3 (from ICU admission) | 5 | not assessed | not assessed | 400 | Duration of infection reduced by 4 days; mortality 1/21 in CCP arm vs. 8/28 | [25] |

RCT, randomized controlled trial; WHO, World Health Organization; nAb, neutralizing antibodies; CCP, COVID-19 convalescent plasma; Ref, reference; BSC, best supportive care; FFP, fresh-frozen plasma; n.a., not assessed; HR, hazard ratio; ARDS, acute respiratory distress syndrome; LDH, lactate dehydrogenase; CRP, C-reactive protein; $PaO_2/FiO_2$, partial pressure of arterial oxygen to fraction of inspired oxygen ratio; ICU, intensive care unit. 'Not assessed' means that antivirus antibodies were assessed only using high-throughput serology.

*The WHO score [20] ranges from 0 to 8: 0: no clinical or virological evidence of infection; 1: no limitations of activities; 2: limitations of activities; 3: hospitalized, no oxygen therapy; 4: oxygen by mask or nasal prongs; 5: non-invasive ventilation or high-flow oxygen; 6: intubation and mechanical ventilation; 7: ventilation + additional organ support - pressors, renal replacement therapy, extracorporeal membrane oxygenation; and 8: death.

Here below, an alternative layout/adaptation of Table 1.

| RCT identifier [Country] | Recruited (out of expected) [randomization strategy] | Control arm components | Median days from symptoms or hospital admission | Baseline recipient WHO score* | Median nAb in CCP units | Median nAb in recipient | Volume of CCP transfused (ml) | Outcome | Refs |
|---|---|---|---|---|---|---|---|---|---|
| ChiCTR2000029757 [China] | 103 (of 200) [1:1] | BSC | 30 (from symptoms) | 5–6 | n.a. | n.a. | 200 | Reduced mortality at day 28 only in WHO score 5 patients (HR 2·5) | [21] |
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| NCT04375098 [Chile] | 58 [1:1] | late CCP | 6 (from symptoms) | 3–4 | ≥1:160 | 59% <1:160 (16% of patients enrolled before day 5 had ≥1:160 vs. 60% of those enrolled after day 6 | 200 + 200 | No benefit at day 30 in death, mechanical ventilation or prolonged hospitalization compared to CCP administration only in case of clinical worsening or >7 days after enrolment | [22] |

**Table 1** (Continued)

| RCT identifier [Country] | Recruited (out of expected) [randomization strategy] | Control arm components | Median days from symptoms or hospital admission | Baseline recipient WHO score* | Median nAb in CCP units | Median nAb in recipient | Volume of CCP transfused (ml) | Outcome | Refs |
|---|---|---|---|---|---|---|---|---|---|
| NCT04479163 [Argentina] | 160 (of 210) [1:1] | Normal saline | ≤ 3 (from symptoms; and >65 years) | 3 | n.a. | n.a. | 250 | Progression to severe COVID-19 was halved at day 30 | [10] |
| NCT04383535 (PlasmAr) [Argentina] | 333 [2:1] | normal saline | 8 (from symptoms) | 5 | 1:300 $IC_{80}$ | n.a. | 500 | No benefit at day 30 (16·2% vs. 31·2%) | [9] |
| CTRI/2020/05/025209 [India] | 80 [1:1] | BSC | 4·2 (from hospital admission) | 5 | n.a. | n.a. | 200 + 200 | Immediate mitigation of hypoxia, reduction in hospital stay as well as survival benefit in severe COVID-19 patients with ARDS aged < 67 years | [11] |
| NCT04356534 [Bahrain] | 40 [1:1] | BSC | n.a. | 4 (95%)<br>5 (5%) | n.a. | n.a. | 200 + 200 | No difference in requirement for ventilation, white blood cell count, LDH, CRP, troponin, ferritin and D-dimer, procalcitonin, mortality rate at 28 days | [23] |
| NCT04346446 [India] | 29 [1:1] | FFP | <3 (from symptoms) | 4–5 | n.a. | n.a. | 250 + 250 | Better median improvement in $PaO_2/FiO_2$ at 48 h (42 vs. 231) and at day 7 | [24] |
| BKH-CT-012 [Iraq] | 49 [1:1] | BSC | <3 (from ICU admission) | 5 | n.a. | n.a. | 400 | Duration of infection reduced by 4 days; mortality 1/21 in CCP arm vs. 8/28 | [25] |

**Table 2** Propensity score-matched studies reported to date

| Type of study | Country | Patients + control | Median days after hospitalization | Baseline recipient WHO score* | CCP volume transfused (ml) | Statistically significant outcomes | Refs |
|---|---|---|---|---|---|---|---|
| Retrospective | Mount Sinai, NY, USA | 39 + 156 | 4 | 5 (87%)<br>6 (10%) | 250 + 250 | On day 14 oxygen requirements worsened in 17·9% of plasma recipients vs. 28·2% of controls (aOR 0·86). Survival improved in plasma recipients (aHR 0·34) | [5] |
| | Providence, RI, USA | 64 + 177 | >2 (<10 from onset of symptoms: median 7) | 4 (70%)<br>5 (30%) | n.a. (2 units) | No significant differences in incidence of in-hospital mortality (12·5% and 15·8%; aHR 0·93) or overall rate of hospital discharge (RR 1·28, although increased among patients > 65 years) | [8] |
| | Montefiore Medical Center, NY, USA | 90 + 258 | <3 (3–7 days from onset of symptoms) | 5–6 (<24 h mechanical ventilation) | 200 | Anti-S IgG titre ≥ 1:2,430 (median 1:47,385)<br>recipients < 65 years had fourfold lower mortality and fourfold lower deterioration in oxygenation or mortality at day 28 | [4] |
| | Washington, USA | 263 + 263 | n.a. | n.a. | 245 (median) | Reduced 7-day (9·1 vs. 19·8%) and 14-day mortality (14·8 vs. 23·6%), but not 28-day mortality, and longer hospital stay | [26] |
| | China | 163 + 163 | n.a. | n.a. | 300 | Hospital stay in the CCP group was significantly longer than in the matched control group ($P < 0·0001$). | [27] |

**Table 2** (Continued)

| Type of study | Country | Patients + control | Median days after hospitalization | Baseline recipient WHO score* | CCP volume transfused (ml) | Statistically significant outcomes | Refs |
|---|---|---|---|---|---|---|---|
| Prospective | Houston, USA | 136 + 251 | n.a. | 3 (9%)<br>4 (63%)<br>5 (18%)<br>6 (10%)<br>7 (1%) | 300 (1–2 units) | Reduction in mortality within 28 days, specifically in patients transfused < 72 h of admission with CCP with an anti-RBD titre ≥ 1:1350 (i.e. ~80% probability of a live virus *in vitro* neutralization titre of ≥1:160 [28]) | [6] |
| | | 341 + 594 | n.a. | | 300 (1-2 units) | Reduced 28-day (aHR = 2·09 for controls) and 60-day (5·7% vs. 10·7%; aHR = 1·82 for controls) mortality in those transfused with anti-RBD ≥ 1:1350 within 72 h post-hospitalization. Optimal window of 44 h to maximize benefit in 60-day mortality (4% vs. 12·3%). 91% received CCP with an anti-RBD titre ≥ 1:1350. Median S/CO ratio = 24 using Ortho Vitros. | [7] |

None of these studies tittered neutralizing antibodies in either the donors or recipients using the plaque reduction neutralization test.

WHO, World Health Organization; CCP, COVID-19 convalescent plasma; Refs, references; aOR, adjusted odds ratio; aHR, adjusted hazard ratio; RR, relative risk; RBD, receptor binding domain; S/CO, significant cut-off.

*The WHO score [20] ranges from 0 to 8: 0: no clinical or virological evidence of infection; 1: no limitations of activities; 2: limitations of activities; 3: hospitalized, no oxygen therapy; 4: oxygen by mask or nasal prongs; 5: non-invasive ventilation or high-flow oxygen; 6: intubation and mechanical ventilation; 7: ventilation + additional organ support - pressors, renal replacement therapy, extracorporeal membrane oxygenation; and 8: death.

## Why you should treat hard

In the previously mentioned subgroup analysis on the EAP, a gradient of mortality was seen in relation to IgG antibody levels in the transfused CCP: 7-day mortality was 8·9% for patients who received high IgG plasma (>18·45 signal cut-off [S/CO]), 11·6% for recipients of medium IgG plasma (4·62 to 18·45 S/CO) and 13·7% for recipients of low IgG plasma (<4·62 S/CO). This unadjusted dose–response relationship with IgG was also observed in 30-day mortality. The pooled relative risk of mortality among patients transfused with plasma units containing high levels of antibodies was 0·65 for 7 days and 0·77 for 30 days compared to units containing low levels [3].

The lack of utility from low-titre (1:40) CCP in moderate COVID-19 was confirmed by the PLACID trial [13]. Similarly, the ConCOVID RCT proved that CCP units with nAb titres similar to those of the recipients (1:160) were useless [14].

Analysis of published and ongoing trials has also revealed the importance of testing the antiviral activity of CCP units within clinical trials with the standard plaque reduction neutralization test (PRNT) rather than with the surrogate high-throughput serological tests [15]. Considering that the qualitative composition of CCP is due to the nAb titre (the higher, the better), its accurate evaluation is particularly critical and could make the difference between clinical efficacy and inefficacy. Thus, although most trials perform a correlation analysis between PRNT and high-throughput serological assays, in many cases, the CCP units are tested only with the latter tests (44% in the PlasmAr trial [3]), with the risk of an incorrect evaluation of the neutralizing CCP activity. One major cause could be that, despite IgM, IgG and IgA all being capable of mediating neutralization, virus neutralization test titres correlated better with binding levels of IgM and $IgA_1$ than IgG [16], which are the only class routinely measured in high-throughput serological assays. In addition, the quaternary structure of the Spike protein available on infected replication-competent cell lines is poorly replicated by recombinant antigens bound on solid substrates.

For the above reason, in the ongoing Italian RCT TSUNAMI (NCT04393727) nAb titration of CCP is mandatory. Only if and when CCP is formally shown to be an effective treatment within clinical trials, could CCP collection be driven by surrogate high-throughput serology, given the hurdles to PRNT scalability.

Finally, in order to collect CCP units with an adequate nAb titre (≥1:160), CCP should preferentially be collected from older male patients who have recovered from a previous symptomatic COVID-19 that required hospitalization, in accordance with the most recent literature data [17,18].

## What are the hurdles to early treatment?

There are several logistical hurdles to early initiation of CCP treatment. First, during a pandemic, there is massive accrual of severely ill patients to emergency departments, and in collapsed health systems, the turnaround time between emergency room admission and admission to a ward can be relevant. Additionally, in the absence of quick (antigenic or molecular) tests for SARS-CoV-2, the turnaround time for final confirmation of diagnosis with polymerase chain reaction tests, usually run in batches, takes from 5 to 10 h. Then, bureaucracy also takes time when it comes to preparing the papers for recruiting a patient within a clinical trial, and there are challenges associated with outpatient transfusion of known infectious individuals. Finally, ABO-compatible CCP units may not be readily available at the local blood bank, and recruited patients are therefore left on the waiting list. All these variables are likely to affect the efficacy of CCP treatment. We suggest wide deployment of quick tests within emergency departments, where CCP could be safely administered even before the patient reaches the final ward.

As suggested by the recently revised European Commission guidelines on CCP, '*evidence suggests that studies should focus on early transfusion of convalescent plasma with high neutralizing antibody titres*'. [19]. In conclusion, CCP is emerging as a new time-sensitive, life-saving treatment.

## Conflict of interest

We declare we have no conflict of interest to disclose.

## Authors contributions

D.F. designed the paper, analysed the data and wrote the first draft. M.F. revised the final version.

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VoxSanguinis
The International Journal of Transfusion Medicine
ISBT International Society of Blood Transfusion
Vox Sanguinis (2021)

COMMENTARY


DOI: 10.1111/vox.13097

# Preventing transfusion-transmitted malaria in France

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Malaria is an infectious disease caused by an a protozoan (haematozoan) parasite of the genus Plasmodium. In malaria non-endemic countries, transfusion-transmitted malaria (TTM) is most often caused by *Plasmodium falciparum* in recipients of red blood cell components.

In mainland France, autochthonous case of malaria is a very rare event while high number of imported cases are recorded yearly: 5570 in 2019 [1]. Among these imported cases, 86.4% relate to patients' natives – or having been living for long periods – in malaria-endemic countries, especially in sub-Saharan regions; the incriminated parasite is *P. falciparum* in 88.5% cases [1].

Between 1960 and 1986, TTM prevention in France was based solely on a 5-year deferral rule for donors reporting malaria occurrence. Despite this deferral, TTM still occurred with nearly 5 cases/year [2]. In 1986, in addition to a new 4-month deferral for donors arriving or returning from endemic countries, an indirect immunofluorescence antibody test (IFAT) was introduced for all donors having arrived or returned from a malaria-endemic country since less than 3 years. This policy change resulted in a steep decrease in TTM cases to 0.3 cases/year on average [3] (Fig. 1).

Nevertheless, to address the persisting occurrence of rare severe TTM after 1986, policies regarding TTM prevention continued to evolve over time, taking into account additional risk factors and novel laboratory methods to detect at risk donors.

Notably, the occurrence in 2002 of a fatal TTM case [4] caused by *P. falciparum* resulted in further modification of the criteria for serological screening. The donor, originating from Ghana, was living in France since 4 years without no reported travel to Africa. According to policy in force at that time (i.e. no deferral and no testing 3 years after return from a malaria-endemic area), the donation was accepted without specific testing. Subsequent work-up that the donor was an asymptomatic parasite carrier, as evidenced by both microscopy (thick and thin slides examination) and PCR. The antibody (Ab) testing was revealed positive as well.

This observation called to a possible risk that *Plasmodium sp* parasitaemia persists longer than 3 years asymptomatically in a so-called state of immunotolerance [5]. This acknowledged risk led authorities to make an additional policy change to introduce Ab testing upon 1st blood donation by donors native of a malaria-endemic country and/or having lived there more than 6 months, whatever the date of arrival in France (Fig. 1). Despite this novel policy change, additional 3 TTM cases (0,2 case/y) were reported by the French haemovigilance system over the next 15 years.

In 2006, a human error resulted in a lethal TTM case [6]. The donor, born in Ivory Coast, was living in France since 5 years and had travelled to his native country 15 months before the blood donation. The malaria risk was not identified at the medical interview step, and malaria serology was thus not prescribed. PCR and Ab testing on archived plasma sample revealed the presence of a *P. falciparum* parasitaemia and Ab at the time of this blood donation.

The next TTM case yet-reported in France occurred in 2012 [7] and involved a donor native of Benin and living in France since 12 years, with no reported intercurrent travelling to malaria-endemic areas. An Ab test (Captia malaria ELISA, Trinity Biotech) performed on a 1st blood donation was negative. Despite this negative result, transfusion of the involved red blood cells concentrate resulted

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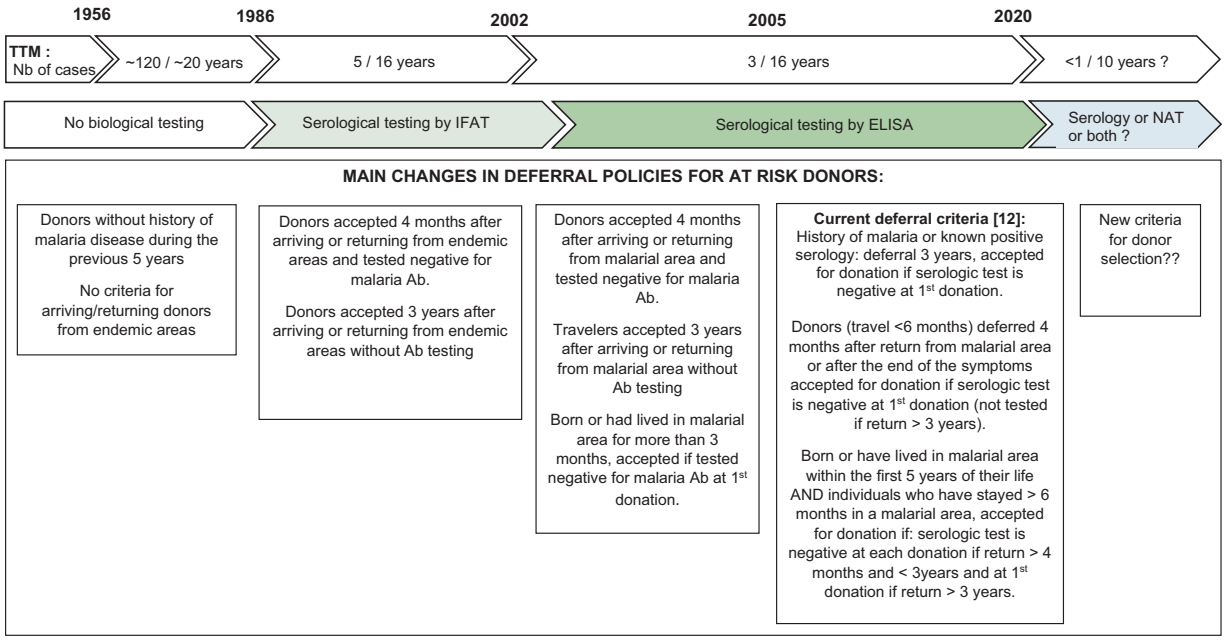


**Fig. 1** Prevention of transfusion-transmitted malaria in France over time. Main changes in blood donor deferral policies, malaria screening and estimates of transfusion-transmitted malaria frequency cases are summarized for different periods from the 1960s to presently.

in a lethal *P. falciparum* infection. Retrospective testing confirmed that the ELISA test at time of donation was negative, though the IFAT was borderline (1/40 on index donation). However, the PCR test was positive for *P. falciparum* with a high cycle threshold (compatible with low parasitaemia).

To our knowledge, such a TTM case in relation with an asymptomatic seronegative blood donor harbouring *P. falciparum* parasitaemia more than 3 years since the last exposure has not been reported yet. This observation could rely on a 'semi-immune' status possibly acquired after repeat exposure to *P. falciparum* during infancy and resulting in a transient dampening of Ab response (likely due to the absence of exposure to recall stimulating antigens). Furthermore, Ab responses to malaria parasites are known to be versatile allowing occasional persistent parasitaemia [8].

The last reported TTM case in France occurred in 2015 [8]. It was caused by *P. malariae,* and the recipient recovered after anti-malarial treatment. The donor, native from the Comorian Islands, had been living in France for more than 3 years with no intercurrent reported travelling to malaria-endemic countries. The Ab testing of the donor at the time of donation was negative (ELISA malaria Lab 21, Biorad, France). Retrospective testing on the same plasma sample was negative for PCR. Archive samples of previous donations were tested negative by ELISA but positive by IFAT. A control sample collected few weeks after index sample was found borderline by Elisa and positive by IFAT (1/160) as well as PCR positive to *P. malariae*.

These four TTM cases were all in relation with donors native to malaria-endemic countries. In the 2002 and 2006 cases, the serological testing would have been efficient to prevent the occurrence of the TTM if prescribed. However, the 2012 and 2015 cases highlight that the current policy may still fail to detect all asymptomatic donors with parasitaemia.

Further optimization of Ab test performances to detect such rare events is challenging as it requires both an increase of sensitivity (to detect very low antibodies titres) and the ability to detect all *Plasmodium* species. Improving the current strategy encompasses the recent new developments in NAT (Nucleic Acid Testing) assays with the high sensitivity detection of total nucleic acid (DNA/RNA) by targeting the Plasmodium 18S rRNA gene on whole blood samples [9].

Indeed, such malaria NAT may be highly contributive for donors with low parasitaemia and negative Abs testing as well for donors infected by species detected only by cross-reactivity when using serological assays. Also, and as recently demonstrated for blood screening of another intra-erythrocytic parasite (Babesia sp), the full automatization of the analytical process allowed for by

NAT testing would allow for its extended use in blood bank laboratories [10].

The main limitation for malaria NAT screening assays is the residual risk that may be associated with insufficient sensitivity when considering the theoretical minimum infectious dose (i.e. 1–10 parasites in a unit of blood) [11]. Consequently, introduction of NAT assay for TTM prevention requires a prior large scale prospective study, in parallel serological testing, to comparatively estimate the frequency of undetected asymptomatic parasitaemic donors with serological and/or molecular assays. Results of such an evaluation, combined with a cost-effectiveness study, will hopefully inform the next steps regarding the prevention of TTM.

Overall, the strategy currently implemented in France combining blood donor interview and serological screening has considerably reduced the frequency of TTM, from 5 cases/year before 1986 to less than 0.2 cases/year in 2019. The current residual TTM risk estimate is of ~1/13 500 000 blood donations.

## Conflict of interests

No conflict of interest to declare.

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VoxSanguinis
The International Journal of Transfusion Medicine
ISBT International Society of Blood Transfusion
*Vox Sanguinis* (2021)

ORIGINAL PAPER


DOI: 10.1111/vox.13100

# An international comparison of anti-SARS-COV-2 assays used for seroprevalence surveys from blood component providers

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**Background and objectives** Access to large pools of healthy adult donors advantageously positions blood component providers to undertake anti-SARS-CoV-2 seroprevalence studies. While numerous seroprevalence reports have been published by blood operators during the COVID-19 pandemic, details on the assay used has not been well documented. The objectives of this study were to evaluate the diversity of assays being used by blood operators and assess how this may affect seroprevalence estimates.

**Materials and methods** We surveyed 49 blood component providers from 39 countries. Questionnaire included information on the number and identity of assays used, the detected immunoglobulin(s) and target antigen, and performance characteristics (sensitivity, specificity).

**Results** Thirty-eight of the 49 contacted blood suppliers provided at least partial responses. The results indicate that 19 commercial and five in-house serology assays have been used by surveyed blood operators. The Abbott SARS-CoV-2 IgG assay was the most commonly used kit and utilized by 15 blood suppliers. Two assays did not detect IgG, but detected either IgM/IgA or IgM. 68·2% of assays targeted the spike protein and 50% the nucleocapsid protein, while 18·2% targeted both viral proteins. The sensitivity and specificity of IgG-specific assays ranged from 71·9% to 100% and from 96·2% to 100%, respectively. As of 18 October 2020, the seroprevalence was below 5% in 10 of 14 countries reporting.

**Conclusion** Our results highlight the diversity of assays being used. Analyses comparing blood donor seroprevalence across countries should consider assay characteristics with optimization of signal/cut-off ratios and consistent methodology to adjust for waning antibody.

*Received: 22 January 2021, revised 25 February 2021, accepted 28 February 2021*

**Key words:** blood component suppliers, blood donors, SARS-CoV-2, seroprevalence, survey.

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## Introduction

With more than 95 million cases and more than 2 million deaths worldwide as of 18 January 2021, the COVID-19 pandemic is by far the most severe global public health crisis of the last 100 years. As treatment is currently limited to supportive care (with the exception of some novel therapies), and since most vaccines are still awaiting regulatory approvals, social distancing, the use of masks during social gatherings, aggressive testing of suspected cases and contact tracing are crucial for limiting the spread of the responsible virus, SARS-CoV-2. Despite strict adherence to social distancing and mask wearing, viral spread can still occur, likely from infected individuals that are asymptomatic or mildly symptomatic [1, 2]. Case data generated from SARS-CoV-2 nucleic acid testing may also be skewed because sampling focusses on outbreaks and contact tracing, or because resources are not available to continue laboratory sampling and/or testing [3]. Thus, measuring the degree of exposure of various populations to the virus through seroprevalence studies is of major importance for determining the level of immunity and the proportion of asymptomatic individuals who have encountered the virus. In fact, several seroprevalence studies published in the past few months revealed that the proportion of the population that has been exposed to the virus is approximately four times greater than the cumulative number of cases confirmed by SARS-CoV-2 nucleic acid amplification testing of respiratory samples and confirmed by national public health authorities [3].

SARS-CoV-2 infection can be detected by either molecular or serological assays. The former detects viral genetic material sampled in the upper and/or lower respiratory tract using real-time reverse–transcriptase PCR (RT-PCR), while the latter reveals the presence of antibodies in blood [4]. From a diagnostic standpoint, RT-PCR has demonstrated superior sensitivity and earlier detection capacity compared to serological assays. In some cases nucleic acid test, results may yield false negatives due to specimen collection timing (e.g. too early or late) or anatomic location of specimen collection [4]. Given that anti-SARS-CoV-2 IgA and IgM antibodies generally appear within the first 7 days after infection while IgG seems to be detectable from 10 days onwards after infection [5], serological assays targeting specific antibodies could be used as a marker of infection. However, since SARS-CoV-2 antibody levels often decline within a 100 days post-infection [6], serological detection of anti-SARS-CoV-2 antibodies (IgA, IgM and IgG) could lose the ability to identify true positives if used as a marker of prior infection. Nonetheless, given the substantial proportion of asymptomatic SARS-CoV-2 infections, as revealed by studies which compared cumulative incidence rates detected by RT-PCR vs. seroprevalence rates [3, 7, 8], the latter could shed light on the 'true' infection prevalence at the population level and informs public health authorities on the degree of exposure of a given population to the virus.

It has only been about 13 months since SARS-CoV-2 emerged, yet numerous commercial and in-house serologic assays have been developed during this interval. In fact, at the time of manuscript submission, more than 60 commercial assays have been approved by the U.S. Food & Drug Administration (FDA) under individual emergency use authorizations [9]. These assays can be classified into two broad categories: qualitative lateral flow immunoassays (LFA) [10] and semi-quantitative enzyme-linked immunosorbent assays (ELISA) [11] and chemiluminescent immunoassays (CLIA) [12]. These assays detect either total or class-specific antibodies (IgM, IgG or IgA). In addition, they recognize different antigen as targets: the nucleocapsid protein, the spike protein or the spike protein receptor-binding domain (RBD). This heterogeneity in assay design leads to variable degrees of sensitivity and specificity. Further to this variability, several systematic reviews have revealed that many assay evaluations are prone to biases as a result of small sample sizes and exclusion of samples from individuals who had experienced asymptomatic or mildly symptomatic COVID-19 [13–16].

Data describing test characteristics continue to be published but it is clear that sensitivity, and specificity vary considerably between assays and with different population. We have previously reported that blood centres around the world are conducting seroprevalence studies to inform public health within their countries [17]. The benefit of these data will be maximized by comparison between countries. Numerous factors will influence the measurement of seroprevalence between countries including timing within the pandemic and donor selection, but variability between assays will be a key consideration.

The aim of this study was to asses the diversity of antibody assays used by blood component suppliers, and to report their seroprevalence estimates, through an e-mail survey.

## Materials and methods

Based on a preliminary survey [17], a list of contacts was compiled from the membership of the International Society of Blood Transfusion (ISBT) Transfusion Transmitted Infectious Diseases Working Party and individuals who volunteered after being contacted by a representative of the European Blood Alliance - Emerging Infectious Disease Monitoring Working Group.

Among the 62 countries who were invited to participate in the first survey, a total of 49 blood component providers (listed in Appendix S1) from 39 countries and six continents which were known for conducting donor seroprevalence studies were contacted by e-mail in September 2020. Prospective survey participants were asked to fill a questionnaire on SARS-CoV-2 seroprevalence studies among their blood donor population and the assays and procedures being followed, details on assay sensitivity and specificity, and the results of seroprevalence estimates. The questionnaire was formatted in an Excel spreadsheet and participants were asked about the region they were reporting for, the number of antibody tests used for the SARS-CoV-2 seroprevalence study, the reason for using more than one serologic assay, details of antibody tests used for SARS-CoV-2 seroprevalence research (name of the assay, supplier, sensitivity, specificity, antigen target and detected antibody class), the use of NAT testing for SARS-CoV-2 and, if so, details on the NAT assay. Finally, blood services were questioned about their SARS-CoV-2 seroprevalence research results and the actual number of cases confirmed by public health authorities in their country/region.

Survey responses were received and compiled until November 2020. Table 1 lists the assays used by survey participants, with details on the assays' respective performance characteristics. In cases where there were discrepancies between the sensitivity and specificity characteristics reported by survey participants vs. those of commercial product inserts or information available from the Food and Administration (FDA) website [18], data from products inserts or the FDA website were reported in Table 1. Finally, some missing survey data on the cumulative incidence of COVID-19 were obtained from the WorldOMeter website [19] for the dates specified by survey participants.

## Results

Out of 49 blood component suppliers that were contacted to answer the survey, 38 provided at least partial responses, representing 27 countries (Fig. 1). Cumulative incidence from participant countries until the end of September 2020 was presented in Fig. 2. The Abbott SARS-CoV-2 IgG assay was by far the most commonly used kit, utilized by 15 blood suppliers, followed by the EUROIMMUN Anti-SARS-CoV-2 ELISA (IgG) (seven blood suppliers), the Beijing Wantai Biological Pharmacy SARS-CoV-2 Total Ab ELISA (six blood suppliers), the F. Hoffmann-La Roche AG Elecsys® Anti-SARS-CoV-2 (four blood suppliers) and the Ortho Clinical Diagnostics VITROS® Anti-SARS-CoV-2 Total test, the DiaSorin S.p.A. LIAISON® SARS-CoV-2 S1/S2 IgG and the Zhuhai Livzon Diagnostics Diagnostic Kit for IgM/IgG Antibody to Coronavirus (SARS-CoV-2) (Lateral Flow) (three blood suppliers each). Notably, five blood suppliers used in-house assays. Only five blood suppliers have indicated that nucleic acid amplification testing of SARS-CoV-2 RNA was done; when performed, this test was done to confirm that the donor was not infectious (Fig. 3 and Appendix S1).

Regarding detected immunoglobulins, target viral protein, sensitivity and specificity of the assays used by blood services, out of 24 assays being used, two assays detected Ig classes other than IgG (e.g. IgA/IgM or IgM), while 68·2% and 50% (15/22 and 11/22 of those who reported data) target the spike or the nucleocapsid viral protein. 18·2% (4/22) of those assays target both the spike and the nucleocapsid proteins. The sensitivity and specificity of IgG-specific assays ranged from 71·9% to 100%, and from 96·23% to 100%, respectively (Table 1). Of the 38 blood component suppliers who partially replied to the survey, only 14 provided seroprevalence data (Appendix S2). Seroprevalence end of sampling dates ranged from May 2020 to October 2021. Ten out of 14 seroprevalence estimates ranged from 0% to 5·6%. The other four were much higher, ranging from 13·48% to as high as 30·89%. Three of these four providers with high seroprevalence rates were from Brazil; the fourth one was from Iran. For at least three of the four reported seroprevalence rates above 10%, the assay that was used targets the nucleocapsid protein; for the fourth seroprevalence value >10%, the information regarding the target viral protein of the assay (Hangzhou AllTest IgG/IgM Rapid Test Dipstick (WB/S/P)) is unknown. Two of the four Brazilian blood component providers used the Abbott SARS-CoV-2 IgG Assay which, as stated earlier, was the most widely used assay among survey participants.

## Discussion

Our results reveal that 19 commercial and five in-house assays have been used by blood component suppliers who replied to the survey. There was substantial variability in the targeted antigen, detected immunoglobulin(s) and overall performances of the assays used by blood centres. These variations could lead to diverging results and interpretations in seroprevalence surveys. Regarding its antigenic determinants, SARS-CoV-2 is an enveloped RNA virus composed of Spike, Envelope, Membrane and Nucleocapsid proteins [20]. As studies have indicated that they are the most immunogenic antigens [21, 22], the spike (which contains the receptor-binding domain (RBD)) and nucleocapsid proteins are prime targets of most serologic assays. In our study, all respondents reported on using

**Table 1** List of assays used by survey participants: detected immunoglobulins, target viral protein, sensitivity and specificity

| Assay name | Supplier | Country/provider | Detected Ig | Target viral protein | % Sensitivity (95% CI) | % Specificity (95% CI) |
|---|---|---|---|---|---|---|
| SARS-CoV-2 IgG assay | Abbott Laboratories (Abbott Park) | IL, USA | IgG | Nucleocapsid | 100 (95·8–100) | 99·6 (99·0–99·9) |
| In-house anti-SARS-CoV-2 ELISA (adapted from Amanat et al. [4] and Stadlbauer et al. [32]) | Héma-Québec | Quebec, Canada | IgA/IgG/IgM | Spike | 98·8 | 98·5 |
| VITROS® Anti-SARS-COV-2 IgG test | Ortho Clinical Diagnostics | NJ, USA | IgG | Spike | 90·0 (76·9–96·0) | 100 (99·1–100) |
| VITROS® Anti-SARS-COV-2 Total test | Ortho Clinical Diagnostics | NJ, USA | IgA/IgG/IgM | Spike | 100 (92·7–100) | 100·0 (99·0–100) |
| Elecsys® Anti-SARS-CoV-2 | F. Hoffmann-La Roche AG | Switzerland | IgA/IgG/IgM | Nucleocapsid | 99·5 | 99·8 |
| Anti-SARS-CoV-2 ELISA (IgG) | EUROIMMUN Medizinische Labordiagnostika AG | Germany | IgG | Spike | 90·0 (74·4–96·5) | 100 (95·4–100) |
| MedTeste Coronavírus (COVID-19) IgG/IgM (Teste Rápido) | Hangzhou Biotest Biotech Co. | China | IgG/IgM | Nucleocapsid | 87 (IgM)<br>84·5 (IgG) | 99·15 (IgM)<br>100 (IgG) |
| DPP® COVID-19 IgM/IgG System | Chembio Diagnostic Systems, Inc. | NY, USA | IgG/IgM | Nucleocapsid | 71·9 (IgM)<br>80·6 (IgG) | NS |
| Diagnostic Kit for IgM/IgG Antibody to Coronavirus (SARS-CoV-2) (Lateral Flow) | Zhuhai Livzon Diagnostics | China | IgG/IgM | NS | 82·58 (75·68–88·20) | 99·54 (98·66–99·90) |
| In-House Virus Neutralization Test (VNT) | Établissement Français du sang (EFS) | France | IgG | NS | NS | NS |
| COVID-19 total Antibody | Fortress Diagnostics | United Kingdom | IgG/IgM | Spike/Nucleocapsid | 94 | 100 |
| SARS-CoV-2 Total Ab ELISA | Beijing Wantai Biological Pharmacy | China | IgA/IgG/IgM | Spike | 98 | 99·6 |
| SARS-CoV-2 IgM ELISA | Beijing Wantai Biological Pharmacy | China | IgM | Spike | 86·9 | 100 |
| Oxford ELISA [5] | University of Oxford | United Kingdom | IgG/IgM | Spike | 85 (70–94) | 100 (93–100) |
| COVID-19 ELISA IgG | Vircell S.L. | Spain | IgG | Spike/Nucleocapsid | 85 | 98 |
| COVID-19 ELISA IgM + IgA | Vircell S.L. | Spain | IgA/IgM | Spike/Nucleocapsid | 88 | 99 |
| LIAISON® SARS-CoV-2 S1/S2 IgG | DiaSorin S.p.A. | Italy | IgG | Spike | 97·6 (87·4–99·6) | 99·3 (98·6–99·6) |
| In-house Spike RBD ELISA developed by Malik Peiris and described in Perera et al., 2020 [33] | Hong Kong Red Cross Blood Transfusion Service | Hong Kong SAR, China | IgG/IgM | Spike | 100 | (Results pending) |
| cPass™ SARS-CoV-2 Surrogate Virus Neutralization Test (sVNT) Kit | GenScript USA Inc. | NJ, USA | IgA/IgG/IgM | Spike | 100 (87·7–100) | 100 (95·8–100) |
| In-house Plaque Reduction Neutralization Test (PRNT) | Hong Kong Red Cross Blood Transfusion Service | Hong Kong SAR, China | NS | NS | 100 (day 28 post-RT-PCT-positive result)<br>99·11 (days 60-209 post-RT-PCT–positive result) | 100 |

**Table 1** (Continued)

| Assay name | Supplier | Country/provider | Detected Ig | Target viral protein | % Sensitivity (95% CI) | % Specificity (95% CI) |
|---|---|---|---|---|---|---|
| Standard Q Covid-19 IgM/IgG Combo | SD Biosensor, Inc. | Gyeonggi-do, South Korea | IgG/IgM | Nucleocapsid | 94·51 | 96·23 |
| ID Screen® SARS-CoV-2-N IgG indirect ELISA | IDVet | France | IgG | Nucleocapsid | 93·3 (78·8–98·2) | 99·9 (99·6–100) |
| In-house ELISA, based on Amanat et al.'s protocol [4] | KEMRI Wellcome Trust Research Programme | Nairobi, Kenya | IgA/IgG/IgM | Spike | NS | NS |
| 2019-nCoV IgG/IgM Rapid Test Dipstick (WB/S/P) | Hangzhou AllTest Biotech Co., Ltd | China | IgG/IgM | Nucleocapsid | 99·9 (IgG)<br>90·9 (IgM) | 98·0 (IgG)<br>97 (IgM) |

NS, Not Specified.

assays that target either the nucleocapsid or spike protein (or both). The available evidence suggests that antibodies against these targets are more generally associated with a protective immune response [20]. Antibody responses against the different SARS-CoV-2 proteins are of major importance since antibody classes have differential dynamics and neutralizing effects [22, 23]. Moreover, estimates of seroconversion (or seroreversion) rates are highly dependent on the assay target, antibody dynamics and the time point at which testing is conducted in the disease course. In support of the latter point, Post et al. [20] have thoroughly described the kinetics of IgG emergence, persistence and slow decline based on a systematic review of the literature. The overall evidence indicates that the IgG response peaks between three- and seven-week post-symptom onset. This acute response is followed by a plateau phase, and then, IgG levels slowly decline, yet persist for up to 12 weeks. Whether SARS-CoV-2-specific IgGs remain detectable beyond that time point is unknown, since studies' follow-up periods were limited in time.

Among the 14 blood suppliers who provided seroprevalence estimates, 10 reported values of 5·6% or less (in Europe, North America and Asia), whereas the other four reported seroprevalence estimates of 13·5% or more (in Brazil and Iran). These values appear to roughly correlate with COVID-19 case counts and/or what is known of the intensity of the regional pandemic. Blood donor studies should be very suitable for international comparison because they are all carefully screened individuals who are reasonably representative of the healthy and well adult population thus similarly selected. Nevertheless, there are some important considerations for international comparison such as the regional selection within country, timing of sample collection within the pandemic, local epidemiology (e.g. re-infection rates and the amplitudes of pandemic waves in particular regions, as well as timings between waves) and as highlighted in our survey, the broad range of assays used for these studies.

While our survey provides an informative overview of blood donor study seroprevalence rates, these data are not appropriate for in depth comparison. There can be broad geographical variability in seroprevalence rates within the same country [24, 25] therefore catchment area of the blood centre must be known. To address differential sampling by demographic variables in donors versus the general population, data need to be sorted by demographics and weighted proportional to the general population. Donor selection and, for example socioeconomic distribution of donors may also differ between countries and complicate generalizability from donors to the general population. Finally, the survey did not include a specific question on the number of samples collected by each blood operator for seroprevalence estimates; the lack

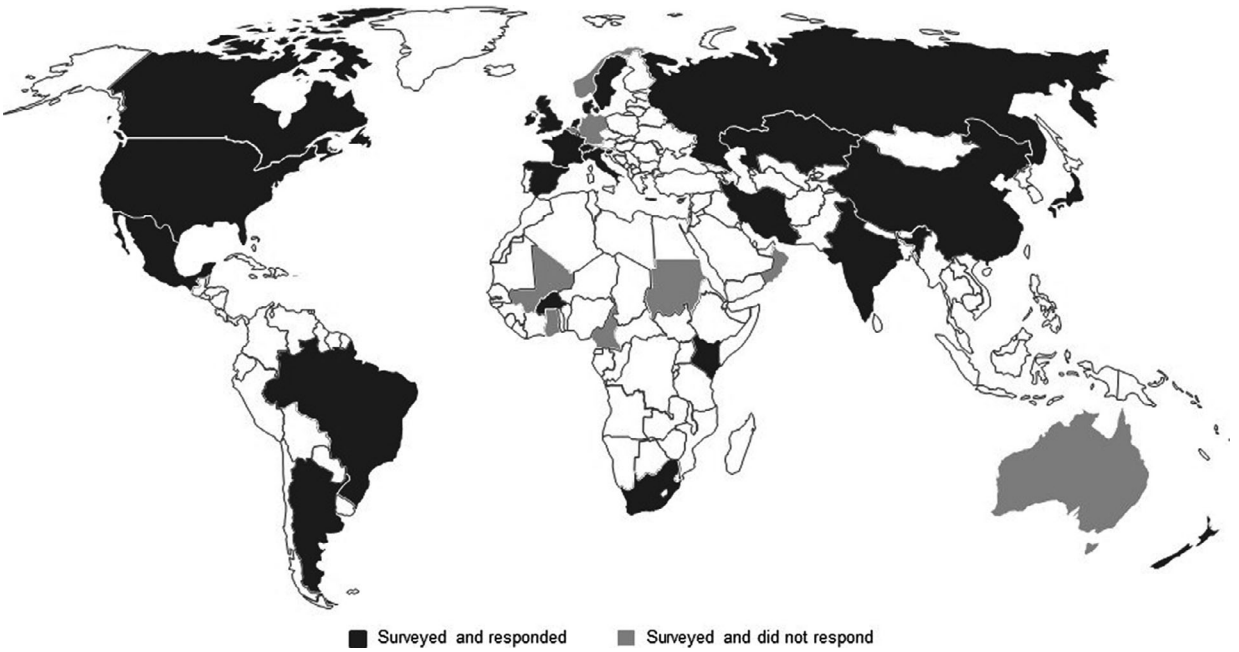


**Fig. 1** Geographical distribution of surveyed blood component providers.

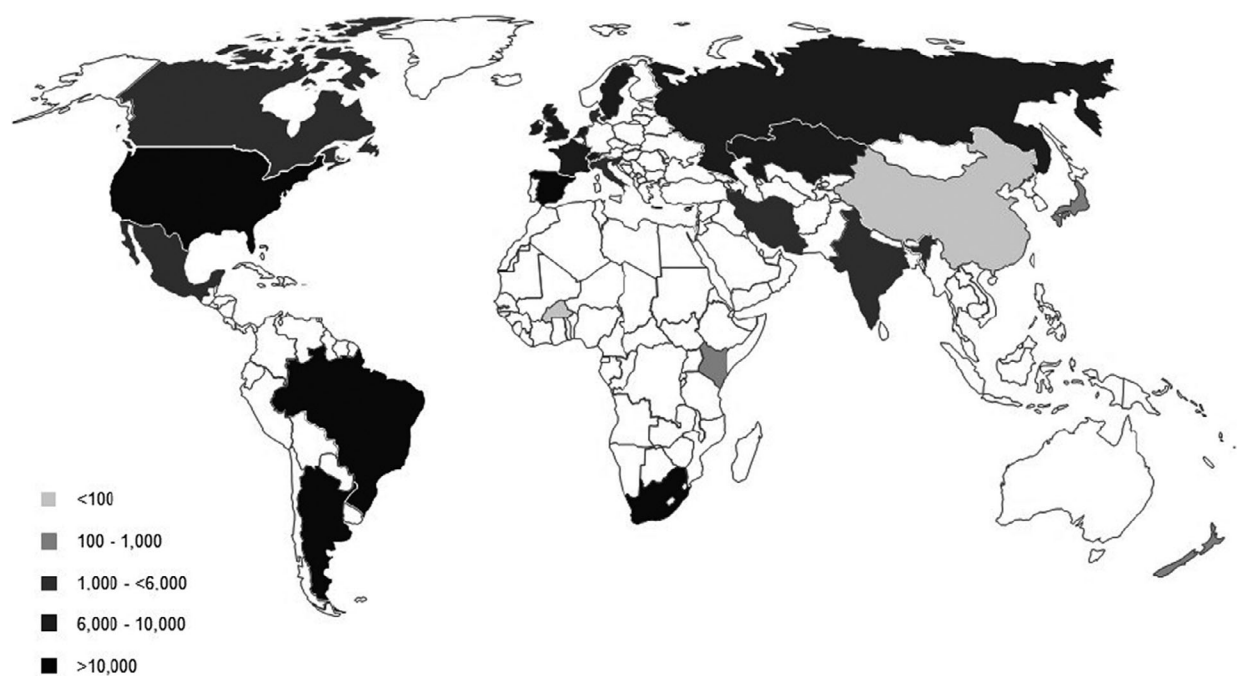


**Fig. 2** COVID-19 cumulative incidence per million population by participating countries as of 30 September 2020.

of sample size information would make any attempt at comparing seroprevalence rates questionable.

The characteristics of the assay should be considered. While there are sensitivity and specificity data for many commercial assays, it needs to be clear how a positive sample was defined. Sensitivity and specificity thresholds are influenced by the time between infection and sample collection, and threshold adjustment could optimize sensitivity and specificity and ultimately assay performance. There is ongoing debate as to what the signal to cut-off

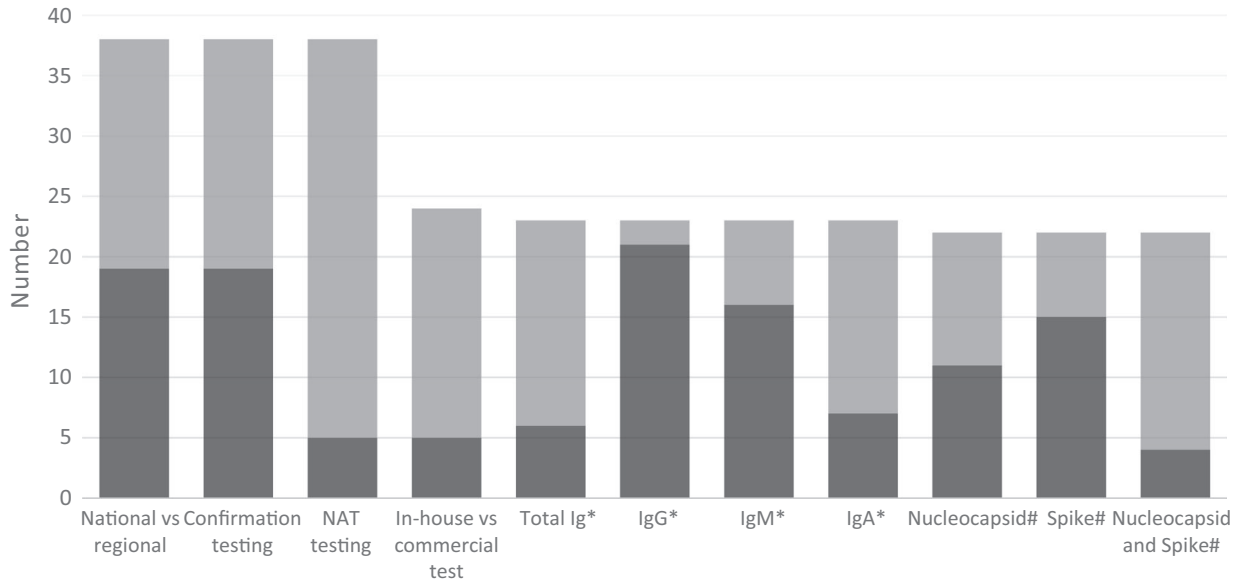


**Fig. 3** Blood operators and assays characteristics *24 assay used in total and one with missing Ig detection information; # 24 assay used in total and two with missing viral protein target information.

(S/Co) ratio should be. The Abbott IgG Assay lists S/Co as 1·4 to define positivity but a grey zone of possible positivity has been proposed for samples as low as S/Co0·49. A head-to-head benchmark evaluation of the sensitivity and specificity of five immunoassays for SARS-CoV-2 (SARS-CoV-2 IgG assay (Abbott, Chicago, IL, USA), LIAISON SARS-CoV-2 S1/S2 IgG assay (DiaSorin, Saluggia, Italy), Elecsys Anti-SARS-CoV-2 assay (Roche, Basel, Switzerland), SARS-CoV-2 Total assay (Siemens, Munich, Germany) and a novel 384-well ELISA (the Oxford immunoassay)) revealed that all assays achieved sensitivity of ≥98% with thresholds optimized to achieve specificity ≥98% on samples collected ≥30 days post-symptom onset [26].

Given the documented waning of antibody levels [6], antibody detection in the days that follow symptom onset could be critical for properly assessing seroprevalence. In support of this notion, a longitudinal analysis of convalescent plasma donors found that 40% of them became seronegative within four months after initial antibody detection [27]. Asymptomatic blood donors experience faster antibody decay compared to individuals with symptomatic disease [28, 29]. Moreover, inconsistent detection between assays was observed in symptomatic COVID-19 patients over around 100 days from acute infection [30]. The Abbott CIMA test seems to show a greater decline in signal after symptom onset compared to the Roche serological assay [30]. Waning antibody should be considered in the estimate of seroprevalence. Mathematical modelling such as stochastic Monte-Carlo approaches would be appropriate but should be applied consistently across the studies being compared.

It is also noteworthy that despite the high sensitivity and specificity of the assays used by blood centres, the infection prevalence has a direct impact on the predictive-positive value (PPV). PPV is the proportion of true-positive results, which is equal to (sensitivity × prevalence)/[(sensitivity × prevalence) + ((1–specificity) × (1–prevalence))] [31]. Given the relatively low prevalence of SARS-CoV-2 infection in many of the studies, even using a highly sensitive test, the PPV will necessarily be imperfect, and a some false-positive results will occur.

In conclusion, aside from a few unusually high numbers, the majority of seroprevalence estimates are consistent with values that have been published in the past few months. Compared to residual samples from patients, often sick people, from medical or commercial laboratories, blood banks are preferred organizations for seroepidemiological studies due to superior sample quality, high sample accessibility and sample representativeness of a generally healthy adult population. Continuous monitoring of seroprevalence among blood donors provides a valuable indication of the level of exposure to SARS-CoV-2, which further informs public health authorities of the extent of the overall immunity against this virus in the general population and assists to evaluate public health interventions. Depending the testing assays used, these studies may become invaluable in monitoring vaccine uptake in the months to come. Blood bank seroepidemiological studies can provide a valuable estimate of

infection across many geographic regions. However, seroprevalence estimation requires careful attention to details including the geographic region within each country, stratification by age with weighting proportionate to the general population and consistency of interpretation of the test result. Our survey shows that there is considerable heterogeneity in assays used for blood donor seroprevalence studies around the world. We believe that comparative studies are best carried out collaboratively with investigators from each seroprevalence study to ensure that data are correctly represented.

## Acknowledgements

We are indebted to the following individuals who provided us with completed questionnaires: Paula Saa, Susan Stramer, Louis Katz, Michael Busch, Paula Loureiro, Cesar de Almeida Neto, Luiz Amorim, Karla Maldonado Silva, Véronique Van Gaever, Boris M. Hogema, Hans L. Zaaijer, Katy Davison, Pierre Gallian, Niamh O'Flaherty, Monique Debattista, Lisa Jarvis, Polonca Mali, Salvador Oyonarte, Jonas Nordberg, Christoph Niederhauser, Crystal Hui Ping, Wai-Chiu Tsoi, Xue Chen, Yongshui Fu, Jinfeng Zeng, Lei Zhao, Hua Shan, Rounak Dubey, Nishith A. Vachhani, Divjot Singh Lamba, Minoko Takanashi, Eugene Zhiburt, Salam Sawadogo, Claude Tayou Tagny, Justina K Ansah, Amadou B. Diarra, Marion Vermeulen, Mahtab Maghsudlu, Arwa Z. Al-Riyami, Christine Van Tilburg.

## Conflicts of interest

The authors have no conflicts of interest to declare.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:
Appendix S1 Participating blood component suppliers and serologic assays used.
Appendix S2 Seroprevalence results.

VoxSanguinis
The International Journal of Transfusion Medicine
ISBT International Society of Blood Transfusion
*Vox Sanguinis* (2021)

DOI: 10.1111/vox.13089

ORIGINAL PAPER

# Application of unsupervised machine learning to identify areas of blood product wastage in transfusion medicine

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Vox Sanguinis

## Abstract

**Background** Wastage of blood products can be a significant cost to blood banks. However, the cause of wastage is often complex and makes it difficult to determine wastage-associated factors. Machine learning techniques may be useful tools to investigate these complex associations. We investigated whether unsupervised machine learning can identify patterns associated with wastage in our blood bank.

**Materials and methods** Data on red blood cells, platelets and frozen products were obtained from the laboratory information system of the Central Zone Blood Transfusion Services at Nova Scotia Health Authority. A total of 879 532 transactions were analysed by association rule mining, a type of machine learning algorithm. Associations with lift scores greater than 25 and with clinical relevance were flagged for further examination.

**Results** Association rule mining returned a total of 3355 associations related to wastage. Several notable associations were identified. For example, certain wards were associated with wastage due to thawing unused frozen products. Other examples included association between smaller blood banks and evening work shifts with product wastage due to excess time outside the laboratory or returning products with high temperatures.

**Conclusion** This paper demonstrates the effective use of unsupervised machine learning for the purpose of investigating wastage in a large blood bank. The use of association rule mining was able to identify wastage factors, which can help guide quality improvement initiatives. This technique can be automated to provide rapid analysis of complex associations contributing to wastage and could be utilized in modern blood banks.

*Received: 16 March 2020, revised 29 January 2021, accepted 1 February 2021*

**Key words:** machine learning, informatics, transfusion medicine, association rule mining, inventory management, product wastage.

## Introduction

Blood bank inventories and procedures are highly monitored, and products are tracked to document the chain of custody between donor and recipient. The electronic storage of this high-resolution blood product data provides an opportunity for machine learning and automated computer algorithms to help optimize blood bank practices. Machine learning has been applied to clinical aspects of transfusion medicine and mainly focus on supervised algorithms [1–3]. Supervised machine learning involves a data set where specific inputs have already been linked to specific outputs. This means there is an entity which first has to link the input with the correct output, called 'labelling the data'. Examples of clinical scenarios where supervised machine learning has been applied to transfusion medicine include ensemble algorithms to predict transfusion-associated cardiac overload

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(TACO) and transfusion-related acute lung injury (TRALI) [3], using supervised machine learning technique to predict when RBC transfusion would be ordered or massive transfusion would be declared [4], and utilizing machine learning to predict transfusion product usage during total knee arthroplasty [5], spine surgery [2] or various elective surgeries [6]. In the laboratory setting, supervised algorithms have been used to identify RhD antigen [7] and predict the quality of stored RBC [8] based on microscopy. Also, the ability of time series analysis has been compared to neural networks to predict quantity of blood supply [9].

Less explored is the use of unsupervised machine learning in transfusion medicine. Unsupervised machine learning is used when the data set provides input data, but there is no corresponding output provided from the data set. Unsupervised algorithms will then attempt to find structure or relationships from the input. Since there is no output required in the data set, unsupervised machine learning does not require an entity to 'label the data'. Unsupervised random forest clustering algorithms have been found to help determine factors that contributed to bleeding and mortality after plasma transfusion [10]. Other computational techniques to reduce wastage have included data visualization strategies, such as process mining which allowed for greater insight into blood bank inventories [11,12].

However, there is a lack of research in using machine learning to identify internal inefficiencies in blood bank practices that can lead to product outdates and wastage.

Traditional methods of assessing internal inefficiencies involve manually reviewing quality assurance indicators to find areas for improvement. This process is labour-intensive and time-consuming, especially if large inventories with multiple product types and many environment-related factors are studied. Additionally, quality assurance indicators that are chosen by subject matter experts may overlook the less obvious causes of laboratory inefficiencies. These obstacles of traditional quality assessment can potentially be minimized by augmenting analysis with unsupervised machine learning algorithms.

Association rule mining is a type of unsupervised machine learning algorithm that involves automated identification of associations between different parameters in a data set. This form of machine learning has been studied in non-transfusion areas of medicine. Association rule mining has been used to analyse medical healthcare records for associations between specific diseases and patient symptoms [13,14], and in identifying associations between medications and specific diseases, and laboratory findings and specific diseases [15].

In this manuscript, we focus on data from our regional transfusion service (Central Zone of the Nova Scotia Health Authority). Our regional transfusion service is composed of four blood banks of varying sizes and transfuses over ten thousand blood products per year (Table 1). We follow the shelf-life guidelines set by Canadian Blood Services. The shelf life for RBC is 42 days, thawed plasma is 5 days and platelets is 7 days. In this manuscript, we assess the use of association rule mining to analyse transfusion data from a regional transfusion service to determine whether machine learning can identify attributes associated with blood product inventory wastage.

## Methods

### Database construction

Transaction data on RBC, platelets and frozen blood product (plasma and cryoprecipitate) usage were queried from Cerner Millennium, the laboratory information system (LIS) of the Nova Scotia Health Authority Central Zone. This LIS (Cerner Millennium) is a single database for all laboratory services at Central Zone in the Nova Scotia Health Authority. The blood transfusion services in the four institutions (hospital complexes) run on this single database. These transactions are instantly and seamlessly recorded into the LIS when a blood product is altered (e.g. thawed, pooled, disposed) or its status changed (e.g. received, available, assigned, issued). The complete data set includes RBC data from 1 January 2014

**Table 1** Blood Bank Information

| Blood bank | Notable clinic /services | Annual RBC transfused (Units) | Annual platelets/ frozen products transfused (units) | Annual number of products disposed (units) | Number of transactions recorded in data set |
|---|---|---|---|---|---|
| Halifax infirmary | Emergency department, trauma centre, surgical wards | 6673 | 1743 | 912 | 541 823 |
| Victoria general | Surgical wards, haematology | 6092 | 3570 | 74·4 | 277 510 |
| Hants community | Emergency department, surgical wards | 425 | 15 | 3 | 16 758 |
| Dartmouth general | Emergency department, surgical wards | 1386 | 172 | 12·8 | 53 450 |

**Table 2** Label names of blood bank data set

| Category Name | Description of category | Examples of options in category (column 1) | Number of options in category and derived options | Example of derived options |
|---|---|---|---|---|
| Product description | Abbreviated name of product | PLT CPD Pooled, APL ACD-A LR IRR, CSP CPD, | 30 | No derived options |
| Product number | Integer unique to each product | 3473560 | Used to derive 'number of transactions'. Contains 5 derived options. | very low, low, medium, high, very high |
| ABO and RH | ABO and RH type of the product | O+, A+, B+, AB+, O–, A–, B–, AB– | 8 | No derived options |
| Special treatments | Standardized descriptions of alterations made to the product | Cryo-reduced, CMV negative, irradiated, apheresis | 1650 | No derived options |
| Status in blood bank | Single word to describe the availability status or outcome of the blood product | Received, available, transferred, transfused, disposed | 13<br>Used to derive 'inventory size'. Contains 6 derived options. | very low, low, medium, high, very high |
| Dispense return reason | The reason why a dispense product is returned to the lab | No consent, issued in error, patient unavailable | 25 | No derived options |
| Transaction location | The hospital where the blood product is located at the time of the transaction | VG BTS, HI BTS, DG BTS | 6 | No derived options |
| Ward location | The hospital unit where the blood is issued to | Emergency, medical day unit, unit 51 | 121 | No derived options |
| Dispose reason | The reason why a product was disposed of or destroyed | Product expired, Excess time outside refrigerator, Received broken | 42 | No derived options |
| Time of transaction entry | Date and time that the transaction was entered into the database | 20-07-15 3:38 | Used to derive 'time of day and seasons, handling time': 12 derived options. | Daytime, evening, overnight, winter, spring, summer, fall, very low, low, medium, high, very high |
| Expiry date and time | Date and time that the product will expiry | 9-06-16 5:32 | Used to derive 'time of day and seasons, time until expiration': 12 derived options. | Daytime, evening, overnight, winter, spring, summer, fall, very short, short, medium, long, very long |
| Username | The account name of the technologist who entered the transaction | MEANEYR | 88 | No derived options |
| Supplier | The location where the blood product was obtained from | CBS – Dartmouth ISBT | 14 | No derived options |

to 31 December 2017 and non-RBC data from 1 January 2015 to 31 August 2018 and is composed of 889 556 transactions involving 20 515 frozen products, 12 056 platelet units and 48 954 red blood cell units. No significant differences in blood bank policies were noted between the study periods covered by the RBC and non-RBC data sets. A summary of the different categories included in this data set is provided in Table 2. The categories are queried without modification from those used in our LIS and were initially adapted from disposition categories used by Canadian Blood Services. Categories which are continuous variables, such as timestamps, were converted into categorical values which are ideal inputs for association rule mining. For example, continuous values were batched into 'very low', 'low', 'medium', 'high' and 'very high' options and included in

our analysis as categorical variables. Likewise, date and timestamps are converted into corresponding seasons and technologist shifts. Two categories were excluded (product handled during downtime and reason for reinstating a product) due to zero products having these attributes during the study period.

Factors where the value differed with every product (such as product identification number) or factors with the same value for all products (such as unit of measurement) were removed since they could not be associated with wastage and would increase the analysis time. Thawing of frozen products and the pooling of thawed cryoprecipitate were recorded in the data set as disposal transactions, and these transactions were removed from the data set since no product was actually disposed or destroyed. A total of 10 024 of these transactions were removed. The remaining 879 532 transactions were divided into sub-groups (frozen products, RBC or platelets). An overview of database construction and analysis is shown in Fig. 1.

## Unsupervised machine learning by association rule analysis

Each sub-group of transactions was analysed using unsupervised machine learning involving association rule mining. The algorithm was written in Python programming language, version 3.6.4 [16]. Additional libraries for analysis included Numpy version 1.14.2, [17] Pandas version 0.23.4 [18] and Mlxtend version 0.13.0 [19].

Association rule analysis evaluates how transactions with sets of defining factors (called the antecedent) correlate with transactions with other sets of factors (called the consequent) [20]. If all the transactions that satisfy the antecedent also satisfy the consequent, then there is likely a high correlation between the antecedent and consequent. The number of defining factors in each set can vary in size; however, when association rule mining was performed on a small subset of the data set, very few clinically significant correlations involved an antecedent or consequent with more than 3 factors. Therefore, analysis of the full data set had a maximum number of factors in the antecedent and consequent limited to 3. In association rule analysis, the support refers to how frequently transactions that fulfill the antecedent criteria appear in the data set [20]. Confidence is the proportion of the transactions in the antecedent that also fulfills the consequent [20]. Lift is a measure that helps differentiate correlations from random associations [21]. A lift value greater than 1 suggests that there is a true association between antecedent and consequent. For this analysis, a minimum confidence and minimum support for the RBC analysis were 0·1 and 0·00001, respectively. Minimum confidence and minimum support for frozen and platelet product analysis were 0·1 and 0·0001, respectively. This minimum support threshold filtered out correlations with less than 6 transactions that meet the antecedent criteria. Correlations with a lift score greater than 25 were further assessed. This lift threshold is similar or higher than the minimum lift score reported in other publications of association rule mining in the medical field, and allowed the analysis to focus on the stronger associations [13–15]. Correlations with greater than 25 lift score were initially classified by one transfusion expert based on whether the association was logical and of clinical significance. The

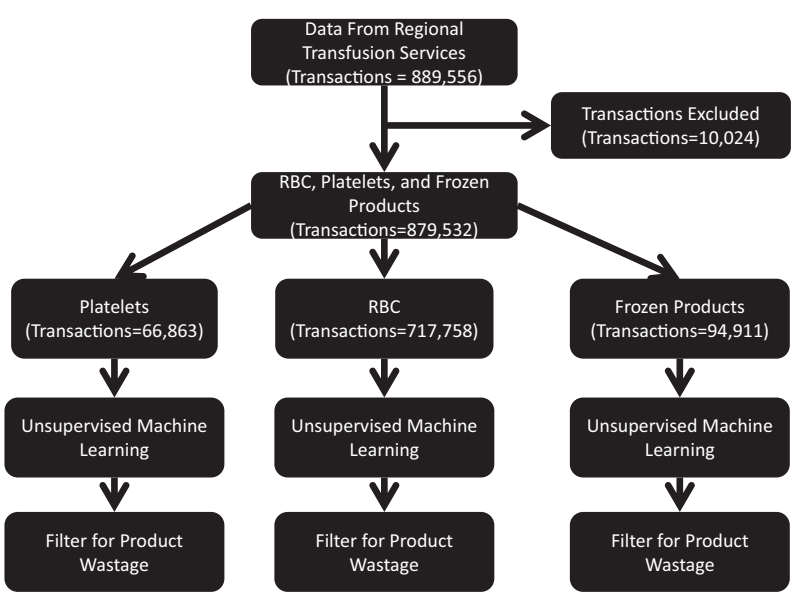


**Fig. 1** Data analysis framework for machine learning using blood product data set.

associations were then reviewed with two other transfusion experts, and a consensus was required to finalize the classification. Correlation which involved wastage and had clinical significance was flagged and reported using the custom filter options in Microsoft Excel (2010) [22]. Wastage associations were filtered for by tagging the associations which included 'STATUS_Disposed' or any of the different 'Dispose_reason' tags. Clinically significant associations were associations that indicate our policies are functioning as intended or unexpected associations that had not previously been considered to be wastage related, which are relevant because they can indicate unexpected wastage. To represent the data graphically, additional variables were calculated including 'proportion ordered but not used', 'proportion excess time outside refrigerator' and 'proportion unacceptable return temperature' (formulae in Fig. 2).

After clinically relevant associations were identified, the blood bank's policies and procedures (such as technologist shift scheduling) were reviewed to hypothesize potential cause of these wastage associations.

## Results

Association rule mining analysis of RBC, frozen products and platelets returned 52 292 associations, 5898 associations and 11 302 associations, respectively. After filtering for associations that involved wastage, a total of 2629 RBCs, 472 frozen products and 254 platelet associations were found.

The associations involving wastage were separated into three categories: logical association with clinical significance, logical associations with no clinical significance and unexplained associations. An example of logical association with no clinical significance would be an association between a product expiring and the age of the product. This association is logical, but it is also obvious and is unlikely to improve blood banking efficiency. An example of an unexplained association would be between unacceptable return temperature and the age of the product, because there is no obvious reason for this association and it is neither logical nor clinically significant. The clinical significance of an association is dependent on the policies and protocols of the blood bank, and will therefore be institution dependent. At our regional transfusion service, we found different combinations of categories gave logical associations that are clinically significant, and other categories gave logical associations with no clinical significance (Table 3). Figure 3 shows the percentage of associations involving wastage that were classified into the three types of associations mentioned above.

An anonymized copy of all the associations for the different products can be found in the Supplemental Materials. Three logical associations with clinical significance are described below. These examples demonstrate associations that were previously unknown to our transfusion management.

### Association of location with wastage of frozen product

The nephrology intermediate care unit (IMCU) was the most likely to dispose of products due to thawing unused

| Derived variable | Formula |
|---|---|
| Proportion ordered but not used | Proportion ordered but not used = Unit of disposed frozen product due to being thawed / Total number of disposed frozen products |
| Proportion unacceptable return temperature | Proportion unacceptable return temperature = Unit of RBC disposed due to unacceptable return temperature / Total number of disposed RBC units |
| Proportion excess time outside refrigerator | Proportion excess time outside refrigerator = Unit of RBC disposed due to excess time outside refrigerator / Total number of disposed RBC units |

**Fig. 2** Derived variables to allow graphical analysis.

**Table 3** Clinical significance of different categories

| Categories and options that return logical association with clinical significance | Categories and options that return logical association with no clinical significance | Categories and options that return unexplained associations |
|---|---|---|
| Category: Status in blood bank<br>Option: Disposed | Category: Supplier | Category: Dispose reason<br>Option: Patient related |
| Category: Status in blood bank<br>Option: Expired | Category: Username | Category: Dispose reason<br>Option: CBS request |
| Category: Status in blood bank<br>Option: Location | Category: Time until expiration | Category: Dispose reason<br>Option: No secure seal |
| Category: Ward location<br>Option: Association location | Category: ABO and RH | Category: Special treatment<br>Option: Various |
| Category: Seasons | Category: Dispose reason<br>Option: Quality control | Category: Transfusion inventory size |
| Category: Time of day | | Category: Reinstate reason |
| Category: Dispose reason<br>Option: Return temperature unacceptable | | Category: Transfusion frequency |
| Category: Dispose reason<br>Option: Exceeds appropriate temperature | | Category: Dispose reason<br>Option: To IWK |
| Category: Dispose reason<br>Option: Thawed but not used | | |
| Category: Special treatment<br>Option: Thawed | | |
| Category: Handling time | | |
| Category: Dispose reason<br>Option: Failed visual inspection | | |
| Category: Dispose reason<br>Option: Security tag missing | | |
| Category: Dispose reason<br>Option: Bag broken | | |
| Category: Size of inventory | | |

frozen product. Wards involved in trauma care, such as the intensive care unit (ICU), emergency department or surgery, were also likely associated with product wastage due to not using a thawed frozen product (Fig. 4). Hospital locations receiving less than 10 units during the course of the data set were omitted since wasting even a single product would heavily skew their proportion of wasted products.

### Hospital-dependent RBC wastage due to inappropriate blood product return

The Nova Scotia Health Authority Central Zone LIS contains transfusion data for 4 different hospitals (Dartmouth General Hospital, Halifax Infirmary, Victoria General Hospital and Hants Community Hospital). RBC returns in these different hospitals were found to have significantly different rates for excess time outside blood bank and unacceptable return temperature (Fig. 5 a and b).

### Inappropriate blood product returns were found to associate with the evening shift

The evening shift (5 pm to 11 pm) was associated with product wastage due to excess time outside refrigerator and product returned at an unacceptable temperature (Fig. 6a and b).

## Discussion

Analysis of our blood bank data set revealed many associations. Filtering using basic spreadsheet functionality was sufficient to identify wastage-related associations. We found that allowing certain categories through our filter would return mostly clinically relevant associations (Table 3). However, if the filter is too stringent, then some clinically relevant associations would be missed because too few categories passed through the filter. On the other hand, increasing the number of categories that are

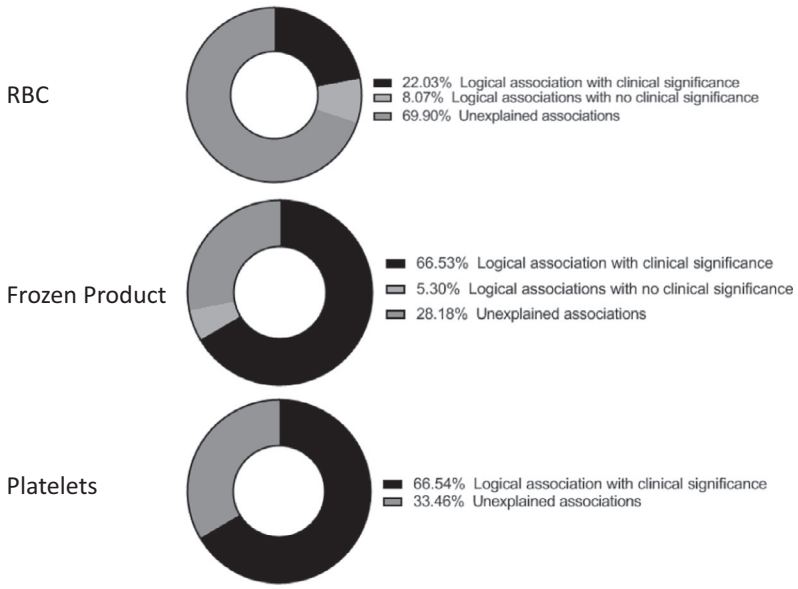


**Fig. 3** Proportion of associations with clinical significance. For each type of blood product, the associations were divided into logical associations with clinical significance, logical associations without clinical significance and unexplained associations, based on the category options in Table 3.

allowed to pass the filter increased the number of associations that need to be analysed and decreased the likelihood of an association being clinically relevant. Different institutions will need to find the balance of how many categories to allow through their filter. The initial filtering process took approximately 12 h, and in future analyses, the time may be shorter since these category filters can be reused.

Unsupervised machine learning identified that wards involved in trauma care were associated with wastage due to thawing products which go unused (Fig. 4). An explanation for this association is that trauma care units are more likely to implement massive transfusion protocols, which have been shown to lead to increase frozen product wastage [23]. However, our algorithm also unexpectedly found that the nephrology IMCU had a higher proportional wastage of thawing unused product than the wards involved with trauma patients. This unexpected finding should prompt a review of the transfusion policies of this unit and also demonstrates that association rule mining can identify unexpected causes of wastage.

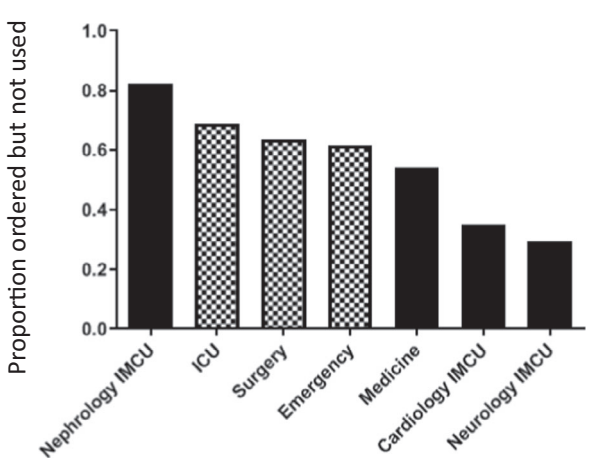


**Fig. 4** Association between different wards and wastage due to unused thawed frozen product. Hospital locations which received less than 10 units during the course of the data set were omitted. Trauma wards are displayed with checkered patterned bars. IMCU, intermediate care unit; ICU, intensive care unit.

Wastage of RBC due to inappropriate product return was found to be hospital-dependent, with the Halifax Infirmary having the lowest relative wastage rate (Fig. 5a and b). In our data set, the Halifax Infirmary is also the largest blood bank with the largest product turnover rate (Table 1), which raises the possibility that nurses and other hospital personnel are more familiar with blood bank policies and more cognizant of returning unused products promptly. Additionally, the blood bank at the Halifax Infirmary is supplying a trauma centre and the blood bank is located 1 floor above the surgical wards which will minimize the time it takes for product transport.

The blood banks in the Central Zone have a day shift, an overnight shift and a swing/evening shift. The unsupervised machine learning algorithm identified that the evening shifts are more often associated with wastage due to inappropriate blood product returns (Fig. 6). There are many possible causes for this wastage. There could be fewer porters or nurses available to transport unused product back to the blood bank in a timely manner.

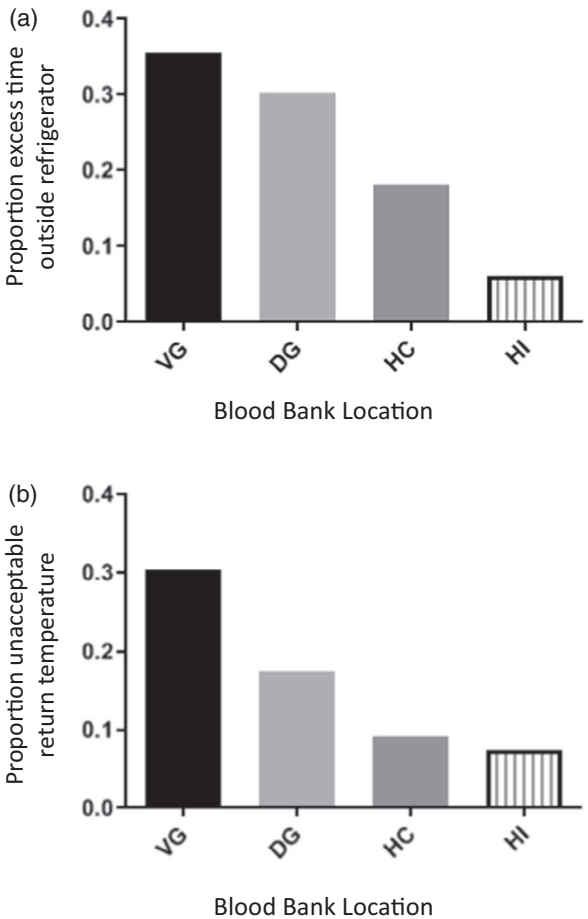


**Fig. 5** Association between blood banks and product return temperature and time outside of refrigerator. (A) Association between different blood banks and wastage due to excess time outside refrigerator. (B) Association between different blood banks and wastage due to high temperature of products when returned to blood bank. VG, Victoria General Hospital; DG, Dartmouth General Hospital; HC, Hann's Community Hospital; HI, Halifax Infirmary.

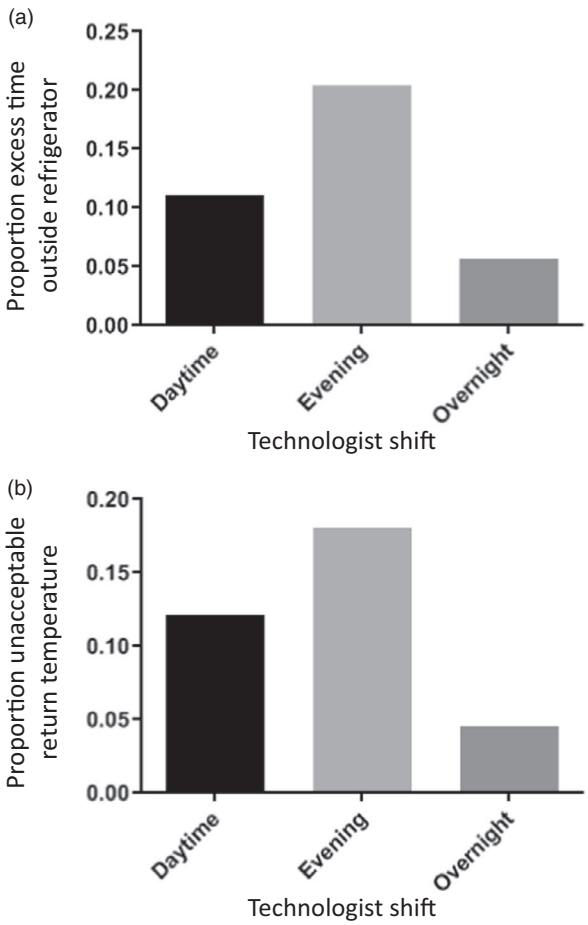


**Fig. 6** Association between technologist shift and product return temperature and time outside of refrigerator. (A) Association between the technologist's shift and wastage due to excess time outside refrigerator. (B) Association between the technologist's shift and wastage due to high temperature of products when returned to blood bank.

Alternatively, unused products could be left on the unit by the day team and only transferred back to the blood bank once the daytime work is complete. This association could be explored via root cause analysis and prompt further investigation by our institutional transport systems.

There were also many associations with wastage without an obviously logical relationship (Supplement Material). This subset of data could be analysed in more depth, since there could be non-obvious relationships which are more likely to be missed by traditional quality assurance indicators.

The total analytical time to process three years of transfusion data, from data set to a list of unsorted associations, was less than 24 h using a standard mid-range desktop computer. The analysis of the data was automatic and only required the user to input the initial data set. A transfusion inventory management committee could take advantage of the speed of unsupervised machine learning to quickly identify potential factors associated with wastage, and then perform targeted root cause analysis and implement solutions to mitigate future wastage. This strategy would be a fast and economical method to help minimize wastage in modern blood banks.

Furthermore, machine learning can possibly also be implemented for real-time interrogation of laboratory wastage. Given the speed of association rule mining, daily automated analysis of blood bank transactions can be performed, and if new associations are discovered, then immediate investigation (i.e. via a Kaizen event) and action plans can be implemented. In principle, an unsupervised machine learning engine could be implemented by hospital blood banks through the use of stand-alone

applications alongside the LIS or via software developers hard-coding it into the LIS software products.

Limitations of using machine learning include the requirement of transfusion personnel with computer science knowledge, or a collaboration between transfusion personnel and computer scientists to implement the analysis. There are large numbers of correlations which do not have clinical relevance and need to be discarded after an expert's review. In this manuscript, the factors that were chosen for analysis were the same factors that are tracked by our LIS. Since our LIS is designed to track factors that are felt to be important for monitoring wastage at our institute, it can increase the likelihood of machine learning to find that these factors are associated with wastage. These algorithms only identify correlations, but not the root cause of these correlations, and therefore, root cause analysis would still have to be implemented. Finally, optimal usage of machine learning requires an LIS that is accurate and contains a complete record of blood bank transactions. In this study, only one LIS was tested and therefore how well association rule mining can be used on other LIS system will need to be verified on an institution by institution basis.

In this manuscript, we analysed a transfusion data set and demonstrated that unsupervised machine learning can provide meaningful additional information about inefficiencies in our transfusion service, including the various consumers of blood products. This novel approach can provide the foundation for using machine learning in managing blood product wastage in transfusion medicine. Future directions for machine learning in transfusion laboratory management can include inventory management, financial forecasting, technologist productivity tracking, and improving donor recruitment and retention. As more data are stored electronically, the implementations of machine learning will expand.

## Acknowledgements

No acknowledgements.

## Conflicts of interest

There are no conflicts of interest relevant to this manuscript submitted to Vox Sanguinis.

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## Supporting Information

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

**Association rule mining results for frozen products.** Each row represents one association between the antecedent and the consequent. The antecedent and the consequent are formatted as "frozenset({<involved factor 1>,<involved factor 2>,<involved factor 3>,...}). Specific antecedent and consequent have been redacted because they contain identifiable information about technologists at our institution. Inf = infinity.

**Association rule mining results for platelets.** Each row represents one association between the antecedent and the consequent. The antecedent and the consequent are formatted in the same way as in supplemental material 1. Specific antecedent and consequent have been redacted because they contain identifiable information about technologists at our institution. Inf = infinity.

**Association rule mining results for RBCs.** Each row represents one association between the antecedent and the consequent. The antecedent and the consequent are formatted in the same way as in supplemental material 1. Specific antecedent and consequent have been redacted because they contain identifiable information about technologists at our institution. Inf = infinity.

VoxSanguinis
The International Journal of Transfusion Medicine
ISBT International Society of Blood Transfusion
*Vox Sanguinis* (2021)

ORIGINAL PAPER


DOI: 10.1111/vox.13098

# Red blood cell storage duration and peri-operative outcomes in paediatric cardiac surgery

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Vox Sanguinis

**Background** Prior research on red blood cell (RBC) storage duration and clinical outcomes in paediatric cardiac surgery has shown conflicting results. The purpose of this study was to evaluate whether blood stored for a longer duration is harmful in these patients.

**Methods** We performed a retrospective cohort study of paediatric patients undergoing cardiac surgery at our institution between January 2011 and June 2015. Patients were stratified based on whether they were transfused RBCs stored for ≤15 days (fresher blood) or >15 days (older blood). The primary outcome was composite morbidity, with prolonged length of stay (LOS) as a secondary outcome. Subgroup analyses were performed after stratification by RBC transfusion volume (≤2 vs. >2 RBC units). Multivariable logistic regression models were used to assess the impact of RBC storage duration on composite morbidity and prolonged LOS.

**Results** Of 461 patients, 122 (26·5%) received fresher blood and 339 (73·5%) received older blood. The overall rate of composite morbidity was 18·0% ($n$ = 22) for patients receiving fresher blood and 13·6% ($n$ = 46) for patients receiving older blood ($P$ = 0·24). In the risk-adjusted model, patients receiving older blood did not exhibit an increased risk of composite morbidity (OR: 0·74, 95% CI: 0·37–1·47, $P$ = 0·40) or prolonged LOS (OR: 0·72, 95% CI: 0·38–1·35, $P$ = 0·30) compared to patients receiving fresher blood. Similar results were seen after stratification by RBC transfusion volume.

**Conclusions** Transfusing RBCs stored for a longer duration was not associated with an increased risk of morbidity or prolonged LOS in paediatric cardiac surgery patients.

*Received: 19 November 2020, revised 24 February 2021, accepted 26 February 2021*

**Key words:** blood, cardiac surgery, length of stay, morbidity, paediatric cardiac surgery, red blood cell storage, transfusion.

## Introduction

Red blood cell (RBC) transfusion is a common intervention in paediatric cardiac surgery [1]. Exposure to allogeneic RBCs occurs for a wide variety of indications in this patient group, including priming of the cardiopulmonary bypass circuit, transfusion during surgery to account for intra-operative blood loss and peri-operative transfusions to restore oxygen carrying capacity. Because the total circulating blood volume in paediatric patients is smaller than that in adults, even a small quantity of allogeneic RBCs can potentially have a large impact on clinical outcomes.

While randomized controlled trials in adult cardiac surgery patients have demonstrated no association between

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RBC storage duration and postoperative outcomes, [2] the data among paediatric cardiac surgery patients are less robust. Much of the research in this patient population has been centred on small retrospective studies, with conflicting results [3–10]. Although some studies suggest poorer outcomes and more peri-operative complications when transfusing RBCs with a longer storage duration, [3–6] others have demonstrated no differences in clinical outcomes [7–10]. These studies, however, have been limited by small sample sizes and differing methodologies in terms of how to best analyse RBC storage duration for patients receiving more than 1 RBC unit [6,7,10]. Likely secondary to these conflicting results, and considering the risks of hyperkalemia, standard practice at most paediatric cardiac surgery centres has been to transfuse RBCs stored for less than 15 days in the youngest patients (under 1 year of age). This practice, however, is not always feasible owing to limitations in blood inventory.

The primary aim of this study was to evaluate the relationship between RBC storage duration and peri-operative outcomes in paediatric patients undergoing cardiac surgery. We hypothesize that transfusing RBCs stored for a longer duration would lead to an increased risk of postoperative complications among paediatric cardiac surgery patients.

## Methods

### Study design

After receiving Institutional Review Board approval with waived informed consent (IRB: NA_00078426), we retrospectively analysed paediatric patients undergoing cardiac surgery at our institution between January 2011 and June 2015. Data were obtained from our institutional patient blood management database, which was created from electronic medical record data acquired through a web-based intelligence portal (Impact Online, Haemonetics, Braintree, MA, USA) and from our hospital billing database [11].

Patients less than 18 years of age who underwent cardiac surgery at the Johns Hopkins Children's Center (with or without the use of cardiopulmonary bypass) and who received at least one RBC unit during their hospitalization were eligible for inclusion in this study. We excluded patients requiring extracorporeal life support (ECMO), as well as those receiving more than 10 RBC units, as these patients likely represented an extreme severity of illness. Their morbidity profiles would be impacted moreso by the disease process and nature of the massive transfusion, rather than the storage duration of the transfused blood. Patients who received RBC units with storage durations of both ≤15 days and >15 days were also excluded, as the transfusion of mixed-age RBCs would have confounded the results of this study [9].

### Bypass practices and transfusion strategies

At our institution, the bypass circuit is primed using whole blood for neonates undergoing complex procedures and with 1 unit of RBCs with 0·5–1 unit of FFP for children less than 7 kg. Modified ultrafiltration is used for children under 20 kg. With respect to transfusion strategies, our primary goal is to keep the haemoglobin (Hb) concentration above 10–12 g/dl in most neonatal surgeries, especially if there is a component of deep hypothermic circulatory arrest, cyanosis or antegrade cerebral perfusion. We do not have a specific paediatric cardiac surgery transfusion algorithm at our institution, but instead focus our intra-operative transfusion efforts on meeting the physiological needs of our patients according to hemodynamic parameters, arterio-venous oxygen differences and cerebral near-infrared spectroscopy readings.

Fresher blood at our institution is primarily given to younger patients. Specifically, ECMO patients, who were excluded from this study, as well as patients in the neonatal intensive care unit (NICU), are preferentially given RBCs stored for <15 days. In the ICU, blood is transfused at a dose of 10–15 ml/kg, whereas in the operating room a full unit of blood is normally provided regardless of the size of the patient. While all RBC units at our institution are pre-storage leukoreduced, they are not routinely washed prior to intra-operative transfusion. Irradiated RBC units are given to children under 6 years of age.

### Baseline characteristics

Demographic and clinical characteristics, such as age, sex, height, weight and the presence of cyanotic disease, were extracted from our institutional patient blood management database. Intra-operative data, including bypass time and cross-clamp time, as well as transfusion data, including RBC storage duration, number of RBC units transfused, starting Hb, nadir Hb and last Hb, were collected through a web-based blood management intelligence portal (Impact Online, Haemonetics). Hb levels were mostly obtained from arterial blood gas samples intra-operatively, and complete blood count laboratory tests in the postoperative setting. The starting Hb refers to the first Hb obtained during the patient's hospitalization, while the last Hb refers to the final Hb obtained prior to discharge. Quality control is routinely performed for these data sources by the Johns Hopkins Clinical Analytics team by comparing electronic medical record data with institutional blood bank data.

### Clinical outcomes and subgroup analysis

In-hospital morbid events were determined by International Classification of Diseases, 9th edition (ICD-9) codes. These included hospital-acquired infections, sepsis, respiratory complications and venous thrombotic events, as we have previously described [12]. A composite of any morbid event (composite morbidity) was designated as the primary outcome. The secondary outcomes were individual morbid events and a prolonged hospital length of stay. The latter was defined as a length of stay longer than the 75th percentile for the entire study population (>11 days).

To account for potential differences based on disease severity, subgroup analyses were performed after stratification by the total number of RBC units transfused. Patients transfused >2 RBC units were classified as high-volume transfusions, while those transfused ≤2 RBC units were classified as low-volume transfusions. This threshold of 2 RBC units was chosen based on prior studies that assessed the impact of RBC storage duration on postoperative outcomes in paediatric cardiac surgery [9].

### Statistical analysis

Patients were divided into two cohorts – those who exclusively received RBC units stored for ≤15 days, and those who exclusively received RBC units stored >15 days. In addition to routine patient characteristics, we used two different scores for the purposes of risk adjustment: the Society of Thoracic Surgeons – European Association for Cardiothoracic Surgery (STAT) category, [13] which was designed to analyse the risk of mortality associated with congenital heart surgery procedures, and the Risk Adjustment for Congenital Heart Surgery (RACHS) score, [14] which was a model developed to predict in-hospital mortality after surgery for congenital heart disease.

JMP version 12 (SAS Institute, Cary, NC, USA) and STATA SE-64 (StataCorp LLC, College Station, TX, USA) were used for statistical analysis. Proportions were compared by chi-square tests and means by Student's *t*-tests. Non-parametric Wilcoxon tests were used to compare medians for data not normally distributed, as determined by histograms. Multivariable logistic regression models were developed to evaluate the association between RBC storage duration and the primary and secondary outcomes after controlling for clinically relevant covariates such as age, sex, height, weight, cardiopulmonary bypass time, cross-clamp time, STAT category and RACHS score. To account for the fact that the presence of cardiopulmonary bypass increases the risk of postoperative complications, additional multivariable analyses were performed solely among patients who underwent surgical procedures involving cardiopulmonary bypass. Due to the small sample size, multivariable logistic regression models could not be used to assess the association between RBC storage duration and individual morbid events after stratification by RBC transfusion volume. Statistical significance was defined as $P < 0{\cdot}05$.

## Results

### Study population

A total of 1155 paediatric patients underwent cardiac surgical procedures at our institution between January 2011 and June 2015, all of whom had complete data on RBC storage duration. Of those, 694 patients were excluded as they received >10 units of blood, or because they were transfused RBCs that belonged to both storage duration groups. The remaining 461 patients met the inclusion criteria, of which 26·5% ($n$ = 122) received blood stored for ≤15 days and 73·5% ($n$ = 339) received blood stored for >15 days (Figure 1).

After stratification by RBC transfusion volume, 64·4% ($n$ = 297) were in the low-volume cohort (≤2 total RBC units) and 35·6% ($n$ = 164) were in the high-volume cohort (>2 total RBC units). Among patients in the low-volume subgroup, 33·3% ($n$ = 99) received blood stored for ≤15 days and 66·6% ($n$ = 198) received blood stored for >15 days. This is in comparison with the high-volume subgroup, where 14·0% ($n$ = 23) received blood stored for ≤15 days and 86·0% ($n$ = 141) received blood stored for >15 days.

### Patient characteristics

Patients who received RBCs stored for a longer duration were older, taller, heavier and had a higher incidence of cyanotic heart disease (Table 1). The two groups did not differ in terms of RACHS score, STAT category or operating times (cardiopulmonary bypass time, cross-clamp time and duration of deep hypothermic arrest). The groups also had similar preoperative Hb levels, although patients in the longer RBC storage duration group had a slightly lower nadir Hb during their hospital stay and a lower last Hb prior to discharge. Patients who were transfused blood ≤15 days old received fewer total RBC units throughout their hospitalization compared to patients who received blood stored for >15 days ($P < 0{\cdot}001$) (Table 1).

After stratification by RBC transfusion volume, patients in both subgroups receiving RBCs stored for >15 days were older, heavier, taller and more likely to have a lower nadir Hb during their hospitalization. In the low-volume

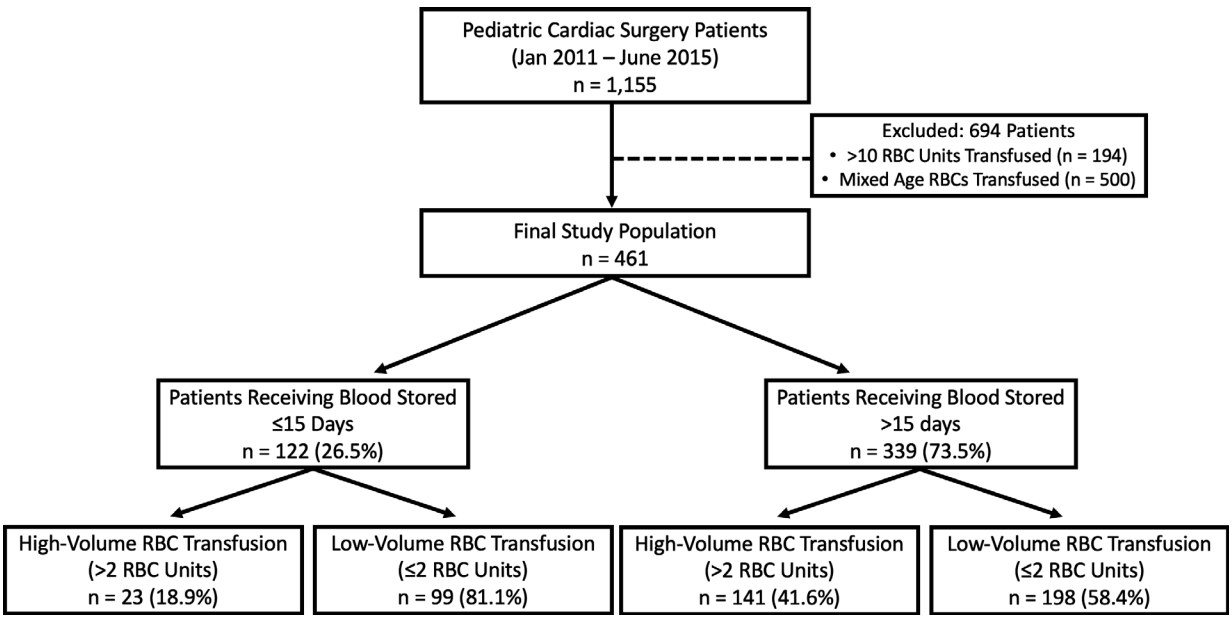


**Figure 1** Flow diagram outlining inclusion and exclusion criteria for our study population of paediatric cardiac surgery patients.

subgroup specifically, patients receiving RBCs stored for >15 days were also more likely to have a higher RACHS score and be in a higher STAT category (Table 2).

## Clinical outcomes

There was no difference in the rate of composite morbidity between patients receiving blood ≤15 days old (22 of 122; 18·0%) and >15 days old (46 of 339; 13·6%) ($P = 0·24$) (Table 3). When analysing individual morbid events, patients receiving blood stored for ≤15 days duration exhibited higher rates of sepsis ($P = 0·0036$) and hospital-acquired infection ($P = 0·048$) compared to those receiving blood stored for >15 days. Median length of stay was not significantly different between patients in the ≤15-day (median: 7, IQR: 5–9) and >15-day (median: 7, IQR: 5–11) ($P = 0·82$) cohorts, and the incidence of prolonged length of stay (>75th percentile [or 11 days]) was similar between the ≤15-day (28 of 122; 23·0%) and >15-day (80 of 339; 23·6%) cohorts ($P = 1·00$) (Table 3).

When stratifying by RBC transfusion volume, the overall rate of composite morbidity for the high-volume transfusion cohort was 19·5% (32 of 164), compared to 12·1% (36 of 297) for the low-volume transfusion cohort ($P = 0·04$). There were no differences in the rate of composite morbidity between the ≤15-day and >15-day cohorts for both the high-volume and low-volume transfusion subgroups (Table 4). However, patients receiving blood stored for ≤15 days in the low-volume transfusion subgroup exhibited a higher incidence of sepsis ($P = 0·0008$), hospital-acquired infection ($P = 0·0011$) and prolonged length of stay ($P = 0·011$) compared to those receiving blood stored for >15 days. These findings were not seen in the high-volume transfusion subgroup.

## Multivariable analysis

Multivariable analysis demonstrated that prolonged RBC storage duration was not associated with an increased risk for composite morbid events (OR: 0·74, 95% CI: 0·37–1·47, $P = 0·40$) or prolonged length of stay (OR: 0·72, 95% CI: 0·38–1·35, $P = 0·30$) (Table 5). There was also no association between RBC storage duration and risk of individual morbid events, such as venous thrombotic events, respiratory complications, sepsis or hospital-acquired infection. These results stayed consistent even after excluding patients who did not have cardiopulmonary bypass (Table 5).

Similar findings were seen after subgroup analysis based on RBC transfusion volume. For both the high-volume (>2 RBC units) and low-volume (≤2 RBC units) transfusion cohorts, multivariable analysis demonstrated that prolonged RBC storage duration was not associated with an increased risk for composite morbid events or prolonged length of stay (Table 6). Once again, there was no association between RBC storage duration and clinical outcomes even after excluding patients who did not have cardiopulmonary bypass (Table 6).

**Table 1** Baseline demographic and clinical characteristics of paediatric cardiac surgery patients both overall and after stratification by red blood cell storage duration

| Characteristic, mean ± SD | All patients (*n* = 461) | ≤15 days RBC storage duration (*n* = 122) | >15 days RBC storage duration (*n* = 339) | *P*-value |
|---|---|---|---|---|
| Age (years) | 4.3 ± 5.4 | 2.1 ± 3.6 | 5.2 ± 5.7 | <0.0001 |
| Age (years), median (IQR) | 1.4 (0.4–6.8) | 0.4 (0.1–2.6) | 2.8 (0.5–8.9) | <0.0001 |
| Sex, *n* (%) | | | | |
| Female | 210 (45.5) | 60 (47.5) | 150 (44.5) | 0.61 |
| Male | 251 (54.5) | 66 (52.5) | 185 (55.5) | |
| Weight (kg) | 17.8 ± 19.7 | 10.1 ± 11.9 | 20.6 ± 21.2 | <0.0001 |
| Weight (kg), median (IQR) | 9.0 (5.8–21.9) | 6.2 (3.3–11.2) | 11.9 (6.4–23.7) | <0.0001 |
| Height (cm) | 90 ± 40 | 70 ± 32 | 98 ± 40 | <0.0001 |
| Height (cm), median (IQR) | 77 (60–119) | 63 (48–84) | 88 (63–126) | <0.0001 |
| Cases on bypass, *n* (%) | 389 (84.4) | 83 (66.4) | 306 (91.1) | <0.0001 |
| Bypass time (min) | 127 ± 55 | 128 ± 53 | 127 ± 56 | 0.81 |
| Cross–clamp time (min) | 65 ± 47 | 68 ± 49 | 65 ± 4 | 0.64 |
| Blood storage duration (days) | 19 ± 6 | 15 ± 1 | 20 ± 5 | <0.0001 |
| Blood storage duration (days), median (IQR) | 16 (15–19) | 15 (15–15) | 18 (16–22) | <0.0001 |
| RBC units transfused, median (IQR) | 2 (1–3) | 2 (1–2) | 2 (1–3) | <0.0001 |
| Starting haemoglobin (g/dL) | 13.0 ± 2.5 | 13 ± 2.7 | 13.0 ± 2.4 | 0.95 |
| Nadir haemoglobin (g/dl) | 9.3 ± 1.9 | 9.9 ± 1.9 | 9.1 ± 1.8 | <0.0001 |
| Last haemoglobin (g/dl) | 11.7 ± 2.0 | 12.2 ± 2.1 | 11.6 ± 1.9 | 0.002 |
| Cyanotic disease, n (%) | 104 (22.5) | 21 (16.9) | 83 (24.6) | 0.023 |
| Univentricular repair, *n* (%) | 69 (15.0) | 16 (12.9) | 53 (15.8) | 0.35 |
| STAT category, *n* (%) | | | | |
| 1 | 170 (36.8) | 42 (33.9) | 126 (37.5) | 0.37 |
| 2 | 201 (43.6) | 63 (50.4) | 138 (40.9) | |
| 3 | 32 (6.9) | 5 (4.0) | 28 (8.4) | |
| 4 | 57 (12.4) | 14 (11.3) | 44 (13.1) | |
| 5 | 1 (0.23) | 1 (0.81) | 0 (0.0) | |
| RACHS score, *n* (%) | | | | |
| 1 | 69 (14.9) | 30 (24.3) | 38 (11.3) | 0.20 |
| 2 | 214 (46.5) | 61 (48.5) | 153 (45.6) | |
| 3 | 163 (35.3) | 30 (24.3) | 132 (39.3) | |
| 4 | 15 (3.2) | 4 (2.9) | 12 (3.6) | |
| 5 | 0 (0.0) | 0 (0.0) | 0 (0.0) | |
| 6 | 0 (0.0) | 0 (0.0) | 0 (0.0) | |

Abbreviations: RACHS score, Risk Adjustment for Congenital Heart Surgery score; RBC, red blood cell; STAT category, Society of Thoracic Surgeons – European Association for Cardiothoracic Surgery category. *P*-values represent comparisons between RBC storage duration cohorts.

**Table 2** Baseline demographic and clinical characteristics of paediatric cardiac surgery patients after stratification by red blood cell storage duration and red blood cell transfusion volume

| | High-volume transfusions (>2 RBC units) | | | Low-volume transfusions (≤2 RBC Units) | | |
|---|---|---|---|---|---|---|
| **Characteristic, mean ± SD** | **≤15 days RBC storage duration (*n* = 23)** | **>15 days RBC storage duration (*n* = 141)** | ***P*-value** | **≤15 days RBC storage duration (*n* = 99)** | **>15 days RBC storage duration (*n* = 198)** | ***P*-value** |
| Age (years) | 2.8 ± 4.3 | 5.1 ± 5.9 | 0.039 | 1.9 ± 3.4 | 5.2 ± 5.5 | <0.0001 |
| Age (years), median (IQR) | 0.6 (0.3–5.1) | 1.2 (0.5–9.4) | 0.049 | 0.4 (0.1–1.9) | 3.2 (0.6–8.3) | <0.0001 |
| Sex, *n* (%) | | | | | | |
| Female | 9 (39.1) | 63 (44.7) | 0.66 | 49 (50.0) | 89 (45.0) | 0.46 |
| Male | 14 (60.9) | 78 (55.3) | | 49 (50.0) | 109 (55.0) | |
| Weight (kg) | 13.7 ± 14.6 | 19.7 ± 19.5 | 0.046 | 9.4 ± 11.3 | 21.1 ± 22.2 | <0.0001 |
| Weight (kg), median (IQR) | 7.2 (5.3–20.0) | 8.9 (6.2–27.7) | 0.12 | 5.7 (2.8–10.0) | 13.5 (6.8–23.1) | <0.0001 |
| Height (cm) | 81 ± 30 | 97 ± 43 | 0.029 | 68 ± 33 | 98 ± 39 | <0.0001 |
| Height (cm), median (IQR) | 68 (60–105) | 76 (63–130) | 0.11 | 60 (45–82) | 91 (64–121) | <0.0001 |
| Cases on bypass, *n* (%) | 23 (100.0) | 128 (90.8) | 0.22 | 57 (57.6) | 181 (91.4) | <0.0001 |
| Bypass time (min) | 164 ± 53 | 154 ± 63 | 0.41 | 113 ± 45 | 108 ± 41 | 0.49 |
| Cross-clamp time (min) | 86 ± 63 | 78 ± 56 | 0.57 | 60 ± 40 | 56 ± 37 | 0.46 |
| Blood storage duration (days) | 14 ± 3 | 21 ± 6 | <0.0001 | 15 ± 1 | 19 ± 6 | <0.0001 |
| Blood storage duration (days), median (IQR) | 15 (15–15) | 18 (16–23) | <0.0001 | 15 (15–15) | 17 (16–21) | <0.0001 |
| RBC units transfused, median (IQR) | 3 (3–4) | 4 (3–5) | 0.15 | 1 (1–2) | 2 (1–2) | 0.46 |
| Starting haemoglobin (g/dl) | 13.8 ± 2.5 | 13.3 ± 2.8 | 0.33 | 12.8 ± 2.7 | 12.8 ± 2.0 | 0.83 |
| Nadir haemoglobin (g/dl) | 10.5 ± 1.8 | 9.1 ± 2.0 | 0.0019 | 9.8 ± 1.9 | 9.1 ± 1.7 | 0.0015 |
| Last haemoglobin (g/dl) | 12.7 ± 2.0 | 12.3 ± 2.0 | 0.36 | 12.2 ± 2.2 | 11.1 ± 1.7 | <0.0001 |
| Cyanotic disease, *n* (%) | 8 (34.8) | 53 (38.1) | 0.87 | 11 (11.2) | 30 (15.5) | 0.38 |
| Univentricular repair, *n* (%) | 7 (30.4) | 37 (26.6) | 0.86 | 8 (8.2) | 16 (8.3) | 1.00 |
| STAT category, *n* (%) | | | | | | |
| 1 | 5 (21.7) | 25 (19.2) | 0.15 | 37 (37.8) | 96 (49.2) | 0.015 |
| 2 | 7 (30.4) | 61 (46.9) | | 54 (55.1) | 72 (36.9) | |
| 3 | 2 (8.7) | 19 (14.6) | | 2 (2.0) | 9 (4.6) | |
| 4 | 9 (39.1) | 25 (19.2) | | 4 (4.1) | 18 (9.2) | |
| 5 | 0 (0.0) | 0 (0.0) | | 1 (1.0) | 0 (0.0) | |
| RACHS score, *n* (%) | | | | | | |
| 1 | 0 (0.0) | 1 (1.0) | 0.90 | 25 (30.1) | 30 (17.3) | 0.036 |
| 2 | 7 (38.9) | 42 (40.8) | | 42 (50.6) | 84 (48.6) | |
| 3 | 9 (50.0) | 53 (51.5) | | 15 (18.1) | 56 (32.4) | |
| 4 | 2 (11.1) | 7 (6.8) | | 1 (1.2) | 3 (1.7) | |
| 5 | 0 (0.0) | 0 (0.0) | | 0 (0.0) | 0 (0.0) | |
| 6 | 0 (0.0) | 0 (0.0) | | 0 (0.0) | 0 (0.0) | |

Abbreviations: RACHS score, Risk Adjustment for Congenital Heart Surgery score; RBC, red blood cell; STAT category, Society of Thoracic Surgeons – European Association for Cardiothoracic Surgery category.
*P*-values represent comparisons between RBC storage duration cohorts.

**Table 3** Clinical outcomes for paediatric cardiac surgery patients both overall and after stratification by red blood cell storage duration

| | All patients ($n$ = 461) | ≤15 Days RBC Storage Duration ($n$ = 122) | >15 days RBC storage duration ($n$ = 339) | $P$-value |
|---|---|---|---|---|
| Composite morbidity | 68 (14.8) | 22 (18.0) | 46 (13.6) | 0.24 |
| Venous thrombotic event | 27 (5.9) | 4 (3.3) | 23 (6.8) | 0.18 |
| Respiratory complications | 24 (5.2) | 9 (7.4) | 15 (4.4) | 0.24 |
| Sepsis | 12 (2.6) | 8 (6.6) | 4 (1.2) | 0.0036 |
| Hospital-acquired infection | 22 (4.7) | 10 (8.2) | 12 (3.5) | 0.048 |
| Prolonged hospital length of stay[a] | 108 (23.4) | 28 (23.0) | 80 (23.6) | 1.00 |

Abbreviations: RBC, red blood cell.

$P$-values represent comparisons between RBC storage duration cohorts.

[a]Prolonged length of stay defined based on 75th percentile of the study population (>11 days).

**Table 4** Clinical outcomes for paediatric cardiac surgery patients after stratification by red blood cell storage duration and red blood cell transfusion volume

| | High-volume transfusions (>2 RBC units) | | | | Low-volume transfusions (≤2 RBC units) | | | |
|---|---|---|---|---|---|---|---|---|
| | All patients ($n$ = 164) | ≤15 days RBC storage duration ($n$ = 23) | >15 Days RBC Storage Duration ($n$ = 141) | $P$-value | All patients ($n$ = 297) | ≤15 days RBC storage duration ($n$ = 99) | >15 days RBC storage duration ($n$ = 198) | $P$-value |
| Composite morbidity | 32 (19.5) | 5 (21.7) | 27 (19.2) | 0.78 | 36 (12.1) | 17 (17.2) | 19 (9.6) | 0.09 |
| Venous thrombotic event | 19 (11.6) | 3 (13.0) | 16 (11.4) | 0.73 | 8 (2.7) | 1 (1.0) | 7 (3.5) | 0.28 |
| Respiratory complications | 7 (4.3) | 2 (8.7) | 5 (3.6) | 0.25 | 17 (5.7) | 7 (7.1) | 10 (5.1) | 0.60 |
| Sepsis | 3 (1.8) | 0 (0.0) | 3 (2.1) | 1.00 | 9 (3.0) | 8 (8.1) | 1 (0.5) | 0.0008 |
| Hospital-acquired infection | 11 (6.7) | 1 (4.4) | 10 (7.1) | 1.00 | 11 (3.7) | 9 (9.1) | 2 (1.0) | 0.0011 |
| Prolonged hospital length of stay[a] | 68 (41.5) | 7 (30.4) | 61 (43.3) | 0.27 | 40 (13.5) | 21 (21.2) | 19 (9.6) | 0.011 |

Abbreviations: RBC, red blood cell.

$P$-values represent comparisons between RBC storage duration cohorts for each subgroup.

[a]Prolonged length of stay defined based on 75th percentile of the study population (>11 days).

**Table 5** Multivariable logistic regression assessing the risk of prolonged red blood cell storage duration (vs. shorter duration) on various clinical parameters, among all patients and the subset undergoing cardiopulmonary bypass

| | Odds ratio | 95% CI | *P*-value |
|---|---|---|---|
| All patients ($n$ = 461) | | | |
| Composite morbidity | 0.74 | 0.37–1.47 | 0.40 |
| Venous thrombotic event | 2.43 | 0.49–11.9 | 0.28 |
| Respiratory complications | 0.42 | 0.16–1.13 | 0.09 |
| Sepsis | 0.19 | 0.03–1.26 | 0.09 |
| Hospital-acquired infection | 0.37 | 0.11–1.20 | 0.10 |
| Prolonged hospital length of stay[a] | 0.72 | 0.38–1.35 | 0.30 |
| Patients undergoing cardiopulmonary bypass ($n$ = 389) | | | |
| Composite morbidity | 0.61 | 0.29–1.38 | 0.23 |
| Venous thrombotic event | 2.19 | 0.54–15.2 | 0.30 |
| Respiratory complications | 0.45 | 0.18–1.19 | 0.10 |
| Sepsis | 0.64 | 0.05–16.3 | 0.74 |
| Hospital-acquired infection | 0.40 | 0.06–3.20 | 0.35 |
| Prolonged hospital length of stay[a] | 2.27 | 0.54–2.70 | 0.70 |

Independent variables included in the multivariable models were age, sex, height, weight, STAT category and RACHS score.

[a]Prolonged length of stay defined based on 75th percentile of the study population (>11 days).

## Discussion

In this study of paediatric cardiac surgery patients, prolonged RBC storage duration was not associated with an increased risk of composite morbidity. There was also no association between RBC storage duration and individual morbid events or prolonged length of stay after risk adjustment for potential confounders. These findings are consistent with randomized trials in adult patients that have shown no adverse effects when transfusing older RBCs [2,15,16]. In the paediatric population, however, the impact of RBC storage duration on clinical outcomes is less well established.

In critically ill paediatric patients, [17] preterm neonates [18] and severely anaemic children with malaria, [19] clinical trials have demonstrated no association between RBC storage duration and clinical outcomes. However, the data in paediatric cardiac surgery are less clear, as there have only been a few retrospective studies published to date, with mixed results. Some have shown adverse effects when transfusing blood stored for a longer duration, [3–6] while others have shown no differences in clinical outcomes [7–10]. Many of these studies have been limited by small sample sizes, [6,10] and unclear methodology regarding how RBC storage duration was classified [7,10]. While there are multiple ways to analyse RBC storage duration for patients who are transfused more than 1 RBC unit, such as categorizing patients based on the oldest RBC unit transfused, [6] or simply averaging the age of the RBC units, [3] each of these methodologies is associated with significant amounts of confounding. The true association between RBC storage duration and clinical outcomes cannot be assessed when patients are transfused both fresher and older blood. Our study only included patients who received blood of a specific storage duration and contributes to the growing body of literature emphasizing that RBC storage duration may not impact clinical outcomes for children undergoing cardiac surgery [7–10].

It is well known that many biochemical changes occur with blood storage, such as decreased cell membrane deformability, decreased 2,3-diphosphoglycerate levels and a left shift of the oxygen dissociation curve, which potentially decreases oxygen delivery at the tissue level [20,21]. Importantly, hyperkalemia can result in cardiac arrest upon RBC transfusion, especially in paediatric

**Table 6** Multivariable logistic regression assessing the risk of prolonged red blood cell storage duration (vs. shorter duration) on various clinical parameters after stratification by red blood cell transfusion volume, among all patients and the subset undergoing cardiopulmonary bypass

| | High-volume transfusions (>2 RBC units) | | | Low-volume transfusions (≤2 RBC units) | | |
|---|---|---|---|---|---|---|
| | Odds Ratio | 95% CI | *P*-value | Odds Ratio | 95% CI | *P*-value |
| All patients ($n$ = 461) | | | | | | |
| Composite morbidity | 0.90 | 0.24–4.00 | 0.88 | 0.48 | 0.17–1.43 | 0.18 |
| Prolonged hospital length of stay[a] | 2.60 | 0.76–11.0 | 0.13 | 0.53 | 0.16–1.84 | 0.31 |
| Patients undergoing cardiopulmonary bypass ($n$ = 389) | | | | | | |
| Composite morbidity | 0.89 | 0.23–4.15 | 0.87 | 0.45 | 0.16–1.33 | 0.15 |
| Prolonged hospital length of stay[a] | 2.64 | 0.76–11.6 | 0.13 | 0.51 | 0.16–1.66 | 0.25 |

Independent variables included in the multivariable models were age, sex, height, weight, cardiopulmonary bypass time, cross-clamp time, STAT category and RACHS score.

Abbreviations: RBC, red blood cell.

[a]Prolonged length of stay defined based on 75$^{th}$ percentile of the study population (>11 days).

patients who require rapid transfusion, such as during cardiac surgery [22]. It has been demonstrated that potassium levels in the storage solution supernatant increase incrementally over the entire 6-week period of FDA-allowed storage [23]. Other RBC storage lesions that may affect clinical outcomes include increased endothelial adherence and increased aggregability [24]. Additionally, continued depletion of nitric oxide causes RBCs to become nitric oxide scavengers, thereby contributing to local vasoconstriction of the microcirculation and impeding oxygen delivery even further [25,26].

In light of these physiologic changes to stored RBCs, it is unknown why many retrospective studies in paediatric cardiac surgery, including ours, have demonstrated negative findings with respect to RBC storage duration and clinical outcomes [7–10]. One hypothesis is that the risks associated with transfusing older blood products may be mitigated by improved peri-operative care. It is also possible that differences exist with respect to patients' illness severity and that patients may be otherwise healthy for blood storage duration to impact clinical outcomes. Moreover, paediatric patients generally receive less blood compared to adults and thus may not experience some of the anticipated effects of older blood due to the smaller total volume. This is likely to only be a minor issue in our study population though, as paediatric cardiac surgery patients tend to receive a large volume of RBCs relative to their body mass to compensate for the volume of the cardiac bypass circuit [27]. Another potential reason for the negative findings in this study was the smaller than anticipated difference in mean RBC storage duration between our fresher and older blood cohorts. It is possible that the metabolic changes associated with prolonged RBC storage duration did not differ significantly between the fresher and older blood, thus preventing us from detecting a difference in clinical outcomes between these two groups.

Nevertheless, on univariate analysis we did find that patients transfused blood stored for <15 days had a higher incidence of sepsis and hospital-acquired infection, particularly among the low-volume transfusion subgroup. While the exact reasons for this finding are unknown, it is likely due to selection bias among our patient population, as these may represent younger patients with a higher severity of illness. It is important to note that after controlling for these relevant characteristics in the multivariable analysis, there was no association between RBC storage duration and individual morbid complications.

Several limitations of this study should be recognized. First, this was a single-centre analysis with a retrospective design, making it susceptible to institutional bias owing to its relatively small sample size. Second, due to the retrospective nature of this study, we were unable to present RBC transfusion volumes as ml/kg, as is sometimes done for neonates. Neonates present a unique situation because they are often transfused fresher blood due to the risk of hyperkalemia [23]. However, only a small proportion (8·2%) of our study population were neonates, and thus, if biases were to exist related to these factors, they would likely be minimal and would not detract from our overall conclusions. Third, the primary outcome of our study, namely composite morbidity, was chosen based on prior studies on RBC storage duration [7,9]. While we recognize that additional outcomes focused primarily on paediatric cardiac surgery, such as arrythmias or haemorrhage, would have been helpful, these variables were unfortunately not available within our database. ICU length of stay in particular could not be assessed due to significant amounts of missing data. Fourth, we were unable to stratify patients based on the type of RBC preservative solution used. Through conversations with experts in our blood bank, we determined that the majority of RBC units were stored in Additive Solution (AS-1), while a smaller proportion were stored in Citrate Phosphate Dextrose Adenine Solution (CPDA). However, more granular data were not available. We recognize that RBCs stored in AS-1 have a longer shelf-life (42 days) compared to RBCs stored in CPDA (35 days), which could be another potential confounder. Fifth, at our institution, irradiated RBCs are given to younger patients. This introduces another potential confounder, since irradiation causes damage to the RBCs, consequently reducing their shelf-life. To address this concern, we performed an additional analysis solely among patients <5 years of age. This subgroup analysis demonstrated no association between RBC storage duration and peri-operative outcomes, suggesting that potential bias associated with irradiated RBCs is unlikely to have influenced the primary findings of our study.

Finally, there is also the issue of what truly constitutes older blood, and how that determination is made. In this study, we chose to treat RBC storage duration as a dichotomous, rather than continuous variable, in order to increase the clinical utility of our findings. We defined older blood as blood stored for greater than 2 weeks, which is consistent with prior studies performed in adults, children and infants [6,10,28]. By using this 15-day cut-off though, it is possible that many of the metabolic changes associated with prolonged RBC storage duration, such as depletion of 2,3-diphosphoglycerate levels, may have already occurred [20,21]. This, in turn, could have impacted our ability to detect a meaningful difference in clinical outcomes between our two storage duration groups.

## Conclusions

In conclusion, we found no clear association between RBC storage duration and peri-operative outcomes among paediatric cardiac surgery patients. These findings add to the growing body of literature emphasizing the safety of transfusing older RBC units and provide anaesthesiologists, surgeons and blood bankers with a useful framework when considering the potential risks and benefits of transfusing fresher versus older blood.

## Sources of funding

J.S. has support from a K08 grant (K08HL145132) awarded by the National Heart, Lung, and Blood Institute.

## Conflict of interests

S.M.F. participates on scientific advisory boards for Haemonetics, Medtronic and Baxter. All other authors declare no conflicts of interest.

## Author contributions

All authors were involved in study design and interpretation of the data. A.P., B.D.L., S.M.F. and J.S. were responsible for analysing the data and drafting the manuscript. All authors critically revised the manuscript and approved of the final version.

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VoxSanguinis
The International Journal of Transfusion Medicine
International Society of Blood Transfusion
*Vox Sanguinis* (2021)

ORIGINAL PAPER


DOI: 10.1111/vox.13085

# Exchange transfusion in the management of critical pertussis in young infants: a case series

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Vox Sanguinis

**Background and objectives** It is proposed that severe leucocytosis mainly contributes to pulmonary hypertension by blocking pulmonary capillaries and restricting blood flow. Exchange transfusion (ET) in pertussis has been demonstrated as a safe and useful technique for depleting the leucocyte mass. We aim to discuss four cases of pertussis-induced respiratory distress and the effectiveness of ET in such a setting.

**Materials and methods** We conducted a retrospective case series at the Infectious Disease Department of Children's Hospital 2 in Ho Chi Minh City, Vietnam, and included four pertussis patients that were confirmed by PCR tests on respiratory secretions, presented with severe leucocytosis and respiratory distress and required mechanical ventilation.

**Results** Among the included patients, three underwent a double volume ET for leucodepletion, two of whom were discharged after the procedure with proper vitals and laboratory test results. On the other hand, one patient died despite ET, performed late in the course of the disease. Exchange transfusion was not performed in the last patient who died as well.

**Conclusion** Early ET may be a useful and rapid life-saving treatment in children with critical pertussis and severe leucocytosis before cardiopulmonary complications appear.

*Received: 12 September 2020, revised 23 January 2021, accepted 23 January 2021*

**Key words:** critical pertussis, exchange transfusion, hyperleucocytosis, pulmonary hypertension.

## Introduction

Pertussis (whooping cough) is an infectious disease caused by *Bordetella pertussis* which is transmitted through respiratory tract secretions. Over the past few years, pertussis has been re-emerging all over the world [1]. It usually occurs in early infancy and can be prevented by adequate vaccination [2]. In 2018, the World Health Organization reported 151 074 pertussis cases, with a 4% mortality rate [3]. In Vietnam, the number of new cases has been recently increasing, from 267 cases (in 2016) to 1013 cases (in 2019) [4]. Critical pertussis is simply defined as a complication of death or intensive care unit (ICU) admission as a result of pertussis infection. Management of pertussis-induced complications can be challenging sometimes. Many articles reporting the admission of patients with pertussis-induced complications to the ICU have been published [5–7]. Critical pertussis, characterized by refractory hypoxaemia, pulmonary arterial hypertension, heart failure, with drastic leucocytosis requires ICU admission [6,8]. These

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clinical manifestations affect only a small fraction of children with pertussis; however, considerations should be made to reduce the high mortality rate [5]. It has been suggested that leucocytosis correlates with high mortality rates in pertussis patients [9]. Accordingly, leucodepletion (which can be achieved by leucapheresis, fluid infusion and exchange transfusion) can be considered a method to improve the prognosis [10,11]. In this article, we present four cases of young infants suffering from critical pertussis and the effectiveness of using blood exchange transfusion (ET) at the Infectious Diseases Department, Children's Hospital 2 (Ho Chi Minh City, Vietnam) in 2019.

## Cases presentation

In this report, we retrospectively reviewed the clinical data of four patients with the following criteria: (1) having a positive pertussis polymerase chain reaction (PCR) result, (2) WBCs count >50 000/$mm^3$ and (3) having severe respiratory distress that required mechanical ventilation and hospitalized at the Infectious Diseases Department, Children's Hospital 2 (Ho Chi Minh City, Vietnam) in 2019. Among the four patients that were included in our study, three underwent a double volume ET once, in three hours through the right femoral vein using a central catheter (Certofix® Duo Paed S408). In each 5–7-minute cycle, 15–40 ml of patients' blood was removed and replaced with a donor's fresh frozen plasma and packed RBCs according to each patient's blood group. The procedure was indicated based on Rowland *et al.* criteria for performing ET in pertussis patients to counteract the leucocytic crisis (WBCs > 50–70 $\times$ $10^3$/ml) and the complications it induces [12]. In the 3rd and the 4th cases, the patients promptly underwent ET when WBCs count reached above 50 $\times$ $10^3$/ml and in the absence of cardiac complications (pulmonary hypertension or cardiac shock). Their WBCs counts decreased remarkably with subsequent rapid respiratory functions improvement (ventilator parameters and arterial blood gas) (Table 2). Although both patients suffered from hospital-acquired pneumonia later, they were successfully discharged from the hospital after achieving full recovery. Patients' demographics and laboratory characteristics are presented in Tables 1 and 2 and Fig. 1. A detailed summary of all cases is described as follows:

### Case 1

A 1-month-old male patient with increasing cough and wheezing for 2 weeks was diagnosed at Dong Nai Children's Hospital with severe pneumonia-induced septicaemia and initially treated with nasal continuous positive airway pressure (NCPAP) ventilation and antibiotics including cefepime, amikacin, imipenem and vancomycin. After 6 days of treatment, with no improvement in his respiratory distress, the child was transferred to Children's Hospital 2. His vaccination included BCG, hepatitis B, but he had not been vaccinated against pertussis, yet. When admitted to the hospital, he was having pneumonia with severe respiratory insufficiency complications. Laboratory test results showed leucocytosis (72·9 $\times$ $10^3$/ml, N 44%, L 32%), toxic granulation in the peripheral blood smear, thrombocytosis (582 $\times$ $10^3$/ml) and normal haemoglobin (Hgb 10·9 g/dl). Arterial blood gases (ABG) test showed respiratory acidosis with concomitant metabolic acidosis, alveolar-capillary barrier damage (pH/$PCO_2$/$PO_2$: 7·16/59 mmHg/169 mmHg), C-reactive protein (CRP) level of 13·2 mg/l with serious hyponatraemia (103 mEq/l). Chest X-ray revealed an underlying alveolar injury and right upper lobe collapse. Echocardiogram revealed pulmonary arterial hypertension (sPAP: 40 mmHg), tricuspid valve regurgitation, mild pericardial effusion, but preserved ejection fraction ratio (EF 75%). Sputum analysis by PCR was positive with *Bordetella pertussis*, *Aspergillus* and *Burkholderia cepacia*. With a diagnosis of severe pneumonia – sepsis – pertussis, he was treated with invasive ventilation, fluid and saline infusion, and antibiotics including meropenem, vancomycin, levofloxacin and azithromycin. However, under the serious lung damage, prolonged hypercapnia, severe hyponatraemia, heart failure with poor response to extreme invasive ventilation, high-dose inotropes/vasopressors (1·5 μg/kg/min), continuous renal replacement therapy (CRRT) was performed to correct hyponatraemia and refractory shock and hopefully eliminate pertussis toxins. However, the patient died 5 days after hospital admission. ET was not conducted with this patient as the attending physicians were not familiar with such cases and due to the late pertussis PCR confirmation.

### Case 2

A 10-month-old female patient was admitted to Children's Hospital 2 after 2 weeks of violent coughing, prolonged wheezing, high fever, breathing difficulty with no improvement after being treated at Binh Phuoc Hospital. The child had not been fully vaccinated (only 1 shot of hepatitis B and BCG when delivered until the time of admission). Physical examination revealed high fever, respiratory failure with cyanosis, decreased $SpO_2$, tachycardia (180 bpm), 3/6 systolic murmur, chest retraction with a respiratory rate of 55 bpm and crackles on auscultation. Laboratory tests showed leucocytosis (43 $\times$ $10^3$/ml) and elevated CRP (90 mg/l). The initial diagnosis was severe pneumonia-induced septicaemia and suspected haematologic malignancies. And she was treated with oxygen therapy, broad-spectrum antibiotics as meropenem,

**Table 1** Demographics and laboratory characteristics of the included patients.

| Characteristics | Case 1 | Case 2 | Case 3 | Case 4 |
|---|---|---|---|---|
| Gender | Male | Female | Female | Female |
| Age (month) | 1 | 10 | 4 | 4 |
| Birth status | Full-term (IVF) | Full-term | Full-term | Full-term |
| Birth weight (g) | 3000 | 3200 | 2800 | 3500 |
| Province | Dong Nai | Binh Phuoc | Gia Lai | Dong Nai |
| Contact with patients having respiratory symptoms | Positive | Positive | Negative | Negative |
| Presenting symptoms | Fever, dyspnoea | Respiratory insufficiency | Fever, cough, dyspnoea | Fever, breathing difficulty |
| Day of onset (day) | 5 | 13 | 3 | 6 |
| Peak WBC ($\times 10^3$/ml) | 72·9 | 108 | 78 | 65 |
| AST/ALT (IU/l) | 46/16 | 66/19 | 34/21 | 19/12 |
| Creatinine (μmol/l) | 29 | 34 | 36 | 40 |
| Serum lactate (mmol/l) | 0·7 | 2·01 | 2·7 | * |
| Troponin I (ng/ml) | 0·065 | 0·0234 | * | * |
| $AaDO_2$ (mmHg) | 99·3 | 444 | 94·8 | 280 |
| sPAP (mmHg) | 40 | 70 | * | * |
| Chest X-ray | Right apex pulmonary collapse | Alveolar damage, air trapping | Bilateral consolidation | Consolidation on upper right lung |
| Combined pathogen | *B. cepacia, Aspergillus* | CMV, *B. cepacia* | Acinetobacter | *E. coli* |
| Maximum parameters of mechanical ventilation (IP/PEEP/$FiO_2$) | 34/10/100% | 24/8/100% -->HFOV (MAP 28) | 18/7/40% | 18/10/60% |
| Time of ET | * | 4 days 5·5 hours | 4 days | 4 days 20 hours |
| Time of CRRT | 29 hours | 6 days | * | * |
| Time of hospital stay (day) | 5 | 8 | 39 | 26 |
| ET details | | | | |
| Packed RBCs (ml) | * | 600 | 250 | 500 |
| Plasma (ml) | * | 600 | 300 | 600 |
| Cycle | | | | |
| Blood volume removed (ml) | * | 40 | 15 | 30 |
| Inserted volumes of packed RBCs/plasma (ml) | * | 20/20 | 7·5/7·5 | 15/15 |
| Outcome | Died | Died | Recovered | Recovered |

$AaDO_2$, alveolar-arterial oxygen gradient; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRRT, Continuous renal replacement therapy; ET, Exchange transfusion; $FiO_2$, fraction of inspired oxygen; HFOV, high-frequency oscillatory ventilation; IP, inspiratory pressure; Peak WBC, peak white-blood-cell count; PEEP, positive end-expiratory pressure; sPAP, systolic pulmonary artery pressure.
*Not performed.

**Table 2** Arterial blood gas and parameters of mechanical ventilation before–after ET

| | Case 1 | | Case 2 | | Case 3 | | Case 4 | |
|---|---|---|---|---|---|---|---|---|
| **Characteristics** | **Before** | **After** | **Before** | **After** | **Before** | **After** | **Before** | **After** |
| pH | 7·24 | * | 7·25 | 7·42 | 7 | 7·6 | 7·35 | 7·45 |
| $PCO_2$ (mmHg) | 43 | * | 52 | 35 | 49 | 28·4 | 62 | 51 |
| $PO_2$ (mmHg) | 124 | * | 52 | 59 | 81 | 226 | 73·8 | 85 |
| BE (mmol/l) | −8·5 | * | −5 | −1·2 | −0 | 6·8 | 4·5 | 10·8 |
| $AaDO_2$ (mmHg) | 99·3 | * | 444 | 612·3 | 95 | 99·8 | 280 | 90·9 |
| $FiO_2$ (%) | 100 | * | 100 | 100 | 40 | 30 | 60 | 40 |
| IP ($cmH_2O$) | 34 | * | 28 | 34 | 18 | 15 | 18 | 16 |
| PEEP ($cmH_2O$) | 10 | * | 8 | 15 | 7 | 6 | 10 | 7 |

BE, Base Excess; $PCO_2$, Partial Pressure of Carbon Dioxide; $PO_2$, Partial Pressure of Oxygen.
*Not performed.

vancomycin, fluid infusion based on the patient's daily demands and nasogastric tube feeding. However, her respiratory symptoms got worse gradually and she was intubated for invasive ventilation. After that, the manifestations got exacerbated with heart failure, dropped $SpO_2$, and subsequently, she was ventilated with high-frequency oscillatory ventilation (HFOV), combined with adrenaline and noradrenaline. Laboratory findings at that time showed hyperleucocytosis ($106 \times 10^3$/ml), pulmonary hypertension (sPAP = 70 mmHg) and positive sputum PCR for *Bordetella pertussis*. Despite leucocyte count did decrease after the procedure, it began to escalate at high speed again (from 29·6 to $49·2 \times 10^3$/ml) (Fig. 1). Hypoxaemia did not improve despite ventilation with high pressure, pulmonary arterial hypertension persisted after ET with poor response to vasodilators, and hypotension did not get better with high-dose vasopressors/inotropes (adrenalin, noradrenalin). CRRT was performed, but she died after 8 days of treatment.

## Case 3

A 4-month-old female patient who complained about four-day symptoms of fever, cough and dyspnoea was treated at Phu Yen Obstetrics and Pediatric Hospital for 12 days with ceftriaxone, ciprofloxacin, vancomycin and azithromycin. After no improvement, she was transferred to Children's Hospital 2. At birth, the child was kept in the neonatal ward for 11 days and did not receive any vaccination. Physical examination showed severe respiratory distress. Laboratory tests indicated extreme leucocytosis and thrombocytosis ($78·1 \times 10^3$/ml and $1348 \times 10^3$/ml, respectively), ABG tests revealed respiratory acidosis ($pH/PCO_2/PO_2$: 7·26/48·8 mmHg/106·6 mmHg), and *Bordetella pertussis* PCR was positive. The diagnosis was severe pertussis-induced critical pneumonia and invasive ventilation, antibiotics, fluid infusion and ET were indicated. After ET, leucocyte depletion was observed ($11·4 \times 10^3$/ml) (Fig. 1), respiratory status improved, and ventilation was withdrawn after 2 days. During her hospitalization, an episode of hospital-acquired pneumonia occurred with bilateral pulmonary consolidation on chest X-ray and positive sputum culture for *Acinetobacter*. Moreover, the patient developed two episodes of seizures (one focal, one generalized). Lumbar puncture was performed, and CSF analysis showed a brain injury status with highly elevated protein (1·93 g/dl), meanwhile, cell count, glucose and lactate levels were normal; epilepsy on EEG was not reported; MRI showed leucomalacia right frontal-occipital areas, possibly related to hypoxia-induced brain damage. However, no neurological complications developed and the patient was treated with antibiotics: meropenem, levofloxacin, colistin, and discharged after 39 days.

## Case 4

A 4-month-old female patient was admitted with a chief complaint of fever and breathing difficulty after 4-day outpatient treatment at Dong Nai Hospital. Clinical symptoms included prolonged coughing which turned her face red and even cyanotic. The patient was inoculated with hepatitis B, BCG vaccines at delivery, but had not received the pentavalent shot. Physical examination revealed moderate chest retractions and crackles on auscultation. After admitted, she developed progressive respiratory distress; a chest X-ray showed alveolar damage and consolidation on the upper right lung. Arterial blood gases indicated respiratory acidosis with hypoxaemia ($pH/PCO_2/PO_2$: 7·315/61·9 mmHg/73·8 mmHg). Endotracheal intubation for invasive mechanical ventilation was carried out. However, after 5 days of treatment with ceftriaxone and

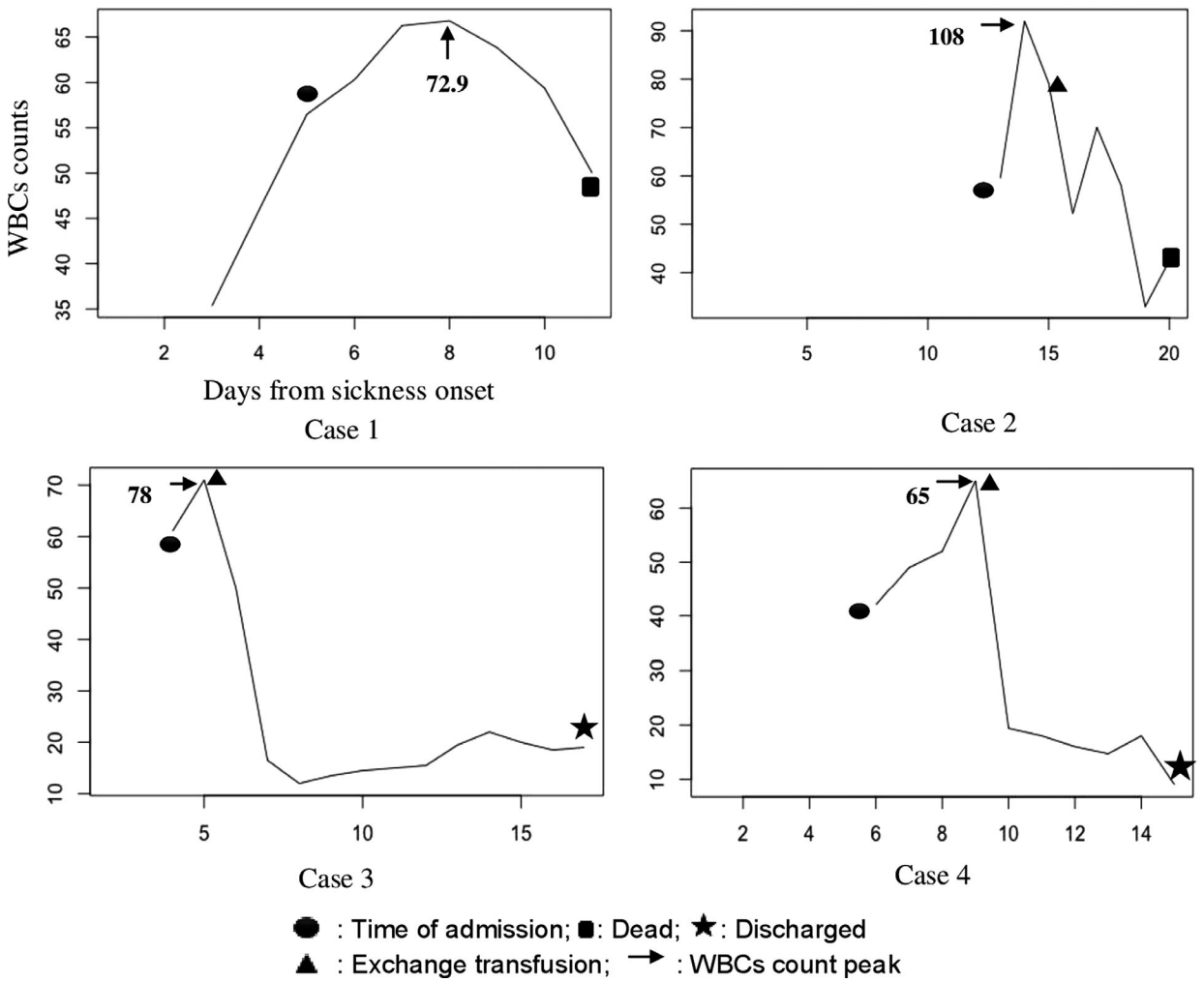


**Fig. 1** White-blood-cell count for all cases before and after admission until recovery/death.

erythromycin, her state did not improve, and complete blood count showed extreme leucocytosis ($65{\cdot}4 \times 10^3$/ml). PCR for *Bordetella pertussis* was positive. After ET, her respiratory distress improved, a depletion in leucocyte count was observed ($19{\cdot}4 \times 10^3$/ml) (Fig. 1), and she was withdrawn from the ventilator. Afterwards, another *E. coli* pneumonia episode occurred, but she finally responded to imipenem and colistin. The patient was eventually discharged after 26 days.

## Discussion

Respiratory failure in pertussis patients usually results from pertussis pneumonia, bacterial superinfection and pulmonary arterial hypertension due to leucocytosis. Leucocytosis is observed in most pertussis cases, and hyperleucocytosis (WBC count is over $100 \times 10^3$/ml) is a rare complication [13]. The pathogenesis of this phenomenon implicates pertussis toxins [9,14]. Monitoring WBC count in early stage and repeating it is crucial in pertussis children's evaluation [15]. Histopathological autopsy findings of leucocyte thrombi in pulmonary vessels have supported the hypothesis that leucocytosis causes pulmonary arterial hypertension in a way of blocking capillaries and restricting blood flow to the lungs due to increased blood viscosity [13,16,17]. As exhibited in these 4 cases, pertussis may be associated with extreme leucocytosis. However, leukaemia is the most common cause of leucocytosis in children, and much less so, pertussis. Therefore, the diagnosis of pertussis is rarely considered in the first place in presence of a critical leucocytosis. Thus, a pertussis PCR test is advised in cases with respiratory failure and abnormal leucocytosis.

Rapid leucodepletion helps to improve the survival of critical pertussis in children [12]. There are several methods to manage leucocytosis, including fluid infusion to dilute blood, and leucapheresis [10]. However,

leucapheresis is a complicated procedure that requires well-trained performers, available high-tech devices (that Children's Hospital 2 did not have), and might have complications [11]. In contrast, ET is a common technique in paediatric ICU, especially in neonates, and rarely leads to serious complications [11]. In 2004, Romano *et al.* published the first report of ET in a severe pertussis case [7]. ET helps to improve the outcome of pertussis patients thanks to (1) leucodepletion that helps to reduce occlusion in pulmonary capillaries, thereby improving pulmonary hypertension; (2) lower the amount of circulating pertussis toxins in the bloodstream so that subsequent leucocytosis could be avoided and airway epithelium could be persevered [9,18]. Besides, according to some reports, if leucocytosis still exists after the first ET, the second round of ET ought to be performed [12,19,20]. In the same context, Rowlands *et al.* [12] reported a reduction in the mortality rate (from 44% to 10%) in critical pertussis infants after severe leucodepletion using ET.

We found that troponin I did not elevate, which suggests that heart failure-induced refractory shock was not the result of a direct myocardial injury caused by bacteria or pertussis toxins but was a consequence of pulmonary hypertension. It is worth mentioning that despite the early use of medication therapy for the first case, the patient died because of refractory cardiogenic shock and unresponsive hyponatraemia. Besides, delays in performing ET might decrease the efficacy of the procedure as observed with the second case, who later had a rapid relapse of hyperleucocytosis which may be the cause of death. This is consistent with Taffarel *et al.* who investigated nine cases suffering from severe pertussis and treated with ET. The authors reported in 4 critically ill patients, ET was done as a last chance approach and all died, while the other 5 received early preemptive ET (when their WBC count was >95 000 $\times$ $10^3$/ml without severe cardiopulmonary compromise) and 4/5 survived [21].

Pertussis patients who undergo mechanical ventilation also have risks related to artificial respiration. Co-infection occurred in all four cases which further complicated the treatment progress and increased medical expenses as reported by previous investigations [3,4]. Moreover, vaccination is extremely crucial, not only for children in preventing whooping cough but also for adults, especially pregnant women in impeding contagion to young children. Consequently, infants should be separated from people who are infected or have acute respiratory symptoms. In our case series, two patients had contacts with patients with respiratory symptoms (Table 1).

## Conclusion

Severe leucocytosis may be associated with critical pertussis, and it can cause death in infants. Prompt notice and management in leucocytosis are crucial to reducing mortality in critical pertussis children. In this scenario, we emphasize the importance of intensive care and ET. Exchange transfusion might be a helpful technique in critical pertussis patients before the progression of cardiopulmonary complications and can be performed in any paediatric intensive care unit without expensive and high-tech devices.

## Acknowledgements

None.

## Conflict of interest

None.

## Ethical approval

Based on the nature of data collection (retrospective), informed consent could not be obtained. However, institutional review board (IRB) approval was obtained from Children's Hospital 2 for conducting this study (ID: 48/20-BVND2).

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VoxSanguinis
The International Journal of Transfusion Medicine
ISBT International Society of Blood Transfusion
*Vox Sanguinis* (2021)

ORIGINAL PAPER


DOI: 10.1111/vox.13087

# Transfusion profile, clinical characteristics, comorbidities and outcomes of 3014 hospitalized patients diagnosed with COVID-19 in Brazil

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Vox Sanguinis

## Abstract

**Background** The novel coronavirus disease-2019 (COVID-19) caused a sudden and unexpected increase in the number of hospital admissions and deaths worldwide. The impact of social distancing on blood stocks was significant. Data on the use of blood products by patients with COVID-19 are scarce.

**Material and methods** A retrospective observational study was conducted by analysing the medical records of 3014 hospitalized COVID-19 patients in 16 Brazilian hospitals. Individual data related to clinical, laboratory and transfusion characteristics and outcomes of these patients were collected. Patients characteristics association with mortality and transfusion need were tested independently by logistic regression models.

**Results** Patients mean age was 57·6 years. In 2298 (76·2%) patients, there was an underlying clinical comorbidity. A total of 1657 (55%) patients required admission to intensive care unit (ICU), and 943 (31%) patients required ventilatory support and orotracheal intubation (OTI). There was a total of 471 (15·6%) deaths among all patients. 325 patients (10·7%) required blood transfusion; 3187 blood products were transfused: 1364 red blood cells in 303 patients, 1092 platelet units in 78 patients, 303 fresh frozen plasma in 49 patients and 423 cryoprecipitates in 21 patients. The mortality among patients who received transfusion was substantially higher than that among the total study population.

**Conclusion** Need for transfusion was low in COVID-19 patients, but significantly higher in patients admitted to ICU and in those who needed OTI. Knowledge of the transfusion profile of these patients allows better strategies for maintaining the blood stocks of hospitals during the pandemic.

*Received: 21 October 2020, revised 3 February 2021, accepted 4 February 2021*

**Key words:**
COVID-19, transfusion, SARS-COV-2, mortality, SARS.

## Introduction

In December 2019, a new disease (COVID-19) caused by a novel coronavirus called SARS-CoV-2 appeared in the city of Wuhan, Hubei Province, China [1,2]. The disease spread rapidly around the world, leading the World Health Organization (WHO) to declare a pandemic on 11 March 2020 [3]. Brazil declared national public health emergency in February 2020 [4].

According to WHO data, up to 14 January 2021, more than 91 million people were infected worldwide, of whom more than 1 970 000 died from complications of the

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disease [5]. In Brazil, the Ministry of Health confirmed the first case of COVID-19 in the city of São Paulo on 26 February 2020. Since then, more than 8·3 million cases and more than 207 000 deaths have been recorded [6]. International authorities have focused their efforts on social isolation measures and detection of new cases of COVID-19, in addition to the search for effective treatments to combat the most severe effects of this disease.

The clinical presentation of COVID-19 ranges from mild and self-limited upper airway inflammation (dry cough, fever, dyspnoea, fatigue, anosmia, ageusia) to severe pneumonia, sepsis, multiple organ failure and death [1,2]. The clinical and epidemiological characteristics, risk factors, laboratory presentation, diagnostic techniques and outcomes have been exhaustively investigated worldwide [7,8]. Many aspects of COVID-19 still need to be elucidated, and advances in its prevention and treatment depend on a greater understanding of its clinical and pathophysiological aspects.

It has been shown that RNA from SARS-COV-2 can be found in the blood of infected patients [9] and even in blood donors [10]. However, there is no evidence to date that it can be transmitted through blood transfusion [11]. With the travel restrictions and recommendations for social isolation, there was a significant and worrying decrease in blood donations and, consequently, in the blood stocks available during the first months of the pandemic. Worldwide, it was necessary to implement additional strategies to attract donors, who were often in social isolation and working from home [12].

The use of blood products in patients hospitalized with COVID-19 also needs to be better investigated and elucidated. The data in the literature on the need for transfusion of blood products in these patients are scarce. Our study describes the transfusion needs, clinical characteristics and outcomes of 3014 patients with COVID-19 in 16 hospitals that serve different regions of Brazil in six states of the country.

## Materials and Methods

We conducted a retrospective observational study by analysing data collected by manual extraction from the medical records of hospitalized patients with a confirmed diagnosis of COVID-19. Diagnosis was made by nucleic acid testing by reverse transcription polymerase chain reaction (RT-PCR) of the sample collected from the nasopharynx/oropharynx or by a reactive antibody result in a blood sample. Infection was considered confirmed if the initial test result was positive or if it was confirmed in a repeated new sample collection. Repeat testing was performed whenever there was a high clinical probability of COVID-19 or if the initial test was considered a probable false-negative due to inadequate sample collection. The patients were admitted to 16 Brazilian hospitals where our transfusion service operates, in six states of Brazil, in the period from 03/01/2020 to 06/30/2020. Individual data related to epidemiological, clinical and laboratory characteristics, treatments performed (including admission to the intensive care unit (ICU) and need for orotracheal intubation (OTI) and mechanical ventilation) and outcome (discharge and death) were collected in 3014 adult patients over 18 years old.

All data regarding the transfusions performed were collected from blood component requests, duly completed and signed by the attending physician and validated by the hemotherapist, the specialist responsible for the transfusion service of the hospital. Written informed consent was obtained from all study participants or their legal representatives, and this study was approved by the ethics committees of the participating hospitals.

Statistical analysis was performed in software R 4.0.2 (R Core Team, Vienna, Austria, 2020). Patients baseline characteristics association with mortality and transfusion need during hospitalization outcomes were tested independently by logistic regression models. A multiple logistic regression model with all baseline variables, except sex (not significantly associated with the outcomes) and blood type (only available to a small subgroup of patients), was adjusted to identify independently associated variables, and adjusted odds ratios were presented with 95% confidence intervals. Area under the ROC curve (AUC) statistics were presented to evaluate predictive performance of the models.

## Results

From March to June 2020, 3014 adult patients were hospitalized in 16 hospitals we evaluated, in six Brazilian states (São Paulo, Rio de Janeiro, Piauí, Pará, Manaus and the Federal District). From these patients, diagnosis was made by RT-PCR in 2951 (97·9%) or a reactive serological test in 63 (2·1%). Table 1 shows the baseline clinical and laboratory characteristics of these patients and compares their distribution between two groups: patients who died and patients discharged, as well as each *odds ratio* related to a specific characteristic. It is notable that the blood type, platelets count and haemoglobin value were not available to all patients. The mean age was 57·6 years (18 to 102) and the male:female ratio, 1·4:1·0 (with 1786 men and 1228 women). Clinical comorbidities were analysed in all patients. Hypertension, diabetes, cancer and pneumopathy were also analysed separately. Age, any comorbidity, hypertension, diabetes, cancer, pneumopathy, low platelets counts and low haemoglobin levels

**Table 1** Patients characteristics and association with death

| Baseline characteristics | Total (n = 3014) | Discharge (n = 2543) | Death (n = 471) | Odds Ratio (95% CI) | *P* value |
|---|---|---|---|---|---|
| Age, years, mean ± SD | 57·6 ± 17·8 | 54·8 ± 16·8 | 73·0 ± 15·3 | 1·067 (1·060; 1·075) | <0·01 |
| Age group | | | | | |
| 18–40 | 595 (19·7%) | 577 (97·0%) | 18 (3·0%) | 1 | |
| 41–60 | 1135 (37·7%) | 1062 (93·6%) | 73 (6·4%) | 2·20 (1·30; 3·73) | <0·01 |
| 61–80 | 899 (29·8%) | 693 (77·1%) | 206 (22·9%) | 9·53 (5·81; 15·62) | <0·01 |
| Over 80 | 385 (12·8%) | 211 (54·8%) | 174 (45·2%) | 26·43 (15·87; 44·03) | <0·01 |
| Sex | | | | | |
| Female | 1228 (40·7%) | 1034 (84·2%) | 194 (15·8%) | | |
| Male | 1786 (59·3%) | 1509 (84·5%) | 277 (15·5%) | 0·98 (0·80; 1·20) | 0·83 |
| Blood type | (n = 340) | | | | |
| A | 137 (40·3%) | 65 (47·4%) | 72 (52·6%) | 1 | |
| AB | 13 (3·8%) | 4 (30·8%) | 9 (69·2%) | 2·03 (0·60; 6·91) | 0·26 |
| B | 32 (9·4%) | 12 (37·5%) | 20 (62·5%) | 1·50 (0·68; 3·32) | 0·31 |
| O | 158 (46·5%) | 75 (47·5%) | 83 (52·5%) | 1·00 (0·63; 1·58) | 1 |
| Any comorbidity | | | | | |
| No | 716 (23·8%) | 657 (91·8%) | 59 (8·2%) | | |
| Yes | 2298 (76·2%) | 1886 (82·1%) | 412 (17·9%) | 2·43 (1·84; 3·27) | <0·01 |
| DM | | | | | |
| No | 2358 (78·2%) | 2031 (86·1%) | 327 (13·9%) | | |
| Yes | 656 (21·8%) | 512 (78·0%) | 144 (22·0%) | 1·75 (1·40; 2·17) | <0·01 |
| HAS | | | | | |
| No | 1812 (60·1%) | 1582 (87·3%) | 230 (12·7%) | | |
| Yes | 1202 (39·9%) | 961 (80·0%) | 241 (20·0%) | 1·72 (1·42; 2·10) | <0·01 |
| Cancer | | | | | |
| No | 2861 (94·9%) | 2438 (85·2%) | 423 (14·8%) | | |
| Yes | 153 (5·1%) | 105 (68·6%) | 48 (31·4%) | 2·64 (1·83; 3·74) | <0·01 |
| Pneumopathy | | | | | |
| No | 2812 (93·3%) | 2388 (84·9%) | 424 (15·1%) | | |
| Yes | 202 (6·7%) | 155 (76·7%) | 47 (23·3%) | 1·71 (1·20; 2·39) | 0·002 |
| Platelets ($\times 10^9$/L) | (n = 2897) | | | | |
| ≥150 001 | 2243 (77·9%) | 1937 (86·4%) | 306 (13·6%) | 1 | |
| 100 001 to 150 000 | 525 (18·2%) | 415 (79·0%) | 110 (21·0%) | 1·68 (1·32; 2·14) | <0·01 |
| ≤100 000 | 111 (3·9%) | 79 (71·2%) | 32 (28·8%) | 2·56 (1·67; 3·93) | <0·01 |
| Haemoglobin (g/L) | (n = 2885) | | | | |
| ≥11·1 | 2483 (86·1%) | 2182 (87·9%) | 301 (12·1%) | 1 | |
| 8·1–11·0 | 315 (10·9%) | 206 (65·4%) | 109 (34·6%) | 3·84 (2·95; 4·98) | <0·01 |
| ≤8·0 | 87 (3·0%) | 48 (55·2%) | 39 (44·8%) | 5·89 (3·8; 9·14) | <0·01 |

Percentages in the total column are variable distribution and in the death column are death incidence.

were associated with death. Sex and ABO blood type were not associated with increased risk of death.

Table 2 shows the events during hospitalization. Transfusion, intensive care unit (ICU) admission and need for orotracheal intubation (OTI) were associated with higher risk of death. There was need for transfusion of any blood component in 325 patients (10·8%). In total, 3187 blood products were transfused, including packed red blood cells (RBCs), random platelet concentrate (RPC), apheresis platelet concentrate (APC), fresh frozen plasma (FFP) and units of cryoprecipitate (CRYO) in 325 patients (mean 9·8 units/patient). A total of 1364 RBCs were transfused in 303 patients (10·0%), with a mean of 4·5 units/patient. A total of 1101 platelet units were transfused in 80 patients (2·6%). In addition, 303 FFPs were used in 49 patients (1·6%) (mean 6·2 units/patient), and 423 CRYO were used in 21 patients (0·7%) (mean 20·1 units/patient). The clinical or laboratory indications for transfusion were not available to most patients.

Table 3 describes patients' characteristics who were transfused and patients who were not transfused. Table 4 describes the need for transfusion based on hospitalization needs. Age (patients older than 60 years), any comorbidity, hypertension, diabetes, cancer, low platelets

**Table 2** Events during hospitalization and outcome

| Events | Total (n = 3014) | Discharge (n = 2543) | Death (n = 471) | Odds Ratio (95% CI) | *P* value |
|---|---|---|---|---|---|
| Transfusion | | | | | |
| No | 2689 (89·2%) | 2404 (89·4%) | 285 (10·6%) | | |
| Yes | 325 (10·8%) | 139 (42·8%) | 186 (57·2%) | 11·3 (8·8; 14·5) | <0·01 |
| ICU | | | | | |
| No | 1357 (45·0%) | 1330 (98·0%) | 27 (2·0%) | | |
| Yes | 1657 (55·0%) | 1213 (73·2%) | 444 (26·8%) | 18·0 (12·4; 27·4) | <0·01 |
| IOT | | | | | |
| No | 2071 (68·7%) | 2006 (96·9%) | 65 (3·1%) | | |
| Yes | 943 (31·3%) | 537 (56·9%) | 406 (43·1%) | 23·3 (17·8; 31·1) | <0·01 |

Percentages in total column are variable distribution and in death column are death incidence.

counts and low haemoglobin levels were associated with need for transfusion. Sex, ABO blood type and pneumopathy were not statistically significant to transfusion need. ICU admission and need for OTI were associated with higher risk for transfusion need.

A multiple logistic regression model with all baseline variables was performed, except sex (not significantly associated with the outcomes) and blood type (only available to a small subgroup of patients). Table 5 shows the results applied to risk of mortality and Table 6 to transfusion need. In this model, age, cancer, low platelets count and low haemoglobin level were independent risk factors for death. Diabetes, hypertension and pneumopathy were not. Considering risk for transfusion, age, hypertension, low platelets count and low haemoglobin level were statistically significant. Diabetes, cancer and pneumopathy were not.

## Discussion

The COVID-19 pandemic brought hundreds of challenges to humanity in all sectors of society. As the number of cases has increased dramatically worldwide, great efforts have been made to better understand the pathophysiology of the disease, its forms of transmission, its clinical behaviour and the search for effective preventive or therapeutic actions were targets of a large part of the global scientific community.

The clinical progression of the disease is the main factor that correlates with the use of blood products. This study shows that the need for transfusions in hospitalized patients with COVID-19 is not high (10·4%). In general, studies show that patients with the disease have normal or slightly decreased haemoglobin and platelet values [13]. However, it has been shown that patients with more severe forms of COVID-19 have lower haemoglobin levels than those with mild forms of the disease [14]. Patients who are admitted to the ICU and/or need OTI have higher mortality rates and require larger amounts of blood products. Our study showed that the main type of blood product used is red blood cells (9·5% of patients), followed by platelets (2·4%), fresh frozen plasma (1·6%) and cryoprecipitate (0·7%).

A previous study showed a higher risk of disease severity for individuals in blood group A and a protective effect in individuals in blood group O [15]. Our study did not observe any effect on mortality related to the ABO blood group. Maybe, one reason for this is the low number of transfused patients and the low percentage of patients with ABO typing available for analysis.

Previous studies have reported that advanced age is an important independent predictor of mortality, both in SARS, MERS and COVID-19 [16-18]. Our study confirms that increasing age is associated with deaths in patients with COVID-19, corroborating other studies that identified this predictor. Age-related defects in T- and B-cell function and excess production of type 2 cytokines can lead to a deficiency in the control of viral replication and to pro-inflammatory activity with longer responses, leading to worse outcomes [19]. Also, laboratory results that indicate more severe clinical conditions, such as low platelet counts and low haemoglobin levels, were also associated with higher mortality rates. Dysregulated inflammation, vascular injury and hypercoagulability are conditions related to COVID-19 pathophysiology that can correlate to these laboratory findings.

Our study identified as risk for transfusion many factors, such as age, clinical underlying comorbidities, low platelet counts, low haemoglobin levels, admission to ICU and OTI need. This is similar to a previous published study, with a lower cohort [20].

It has been previously demonstrated that hospitalized patients with COVID-19 have lower blood transfusion utilization rates than hospitalized patients without COVID-19 [21]. Our study, which included a large number of patients, also found that these patients had low rates of

**Table 3** Patient characteristics and association with transfusion

| Baseline characteristics | Total (n = 3014) | No transfusion (n = 2699) | Transfusion (n = 325) | Odds Ratio (95% CI) | *P* value |
|---|---|---|---|---|---|
| Age, years, mean ± SD | 57·6 ± 17·8 | 56·6 ± 17·7 | 65·6 ± 17·1 | 1·03 (1·02;1·04) | <0·01 |
| Age group | | | | | |
| 18–40 | 595 (19·7%) | 555 (93·3%) | 40 (6·7%) | 1 | |
| 41–60 | 1135 (37·7%) | 1065 (93·8%) | 70 (6·2%) | 0·91 (0·61; 1·36) | 0·65 |
| 61–80 | 899 (29·8%) | 755 (84·0%) | 144 (16·0%) | 2·65 (1·83; 3·82) | <0·01 |
| Over 80 | 385 (12·8%) | 314 (81·6%) | 71 (18·4%) | 3·14 (2·08; 4·73) | <0·01 |
| Sex | | | | | |
| Female | 1228 (40·7%) | 1099 (89·5%) | 129 (10·5%) | 1·05 (0·83;1·33) | 0·68 |
| Male | 1786 (59·3%) | 1590 (89·0%) | 196 (11·0%) | | |
| Blood type | (n = 340) | | | | |
| A | 137 (40·3%) | 18 (13·1%) | 119 (86·9%) | 1 | |
| AB | 13 (3·8%) | 2 (15·4%) | 11 (84·6%) | 0·83 (0·17; 4·06) | 0·82 |
| B | 32 (9·4%) | 1 (3·1%) | 31 (96·9%) | 4·69 (0·60; 36·50) | 0·14 |
| O | 158 (46·5%) | 16 (10·1%) | 142 (89·9%) | 1·34 (0·66; 2·75) | 0·42 |
| Any comorbidity | | | | | |
| No | 716 (23·8%) | 682 (95·3%) | 34 (4·7%) | 2·92 (2·05;4·26) | <0·01 |
| Yes | 2298 (76·2%) | 2007 (87·3%) | 291 (12·7%) | | |
| DM | | | | | |
| No | 2358 (78·2%) | 2125 (90·1%) | 233 (9·9%) | 1·49 (1·14;1·92) | <0·01 |
| Yes | 656 (21·8%) | 564 (86·0%) | 92 (14·0%) | | |
| HAS | | | | | |
| No | 1812 (60·1%) | 1657 (91·4%) | 155 (8·6%) | 1·77 (1·40;2·22) | <0·01 |
| Yes | 1202 (39·9%) | 1032 (85·9%) | 170 (14·1%) | | |
| Cancer | | | | | |
| No | 2861 (94·9%) | 2573 (89·9%) | 288 (10·1%) | 2·86 (1·91;4·17) | <0·01 |
| Yes | 153 (5·1%) | 116 (75·8%) | 37 (24·2%) | | |
| Pneumopathy | | | | | |
| No | 2812 (93·3%) | 2516 (89·5%) | 296 (10·5%) | 1·42 (0·93;2·12) | 0·09 |
| Yes | 202 (6·7%) | 173 (85·6%) | 29 (14·4%) | | |
| Platelets (x$10^9$/L) | (n = 2897) | | | | |
| ≥150 001 | 2243 (77·9%) | 2035 (90·7%) | 208 (9·3%) | 1 | |
| 100 001 to 150 000 | 525 (18·2%) | 456 (86·9%) | 69 (13·1%) | 1·48 (1·11; 1·98) | 0·01 |
| ≤100 000 | 111 (3·9%) | 80 (72·1%) | 31 (27·9%) | 3·79 (2·45; 5·88) | <0·01 |
| Haemoglobin (g/L) | (n = 2885) | | | | |
| ≥11·1 | 2483 (86·1%) | 2320 (93·4%) | 163 (6·6%) | 1 | |
| 8·1 to 11·0 | 315 (10·9%) | 231 (73·3%) | 84 (26·7%) | 5·18 (3·85; 6·96) | <0·01 |
| ≤8·0 | 87 (3·0%) | 24 (27·6%) | 63 (72·4%) | 37·36 (22·75; 61·37) | <0·01 |

Percentages in total column are sample distribution and in transfusion column are transfusion need during hospitalization.

**Table 4** Events during hospitalization and transfusion need

| Hospitalization variables | Total (n = 3014) | No transfusion (n = 2699) | Transfusion (n = 325) | Odds Ratio (95% CI) | *P* value |
|---|---|---|---|---|---|
| ICU | | | | | |
| No | 1357 (45·0%) | 1328 (97·9%) | 29 (2·1%) | | |
| Yes | 1657 (55·0%) | 1361 (82·1%) | 296 (17·9%) | 9·97 (6·87;15·00) | <0·01 |
| IOT | | | | | |
| No | 2071 (68·7%) | 1992 (96·2%) | 79 (3·8%) | | |
| Yes | 943 (31·3%) | 697 (73·9%) | 246 (26·1%) | 8·94 (6·84;11·69) | <0·01 |

Percentages in total column are sample distribution and in transfusion column are transfusion need during hospitalization.

**Table 5** Multiple logistic regression model for mortality

| Coefficient | Estimate | Std. Error | Odds Ratio (95% CI) | *P* value |
|---|---|---|---|---|
| Age, years | 0·06 | 0·003 | 1·06 (1·05; 1·07) | <0·01 |
| DM | 0·25 | 0·13 | 1·28 (0·99; 1·66) | 0·06 |
| HAS | -0·01 | 0·12 | 0·99 (0·78; 1·26) | 0·94 |
| Cancer | 0·42 | 0·22 | 1·52 (0·99; 2·33) | 0·05 |
| Pneumopathy | 0·12 | 0·21 | 1·12 (0·74; 1·70) | 0·58 |
| Platelets (100 001 to 150 000 × $10^9$/L) | 0·53 | 0·14 | 1·70 (1·30; 2·24) | <0·01 |
| Platelets (<100 000 × $10^9$/L) | 0·45 | 0·25 | 1·57 (0·96; 2·58) | 0·07 |
| Haemoglobin (8·1–11·0 g/L) | 0·85 | 0·15 | 2·33 (1·74; 3·13) | <0·01 |
| Haemoglobin (<8·0 g/L) | 1·41 | 0·26 | 4·11 (2·48; 6·81) | <0·01 |
| AUC: 81·3% | | | | |

**Table 6** Multiple logistic regression model for transfusion need

| Coefficient | Estimate | Std. Error | Odds Ratio (95% CI) | *P* value |
|---|---|---|---|---|
| (Intercept) | –4·03 | 0·26 | 1 | - |
| Age, years | 0·02 | 0·004 | 1·02 (1·01; 1·03) | <0·01 |
| DM | 0·08 | 0·16 | 1·09 (0·80; 1·48) | 0·59 |
| HAS | 0·42 | 0·14 | 1·53 (1·15; 2·03) | <0·01 |
| Cancer | 0·34 | 0·26 | 1·41 (0·85; 2·33) | 0·18 |
| Pneumopathy | 0·13 | 0·25 | 1·14 (0·70; 1·85) | 0·61 |
| Platelets (100 001 to 150 000 × $10^9$/L) | 0·53 | 0·16 | 1·71 (1·25; 2·34) | <0·01 |
| Platelets (<100 000 × $10^9$/L) | 0·64 | 0·28 | 1·90 (1·10; 3·27) | 0·02 |
| Haemoglobin (8·1–11·0 g/L) | 1·43 | 0·16 | 4·17 (3·05; 5·69) | <0·01 |
| Haemoglobin (<8·0 g/L) | 3·40 | 0·27 | 30·04 (17·83; 50·59) | <0·01 |
| AUC: 77·1% | | | | |

blood utilization. With the pandemic and the consequent suspension of elective surgeries, the number of transfusions also naturally drops in hospitals. Thus, with these two factors, the impact caused by the pandemic on the low rates of blood donations with a consequent decrease in blood stocks is lower. Future studies that better elucidate the pathophysiology of the disease may guide us better regarding the use of blood products by patients with COVID-19 and aid in outlining specific strategies for blood stock maintenance in this new hospital scenario.

## Conflict of Interest

The authors have no conflict of interest.

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VoxSanguinis
The International Journal of Transfusion Medicine

ISBT International Society of Blood Transfusion

*Vox Sanguinis* (2021) **116**, 990–997

ORIGINAL PAPER


DOI: 10.1111/vox.13090

# Exchange transfusions in severe Rh-mediated alloimmune haemolytic disease of the foetus and newborn: a 20-year overview on the incidence, associated risks and outcome

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Vox Sanguinis

## Abstract

**Background and objectives** Guidelines and indications for exchange transfusion in haemolytic disease of the foetus and newborn (HDFN) have changed drastically in the past decades, causing a decline in exchange transfusion rate. This study aims to evaluate the incidence of exchange transfusions (ETs) in neonates with Rh-mediated HDFN over the past 20 years at our centre, and report potentially ET-related complications as well as indicators for bilirubin encephalopathy.

**Material and methods** In this observational study, 438 neonates were included with HDFN, born ≥ 35 weeks gestational age at the Leiden University Medical Centre between January 2000 and July 2020. The incidence of ET and procedure-related complications were assessed in three consecutive time periods determined by changes in guidelines and indications for ET.

**Results** The incidence of ET in our centre declined from (104/156) 67% (time period 2000–2005), to (39/181) 22% (2006–2015) and to (10/101) 10% (2015–2020, $p < 0{\cdot}001$). The maximum bilirubin levels in neonates after birth increased from 13·6 mg/dL (or 233 μmol/L), to 15·0 mg/dL (257 μmol/L) and to 15·3 mg/dL (263 μmol/L). The incidence of complications associated with the use of ET (including sepsis, haematologic disorders and respiratory failure) remained stable throughout the years, and no neonates died during the study period.

**Conclusion** Exchange transfusion incidence declined significantly over the past two decades. Decrease in ET incidence, and concomitant decrease in exposure and expertise, was not associated with an increase in procedure-related complications.

**Key words:** haemolytic disease of the foetus and newborn, exchange transfusion, alloimmunization, hyperbilirubinaemia.

*Received: 25 October 2020,*
*revised 5 February 2021,*
*accepted 5 February 2021,*
*published online 17 March 2021*

## Introduction

Haemolytic disease of the foetus and newborn (HDFN) is caused by an incompatibility between maternal and foetal red blood cell antigens. Destruction of the foetal red blood cells (RBC) by maternal alloantibodies results in

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foetal and neonatal haemolytic anaemia, which can be treated antenatally with intrauterine transfusions (IUTs) and post-natally with RBC transfusions. Haemolysis may also lead to hyperbilirubinaemia and can result in acute and chronic bilirubin encephalopathy.

Hyperbilirubinaemia is treated with intensive phototherapy and exchange transfusion (ET). ET is recommended for infants whose bilirubin levels continue to rise to exchange levels, despite intensive phototherapy [1]. ET removes excess bilirubin from the neonatal blood circulation as well as maternal antibodies and antibody-coated erythrocytes [1–3]. Approximately 85% of the neonatal blood is replaced by irradiated donor blood by double-volume exchange transfusion [3,4]. While being effective in the acute treatment of hyperbilirubinaemia, ET is an invasive procedure requiring central lines and has potentially severe side effects. Mortality rates around 0·3% are reported in term neonates, but increase above 10% in preterm neonates [4–6]. Morbidity rates are reported up to 24% and include cardiorespiratory instability, catheter-related complications, thrombocytopenia and sepsis [7–9].

Data on changes in the incidence and complications of ET in severe HDFN are limited. In our centre, we noticed a reduction in the need for ET in the past decades, partly related to changes in our guidelines and indications for ET, which have become more restrictive over the years. Whether a reduction in exposure and expertise in performing an invasive and complex procedure as an ET is also associated with an increased risk of complications, is not well known.

The aim of this study is to give an overview of the incidence of exchange transfusions in neonates with severe Rh-mediated HDFN over the past 20 years at our centre and to report potentially ET-related complications, as well as indicators for bilirubin encephalopathy.

## Methods

### Study design and population

This is an observational cohort study conducted at the Leiden University Medical Centre (LUMC), the Dutch National Referral centre for severe HDFN and foetal therapy. In the Netherlands, all pregnant women with RhD immunization and an ADCC (antibody-dependent cell-mediated cytotoxicity) of >50% and/or antibody titre ≥ 16, as well as with Rh immunization other than D and ADCC > 30% and/or antibody titre ≥ 16, are referred to the LUMC [10]. Subsequently, these high-risk pregnancies are monitored by serial Doppler measurements to assess the velocity of the blood flow in the middle cerebral artery. If this velocity exceeds 1·5 multiples of the median or if signs of hydrops are present, the treatment with IUT is indicated. IUTs can be administered until 34–35 weeks of gestation, after which induced delivery is preferred to IUT treatment. The IUT technique used in the Netherlands has been previously described [11].

All (near-) term neonates (≥35 weeks of gestation) with HDFN due to maternal red cell alloimmunization against Rh antigens (D, C, c, Cw, E and e) admitted to the LUMC between 1 January 2000 and 31 June 2020 were eligible for this study.

Neonates with blood group alloimmunization caused by non-Rh antigens were excluded as these antigens show different pathophysiological characteristics and great variation in exchange transfusion risk after birth [12]. Neonates born <35 weeks of gestation were excluded as prematurity itself is a major risk factor for hyperbilirubinaemia and ET treatment and is associated with greater odds of death following ET compared to term infants [13]. In addition, neonates who received intravenous immunoglobulins (IVIg) (n = 41) as part of a randomized controlled trial (RCT) (LIVIN trial, identifier ISRCTN14013064) between 2006 and 2010, were excluded as IVIg is not a standard practice at the LUMC.

The study cohort was divided into three different time periods according to changes in ET guidelines:

Group I: *1 January 2000 to 31 December 2005*

In this first group, the bilirubin threshold for ET was a total serum bilirubin level at birth > 3·5 mg/dL (measured in umbilical cord blood, or, more often, in neonatal blood at birth) and/or a rise of bilirubin > 0·5 mg/dL/h despite intensive phototherapy [14]. In all neonates, bilirubin levels were measured every 2–3 hours according to protocol during the first few days after birth. The differences in bilirubin thresholds have been previously described [9].

Group II: *1 January 2006 to 31 March 2015*

On 1 January 2006, we implemented new, more restrictive, ET guidelines based on the updated guideline of the American Academy of Pediatrics (AAP) [2]. After the guideline change, the new criteria for ET were: (1) total serum bilirubin above thresholds according to the AAP guideline, and/or (2) rise of bilirubin > 0·5 mL/dL/h despite intensive phototherapy, and/or (3) clinical symptoms of acute bilirubin encephalopathy regardless of bilirubin level [9].

Group III: *1 April 2015 to 31 June 2020*

This third group encloses the years after the most recent guideline change at our centre, implemented in April 2015. The AAP guideline advises to classify proven blood group alloimmunization as 'high risk' for the rise of bilirubin to levels that might cause bilirubin encephalopathy [2]. After a consensus meeting at our centre, we decided not to categorize proven blood group antagonism as an extra risk factor as the blood-brain barrier does not decrease in function with the presence

thereof. Neonates with proven blood antagonism born after April 2015 were therefore categorized as 'standard risk' when choosing which threshold curve (low, standard or high risk) to use for phototherapy and ET.

Throughout the study period, ET was performed with blood exchange volumes of 100–200 mL/kg. The irradiated blood product consists of leukocyte-reduced erythrocytes and plasma of two donors, less than 5 days old with a haematocrit of 0·50–0·65 g/L. No albumin or calcium infusions were given prior or during ET.

### Outcome measures

The primary outcome was the incidence of ETs. Secondary outcomes included the timing of the first ET in hours, the duration of phototherapy in days, post-natal RBC transfusion dependency, length of stay at the neonatal intensive care unit (NICU) and ET-related complications.

### Data collection

The following obstetric and neonatal data were directly recorded at our centre and obtained from patient's medical files: foetal haemoglobin at first IUT and number of IUTs, gestational age and weight at birth, gender, mode of delivery, haemoglobin and bilirubin level at birth (conjugated and unconjugated), type of alloimmunization, maximum bilirubin level during admission, number of ETs and timing of ETs, post-natal RBC transfusions (not including RBCs given as part of ET), respiratory distress (defined as need for mechanical ventilation), umbilical vein catheterization, number of days of phototherapy before discharge home or transfer to another hospital, results from standard cerebral ultrasound (intraventricular haemorrhage graded according to the grading system of Papile [15] and periventricular leukomalacia (PVL) according to de Vries et al. [16]), results from standard hearing screening and total length of hospital stay. The following complications of ET were recorded: proven sepsis (defined as clinical symptoms of infection combined with positive blood culture after the moment of ET), thrombosis (defined as the detection of a vascular thrombosis on ultrasound examination), mechanical ventilation, leukopenia (defined as leukocytes $< 5{\cdot}10^9$/L), thrombocytopenia (defined as platelets $< 100{\cdot}10^9$/L), platelet transfusion rate, hypocalcaemia (defined as calcium < 8·0 mg/dL), hyperkalaemia (defined as potassium > 6·5 mEq/L) and neonatal mortality [17]. Follow-up data on RBC transfusions after discharge from our centre were collected from referral hospitals with written consent from parents or caregivers. RBC transfusions were administered in term neonates with HDFN when haemoglobin levels fall below 10·5 g/dL for day 0–6, below 8·9 g/dL for day 7–13 and below 7·2 g/dL from day 14 onwards. Before February 2014, haemoglobin thresholds for transfusion were 9·6 g/dL for days 7–13 and 8·0 g/dL from day 14 onwards. A transfusion of 15 mL/kg irradiated packed erythrocytes less than 5 days old was advised throughout the study period, with a haematocrit of 0·50–0·65 g/L.

### Statistical analysis

Data was reported as means and standard deviations (SD), or as medians and interquartile range (IQR), when appropriate. The primary outcome was tested by $\chi^2$ test. Statistical analysis was performed using IBM SPSS Statistics version 26.

### Ethical considerations

Due to the non-invasive nature of this study, a waiver of consent was granted by the medical ethics committee of our centre.

## Results

During the study period of 20 years, 612 neonates with severe HDFN were admitted to the neonatal intensive care unit (NICU) of the LUMC, and 438 neonates met the inclusion criteria and were eligible for this study. We excluded 87 neonates due to HDFN primarily caused by non-Rh antibodies, 56 neonates with a gestational age < 35 weeks, 10 neonates who fulfilled both exclusion criteria and 41 neonates treated with IVIg as part of the LIVIN trial (Fig. 1).

Baseline characteristics for each of the three cohorts are shown in Table 1. RhD was the most common causative Rh antigen of HDFN in all three groups (89%, 85% and 92%, respectively), followed by Rhc. The occurrence of multiple Rh-alloantibodies was 51% in group I, 43% in group II and 39% in group III. The occurrence of a non-Rh alloantibody, besides a primary Rh-alloantibody, was, respectively, 7% in group I, 6% in group II and 3% in group III. This most often involved alloantibodies against Kell or Jka antigens.

Neonatal treatment data and outcome measures are presented in Table 2. The ET incidence decreased significantly from 104/156 (67%) in group I (before the AAP guideline change) to 39/181 (22%) in group II (after the AAP guideline change) and was further reduced since then to 10/101 (10%) in the most recent group III ($p < 0{\cdot}001$). Numbers of ET per year are shown in Fig. 2.

In group I, 106/156 (69%) neonates were treated with post-natal RBC transfusion(s), 37/156 (81%) in group II and 68/101 (74%) neonates in group III. The maximum bilirubin reached levels > 25·0 mg/dL in eight neonates: one (1%) in group I, six (3%) in group II and one (1%) in

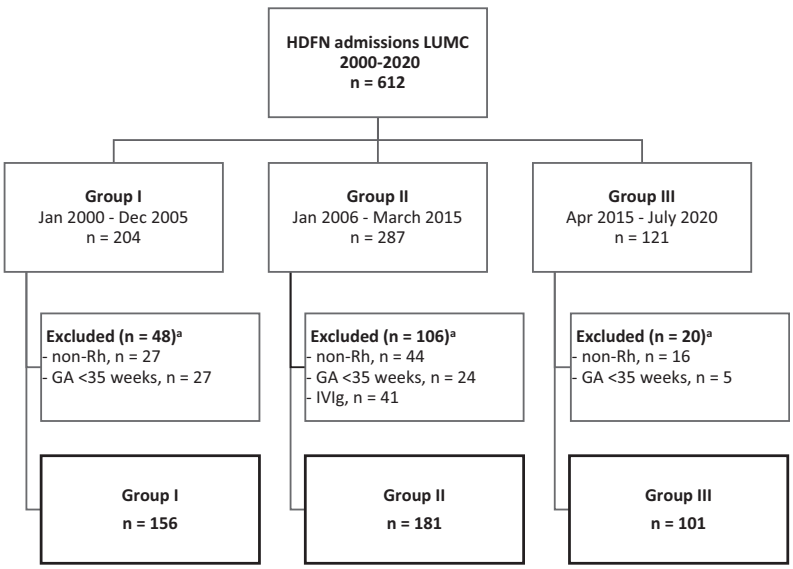


**Fig. 1** Flowchart of study participants. [a]Some neonates meet more than one exclusion criteria; hence, combined numbers per criterion can exceed the total number of exclusions. GA, gestational age; IVIg, intravenous immunoglobulin.

**Table 1** Baseline characteristics.

| | Group I n = 156 | Group II n = 181 | Group III n = 101 |
|---|---|---|---|
| Neonates treated with IUT(s) - n (%) | 99 (63) | 102 (56) | 61 (60) |
| Number of IUT(s) per neonate[a] | 3 (2–4) | 2 (2–4) | 2 (1–3) |
| Gestational age at birth - weeks[a] | 37 (36–37) | 37 (36–37) | 37 (36–37) |
| Birthweight - grams[b] | 2972 ± 443 | 2987 ± 470 | 2913 ± 348 |
| Caesarean delivery - n (%) | 48 (31) | 41 (23) | 27 (27) |
| Male gender - n (%) | 87 (56) | 114 (63) | 47 (47) |
| Primary type of alloantibodies | | | |
| Rh D - n (%) | 139 (89) | 153 (85) | 93 (92) |
| Rh C - n (%) | 1 (1) | 2 (1) | 0 (0) |
| Rh c - n (%) | 13 (8) | 17 (9) | 8 (8) |
| Rh Cw - n (%) | 0 (0) | 2 (1) | 0 (0) |
| Rh E - n (%) | 3 (2) | 7 (4) | 0 (0) |
| Additional non-Rh alloantibodies - n (%) | 11 (7) | 10 (6) | 3 (3) |
| Haemoglobin level at birth - g/dL[b,c] | 11·6 ± 2·6 | 13·4 ± 3 | 13·5 ± 2·6 |
| Unconjugated bilirubin level at birth - mg/dL[b,d] | 5·5 ± 2·8 | 5·7 ± 2·9 | 5·1 ± 2·3 |
| Unconjugated bilirubin level at birth - μmol/L[b,d] | 94 ± 48 | 97 ± 49 | 87 ± 39 |
| Conjugated bilirubin level at birth - mg/dL[a,e] | 0·5 (0·4–0·8) | 0·6 (0·5–0·8) | 0·5 (0·4–0·6) |
| Conjugated bilirubin level at birth - μmol/L[a,e] | 9 (6–14) | 10 (8–13) | 8 (6–11) |

IQR, interquartile range; IUT, intrauterine transfusion; SD, standard deviation.

[a]Median (IQR).

[b]Mean ± SD.

[c]0 missing values in group I, five missing values in group II, one missing value in group III.

[d]one missing value in group I, five missing values in group II, one missing value in group III.

[e]17 missing values in group I, 35 missing values in group II, five missing values in group III.

group III. In group I, 129/156 (83%) neonates had an umbilical venous catheter, 68/156 (38%) in group II and 39/101 (39%) in group III (Table 2).

Characteristics and complications in subgroup of neonates treated with ET are presented in Table 3. The number of ETs per neonate did not differ between groups

**Table 2** Neonatal outcomes.

| | Group I n = 156 | Group II n = 181 | Group III n = 101 |
|---|---|---|---|
| Neonates treated with ET(s) - n (%)[a] | 104 (67) | 39 (22) | 10 (10) |
| Maximum unconjugated bilirubin level - mg/dL[b] | 13·6 ± 4·7 | 15·0 ± 5·2 | 15·3 ± 4·7 |
| Maximum unconjugated bilirubin level - μmol/L[b] | 233 ± 80 | 257 ± 89 | 262 ± 80 |
| Bilirubin > 25·0 mg/dL - n (%)[c] | 1 (1) | 6 (3) | 1 (1) |
| Umbilical venous catheter - n (%) | 129 (83) | 68 (38) | 39 (39) |
| Duration of phototherapy - days[d,e] | 4 (3–5) | 5 (3–6) | 5 (4–6) |
| Neonates receiving RBC transfusion(s) - n (%)[f] | 106 (69) | 137 (81) | 68 (74) |
| Number of RBC transfusions per neonate[d,g] | 2 (1–3) | 2 (1–3) | 2 (1–3) |
| Mechanical ventilation - n (%) | 6 (4) | 1 (1) | 1 (1) |
| Proven sepsis - n (%)[h] | 6 (4) | 6 (4) | 5 (5) |
| Duration of NICU admission - days | 6 ± 3 | 7 ± 3 | 7 ± 2 |
| Mortality - n (%) | 0 (0) | 0 (0) | 0 (0) |

ET, exchange transfusion; IQR, interquartile range; IUT, intrauterine transfusion; NICU, neonatal intensive care unit; RBC, red blood cell; SD, standard deviation.

[a] $p$-value < 0·001.

[b] Mean ± SD.

[c] Absolute 'medical emergency' value indicating direct need for intensive phototherapy as recommended by the AAP [2], equal to 428 μmol/L.

[d] Median (IQR).

[e] 22 missing values group I, one missing value in group II, 0 missing values in group III.

[f] Two missing values in group I, 12 missing values in group II, nine missing values in group III.

[g] Two missing values in group I, three missing values in group II, six missing values in group III.

[h] 0 missing values in group I, eight missing values in group II, one missing value in group III.

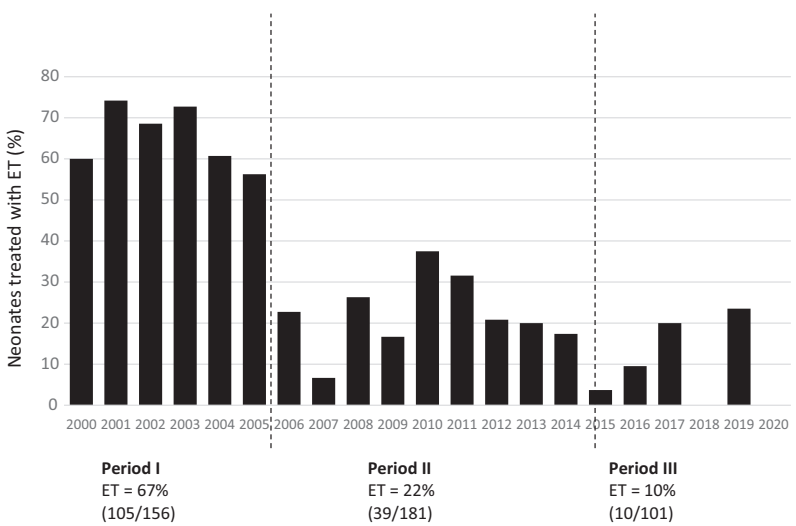


**Fig. 2** Incidence of exchange transfusion throughout the years. ET, exchange transfusion.

(median one ET per neonate); the time after birth before an ET was performed increased from a median of 6 hours in group I to a median of 50 hours in group III.

In group I, four neonates required mechanical ventilation in relation to ET treatment, none in the other groups. Sepsis after the first or following ETs occurred in 6% of group I, respectively, 11% and 10% in groups II and III. All neonates received the ET through an umbilical vein catheter. Umbilical vein thrombosis was diagnosed in one case, which was detected after an ultrasound examination was performed due to persisting thrombocytopenia. Leukocytopenia occurred in 63% of the cases in group I, 71% in group II and 33% in group III. Thrombocytopenia < $100{\cdot}10^9$/L occurred in almost all neonates that

**Table 3** Characteristics and complications in subgroup of neonates treated with ET.

| | Group I n = 104 | Group II n = 39 | Group III n = 10 |
|---|---|---|---|
| Number of ET(s) per neonate[a] | 1 (1–2) | 1 (1–2) | 1 (1–1) |
| Time to first ET - hours after birth[a] | 6 (5–9) | 31 (15–55) | 50 (18–65) |
| Mechanical ventilation - n (%) | 4 (4) | 0 (0) | 0 (0) |
| Proven sepsis as related to ET - n (%)[b] | 6 (6) | 4 (11) | 1 (10) |
| Umbilical vein thrombosis - n (%)[c] | 1 (1) | 0 (0) | 0 (0) |
| Leukocytopenia < $5{\cdot}10^9$/L - n (%)[d] | 66 (63) | 27 (71) | 3 (33) |
| Thrombocytopenia < $100{\cdot}10^9$/L - n (%)[e] | 101 (98) | 36 (97) | 10 (100) |
| 25-49·$10^9$/L | 41 (40) | 19 (50) | 3 (30) |
| <25·$10^9$/L | 21 (20) | 5 (13) | 0 (0) |
| Neonates receiving platelet transfusion(s) - n (%) | 56 (54) | 23 (59) | 3 (30) |
| Hypocalcaemia < 8·0 mg/dL - n (%) | 12 (12) | 6 (15) | 0 (0) |
| Hyperkalaemia > 6·5 mEq/L - n (%) | 1 (1) | 1 (3) | 0 (0) |
| Hearing screening performed - n (%) | 43 (41) | 32 (82) | 8 (80) |
| Hearing screening passed - n (%)[f] | 43 (100) | 32 (100) | 8 (100) |
| Cerebral ultrasound performed - n (%) | 51 (49) | 35 (90) | 10 (100) |
| Minor IVH, grade I or II - n (%) | 0 (0) | 0 (0) | 0 (0) |
| Major IVH, grade III or IV - n (%) | 0 (0) | 1 (3) | 0 (0) |
| Cystic PVL - n (%) | 0 (0) | 1 (3) | 0 (0) |
| Mortality - n (%) | 0 (0) | 0 (0) | 0 (0) |

ET, exchange transfusion; IQR, interquartile range; IUT, intrauterine transfusion; IVH, intraventricular haemorrhage; PVL, periventricular leukomalacia; SD, standard deviation.

[a]Median (IQR).

[b]Sepsis defined as symptomatic infection with positive blood culture, onset later than first ET.

[c]Only one neonate underwent a catheter ultrasound.

[d]one missing value in group II.

[e]three missing values, one in each group.

[f]61 missing values in group II, seven missing values in group II, two missing values in group III.

underwent at least one ET, but severe thrombocytopenia < $25{\cdot}10^9$/L was rare (21 neonates (20%) in group I, 5 neonates (13%) in group II and none in group III. The occurrence of hypocalcaemia < 8·0 mg/dL after ET was 12% in group I, 15% in group II and did not occur in group III. Hyperkalaemia > 6·5 mEq/L after ET occurred in only two cases, one neonate in group I and one neonate in group II.

Hearing screening was conducted in a great majority of neonates in groups II and III, but only in 43 (41%) of group I. All neonates tested passed the screening. Cerebral ultrasound was conducted in a great majority of group II (90%) and group III (100%), but only in half of the neonates (49%) of group I. Ultrasound showed one case of severe IVH (grade 3) and one case of cystic PVL in group II. No neonates died in our study population.

## Discussion

In this study, we showed a significant decline of ET incidence in neonates with severe HDFN due to severe Rh-mediated HDFN over the past 20 years. The incidence of ET treatment declined from 67% to 10%. The time to first ET after birth was postponed from 6 to 50 hours. This impressive decline in ET can be attributed to the changes and implementation of increasingly restrictive ET guidelines as well as to improved use of intensive phototherapy. Importantly and reassuringly, the strong decline in ET incident and thus decreased exposure and expertise with this complex procedure was not associated with an increase in procedure-related complications. In the Netherlands, this is presumably due to the centralization and thus specialization of care for neonates with HDFN.

Only few studies have evaluated the changes in the use of ET over the years [8,13,18–21], and although accurate comparison is not possible, these studies also show an overall sharp decline in ETs in HDFN without increase in adverse events related to ET. Comparison with other centres is complicated due to various time cohorts chosen, different study populations as definitions of (severe) HDFN vary greatly, as do local phototherapy and ET protocols and guidelines. A study from the USA compared 71 infants treated with ET between 1986 and 1995 to 36 infants treated with ET between 1996 and 2006

(overall decline of 35/71, 49%). The ET-related complications' incidence was 14% vs. 7% in the two cohorts ($p = 0{\cdot}270$). Of the studied cases, only 41% was treated with ET due to hyperbilirubinaemia caused by Rh-mediated HDFN and an additional 28% due to ABO incompatibility [8]. A study from Norway showed a decrease in ET incidence from (39/80) 49% in the period 1993–1998 to (12/96) 12% in the period 1999–2003 [18]. The study group included infants with Rh-mediated HDFN, as well as ABO incompatibility. This decline was attributed to the introduction of standard IVIg treatment in 1998. A study in India reported a decline of ET incidence from 6·8% of the total neonatal admissions in 2006 to 0·3% in 2016, but did not report on underlying indications for ET [19]. A recently published large multicentre cohort study assessing the prevalence of ET and ET-associated morbidity and mortality in the USA between 1997 and 2016 reported a decline in ETs of 0·3% to 0·05% among all NICU admissions and found similarly high numbers of thrombocytopenia and leukopenia. However, the study also did not report on underlying causes of hyperbilirubinaemia and included neonates as young as 23 weeks of gestation, complicating accurate comparison [13].

For an increasingly rare disease as HDFN, international research would be highly recommendable to register and address these major differences between countries and treatment centres. It is important to have seemingly simple numbers as the current ET incidence available for counselling of parents and caregivers, organization of care and for further research in which ET incidence can be a potential outcome measure.

In our cohort, no cases of acute transfusion reactions such as transfusion-related lung injury and transfusion-associated circulatory overload were reported. The features of these reactions are presumably less outspoken and therefore under recognized in neonates [22].

Several studies that describe the decline of ETs during the last three decades suggest that the decrease in ETs could be explained by the administration of IVIg to neonates [18]. A Cochrane review by Zwiers et al. [23] was inconclusive on this subject since the studies that showed a reduction in ETs after administration of IVIg were of low quality and of high risk of bias. The only two high-quality placebo-controlled randomized studies did not provide any evidence that administration of IVIg reduced the need for ETs [24,25]. In our centre, we do not administer IVIg (except during the study period of the aforementioned LIVIN study [24]), and therefore, the observed decline in ETs in our cohorts cannot be explained by IVIg treatment.

Earlier work by our study group showed a tendency towards an increase in the post-natal RBC transfusion rate and correlated with a decrease in ET incidence in neonates with severe HDFN. This effect was attributed to the removal of antibodies and IgG-coated erythrocytes during ET, hence reducing the haemolytic process [10,18,23]. Although morbidity of RBC transfusion (reported between 0·014 and 0·04% [26]) is much less than the morbidity of ET treatment (between 7 and 24% [9]), it is still of interest that in the current study, no further increase in neonatal RBC transfusions was observed. A possible explanation could be the implementation of a more restrictive RBC transfusion threshold at our department in February 2014.

This study has several strengths and limitations. One of the major strengths is the setting of one national referral centre for severe HDFN, providing us with a homogenous and near-complete collection of data and follow-up records of an increasingly rare disease. However, the results must be carefully interpreted in the context of our study population, as it is a selection of (very) severe HDFN cases as result of the referral guidelines in the Netherlands. Neonates not treated with IUT, particularly the less severe cases that did not require foetal therapy, were probably more likely to have been admitted to other centres.

Another major limitation of this study, and of the research in the field of HDFN in general, is the lack of long-term outcome results of these neonates. Although cerebral ultrasound and hearing screening are now part of routine care after IUT treatment and ET treatment at our centre and no neonates in this study showed abnormalities in these tests, the occurrence of long-term (mild) symptoms of bilirubin encephalopathy is unknown, as well as clear, absolute bilirubin cut-off values which may give rise to such symptoms.

In conclusion, the need for ET in neonates with severe HDFN admitted to our centre has gradually decreased and has now become relatively rare. Reduction in ET incidence and therefore in expertise in performing this complex procedure was not associated with an increase in procedure-related complications. Nevertheless, if the exposure of physicians to ET treatment will continue to decline, centralization of this procedure in specialized tertiary care centres may be necessary to maintain sufficient experience.

## Conflicts of interest

There are no conflicts of interest to report.

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VoxSanguinis
The International Journal of Transfusion Medicine
ISBT International Society of Blood Transfusion
*Vox Sanguinis* (2021)

ORIGINAL PAPER


DOI: 10.1111/vox.13095

# Activity-based cost of platelet transfusions in medical and surgical inpatients at a US hospital

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Vox Sanguinis

**Background and objectives** Previous studies by the Cost of Blood Consensus Conference (COBCON) have used a comprehensive, standardized and generalizable activity-based costing (ABC) model to estimate the cost of red blood cell transfusions and plasma transfusion. The objective of this study was to determine the total cost of platelet transfusions in a real-world US hospital inpatient setting.

**Materials and methods** This database analysis study retrospectively collected costs for all activities related to platelet transfusion in a single-acute care US teaching hospital in 2017. Costs were collected in a stepwise manner using a custom ABC model which mapped the technical, administrative and clinical processes involved in the transfusion of platelets.

**Results** For the 15 024 inpatients included in the analysis, 6335 (42·2%) were given a blood type and screen, and 941 (6·3%) received a transfusion of one or more blood products. A total of 333 platelet units were transfused in 131 patients (mean 2·54 units per patient): 211 (63·4%) units in medical inpatients and 122 (36·6%) in surgical inpatients. The total cost was $1359·99 per platelet unit, corresponding to $3457·06 per inpatient. Acquisition costs made up the largest proportion of the total cost (45·1%) followed by direct and indirect overheads (38·7%) and hospital processes costs (16·3%).

**Conclusion** This is the first study to use an ABC costing model to determine the full cost of platelet transfusions within a US inpatient setting. This provides a useful reference point for comparisons with other transfusion products, and considerations for cost reduction.

*Received: 25 September 2020, revised 11 February 2021, accepted 17 February 2021*

**Key words:** platelet transfusion, transfusion medicine, transfusion strategy, transfusion therapy.

## Introduction

Platelets are used therapeutically for the treatment of acute haemorrhage in patients who develop thrombocytopenia (low platelet count), or prophylactically to reduce the risk of bleeding for high-risk patients undergoing surgical procedures [1, 2]. The two main types of platelet products currently available for use are pooled donor platelets, which contain products from multiple donors, and apheresis platelets which are harvested from a single donor [3]. The 2017 National Blood Collection and Utilization Survey (NBCUS) found that 1 937 000 (95% CI, 1 794 000–2 079 000) platelet units were transfused in the United States in 2017, while 2 560 000 (95% CI, 2 391 000–2 730 000) platelet units were distributed over the same time period. This represents a 5·1% increase in

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distributed platelets and a 2·3% decrease in transfused platelets since the data collected in 2015, suggesting a continued but slowing decline in demand for blood products [4].

Platelet transfusions are indicated for the treatment of patients with thrombocytopenia or platelet function disorders [5] and are associated with higher cost compared with other blood products such as fresh-frozen plasma (FFP) or red blood cells (RBC). The most prevalent issues associated with platelet distribution and transfusion are concerned with storage time, pathogen risks, transfusion refractoriness and supply issues [6]. Platelets can be stored for up to 5 days postcollection, but may become deactivated or dysfunctional towards the end of this storage time [7]. In platelet products which are stored at room temperature, there may be the risk of pathogen contamination [8]. The process of photochemical pathogen reduction, which involves inactivating pathogens in the product, can reduce pathogen load, but has not been shown to decrease the risk of platelet refractoriness, and there are varying results on whether pathogen inactivation reduces all-cause mortality [9, 10]. Between 2011 and 2015, a decline in demand for blood products was seen in the United States, which may be due to guidelines suggesting more conservative thresholds for transfusion, and the implementation of blood management programmes designed to minimize patient transfusion needs [11]. This decline has slowed in recent years, although data from 2017 still showed fewer platelet transfusions compared with 2015 [4].

In discussions regarding the effectiveness of prophylactic therapy, or clinical thresholds for platelet transfusions, it is important to be able to accurately assess the risk benefit and cost benefit of transfusions. To gain the most accurate cost benefit assessments, a costing model needs to include all costs related to platelet transfusion, not just the cost of the platelet units alone. Costs should include direct and indirect hospital overheads, and all processes involved in patient testing, decision making, consent, and administration and monitoring of platelet transfusion. By providing a full analysis of all platelet-related costs, this model will allow accurate comparisons of platelets with other haemorrhage therapies, and a better understanding of the cost benefit of prophylactic treatment.

Previous studies by the Cost of Blood Consensus Conference (COBCON) have used a comprehensive, standardized and generalizable activity-based costing (ABC) model to estimate the cost of red blood cell transfusions [12] and plasma transfusions [13]. In this study, we used the ABC method to map all technical, administrative and clinical processes involved in the cost of platelet use in real-world US medical and surgical inpatients.

## Materials and methods

The objective of the study was to determine the total cost of platelet transfusions in an inpatient setting in a US acute care teaching hospital. Data were collected from Englewood Hospital, Englewood, New Jersey, during the calendar year 2017. The study used an activity-based costing (ABC) process flows model, and custom ABC software, developed by the COBCON group [14] and previously described by Shander *et al.* [12]. Ethical approval for the study was provided by the Englewood Hospital (EH) institutional review board.

The data collected for this study were observed and recorded by trained study staff. Data were captured retrospectively from the hospital database in a stepwise manner and used to populate the modules of the custom ABC software as described by Shander *et al.* [12]. Total cost was specified to include the following: all product acquisition costs, both for products used and products wasted; transfusion-related hospital processes, including patient admission, screening and testing, logistics, staff support, administration and consent; and direct and indirect overhead costs. Direct overhead costs were either added directly from the hospital finance department or estimated, while indirect overhead costs, such as day-to-day hospital operations, administration and central services, were added as a percentage of the overall hospital process costs. The percentage of indirect overhead costs relative to the direct process costs were calculated from the overall hospital ratio between indirect (e.g. non-patient-related) costs and direct or operational costs, as described more fully in Shander *et al.* [12]. Costs relating to long-term complications, patient rehabilitation, litigation and indemnification were excluded and the impact of platelet transfusions on hospital length of stay was also not assessed by the model. A full list of all in-hospital processes and process cost captured and the direct overhead cost is given in Table S1, while a list of all indirect hospital processes used for the calculation of indirect cost is given in Table S2.

## Results

### Hospital characteristics

A total of 402 014 patients were recorded at the study hospital over the calendar year 2017. Of these, 18 512 (4·6%) were inpatients, of which 15 024 (3·7%) had a diagnostic-related group (DRG) recorded and were included in the cost analysis. Of all inpatients included in the analysis, 9384 (62·5%) were medical inpatients, 5012 (33·4%) were surgical inpatients, and 628 (4·2%) were other inpatients.

A summary of the inpatient blood product usage is shown in Table 1. In total, 425 platelet units were transfused, all of which were single donor apheresis platelets. Of these, 333 (78·4%) were given to inpatients. Of these 333 platelet units, 211 (63·4%) were transfused in medical inpatients and 122 (36·6%) in surgical inpatients.

Transfusion-related statistics for the 15 024 inpatients, including screening tests performed and the number of units transfused, are shown in Table 2. While 10 752 (71·6%) of inpatients were given some form of transfusion-related blood test, including 6335 (42·2%) who had a blood type and screen, only 941 (6·3%) of patients received a transfusion of one or more blood products. A total of 333 platelet units were transfused in 131 inpatients, giving a mean of 2·54 units per patient. The mean platelet units transfused per patient was higher for medical inpatients (2·97) than for surgical inpatients (2·03). Patients required platelet transfusions for a number of clinical indications, the most common being: cardiac valve and other major cardiothoracic procedures, septicaemia or severe sepsis, ECMO or tracheostomy, coronary bypass (without cardiac catheterization), gastrointestinal haemorrhage, major haematological and immunological disorders, lymphoma and non-acute leukaemia, and pleural effusion (see Table S3). 4·49% of the platelets were discarded.

### Cost results

The average cost of platelets estimated over the course of the study was $1359·99 per platelet unit in the inpatient setting, corresponding to an average of $3457·06 per inpatient transfused with platelets (Fig. 1). This total includes costs from direct and indirect overheads, costs from in-hospital processing related to platelet transfusion and the cost of platelet acquisition (see Table S1 for a full list of the processes recorded). Acquisition costs and overheads made up the largest proportion of the costs.

The breakdown of the total cost per patient and cost per transfused platelet unit of the acquisition, hospital processes and overhead costs is shown in Table 3. Total platelet acquisition cost was $204 115·25 with an average cost of $585·41 per transfused unit and $1488·10 per patient and made up the largest proportion (45·1%) of the total cost. Total hospital processes costs were $73 631·98 and comprised the smallest proportion (16·3%) of the total cost. The largest contributors to the in-hospital processing costs were as follows: pre-transfusion processes and patient blood testing, and the administration and monitoring of transfusions (Fig. 2). Overhead costs, including staff, staff support and equipment costs, were a total of $175 128·13 corresponding to 38·7% of the total costs. Indirect overheads were responsible for 95·0% of the total overhead costs, while direct overheads made up the remaining 5·0% (Fig. 2).

**Table 1** Characteristics of blood product usage

| | All patients N (%) | Inpatients N (%) |
|---|---|---|
| Number of patients | 402 014 (100·0) | 18 512 (4·6) |
| Units of BP charged | 3991 (100·0) | 3073 (77·0) |
| Red blood cells | 2958 (100·0) | 2150 (72·7) |
| Platelets | 425 (100·0) | 333 (78·4) |
| Fresh-frozen plasma | 529 (100·0) | 513 (97·0) |
| Cryoprecipitate | 79 (100·0) | 77 (97·5) |

BP, blood products.

## Discussion

From the authors understanding, this is the first time that the ABC methodology has been applied in a hospital setting to determine the full cost of platelet transfusion. Overall, the study showed that out of the 15 024 inpatients, there was an associated cost of $1359·99 per platelet unit. This corresponds to a cost of $3457·06 per patient transfused.

Previous studies have used the ABC methodology to explore the total cost of RBC [12] and FFP transfusions [13]. The RBC costs were explored for four different hospitals while the FFP analysis was carried out in the same hospital as the platelet analysis reported here. Overall, the cost per unit and per patient was three times higher for platelets than for FFP, with platelet costs of $1359·99 per unit versus $409·62 for FFP and $3457·06 per patient versus $1608·37 for FFP [13]. Platelet costs were also higher than the total costs for RBC transfusions which came to $760·82 (standard deviation [SD]: $293·74) per RBC unit. Comparing the cost breakdowns between the different blood products showed that acquisition costs made up a much higher proportion of the total cost (46%) for platelet transfusion compared with red blood cells (20–30%) [12] and FFP (11%) [13], which reflects the higher cost of platelet units.

A full understanding of the cost benefits of platelet transfusion is advantageous for clinical practice, and other studies have also made estimates for total platelet cost. A conceptual framework using a literature search to identify direct, indirect and intangible platelet transfusion costs (including those identified through the COBCON group) gave an average cost of $5258 to $13 117 per patient in patients with chronic liver disease and associated thrombocytopenia undergoing elective procedures [15]. While this framework does cover a full range of costs, including costs associated with transfusion-related

**Table 2** Inpatient transfusion-related characteristics

| | All inpatients ($n$ = 15 024) N (% of total) | Medical inpatients ($n$ = 9384) N (% of medical inpatients) | Surgical inpatients ($n$ = 5012) N (% of surgical inpatients) | Other inpatients ($n$ = 628) N (% of other inpatients) |
|---|---|---|---|---|
| Number of patients undergoing transfusion-related blood tests | 10 752 (71·6) | 6694 (71·3) | 4055 (80·9) | 3 (0·5) |
| Type and screen | 6335 (42·2) | 3108 (33·1) | 3225 (64·3) | 2 (0·3) |
| PTT | 5443 (36·2) | 3629 (38·7) | 1813 (36·2) | 1 (0·2) |
| PT | 6674 (44·4) | 4565 (48·6) | 2108 (42·1) | 1 (0·2) |
| Plavix function test* | 170 (1·1) | 22 (0·2) | 148 (3·0) | 0 (0·0) |
| Platelet function test† | 23 (0·2) | 12 (0·1) | 11 (0·2) | 0 (0·0) |
| Number of patients transfused | | | | |
| ≥1 BP | 941 (6·3) | 552 (5·8) | 389 (7·7) | 0 (0·0) |
| Platelet only | 28 (0·2) | 20 (0·2) | 8 (0·2) | 0 (0·0) |
| Platelet or other BP including platelet | 131 (0·9) | 71 (0·8) | 60 (1·2) | 0 (0·0) |
| Other components only | 810 (5·4) | 481 (5·1) | 329 (6·6) | 0 (0·0) |
| Number of units transfused | | | | |
| All BP | 3079 (100·0) | 1682 (100·0) | 1397 (100·0) | 0 |
| RBC | 2156 (70·0) | 1179 (70·1) | 977 (69·9) | 0 |
| Platelets | 333 (10·8) | 211 (12·5) | 122 (8·7) | 0 |
| FFP | 513 (16·7) | 259 (15·4) | 254 (18·2) | 0 |
| Cryoprecipitate | 3079 (100·0) | 1682 (100·0) | 1397 (100·0) | 0 |

BP, blood products; FFP, fresh-frozen plasma; PT, prothrombin time; PTT, partial thromboplastin time; RBC, red blood cells.

*The institution uses VerifyNow, which provides platelet reactivity information to guide antiplatelet therapy P2Y12 inhibitors such as clopidogrel.

†Platelet function testing was carried out with the PFA-100 analyser system.

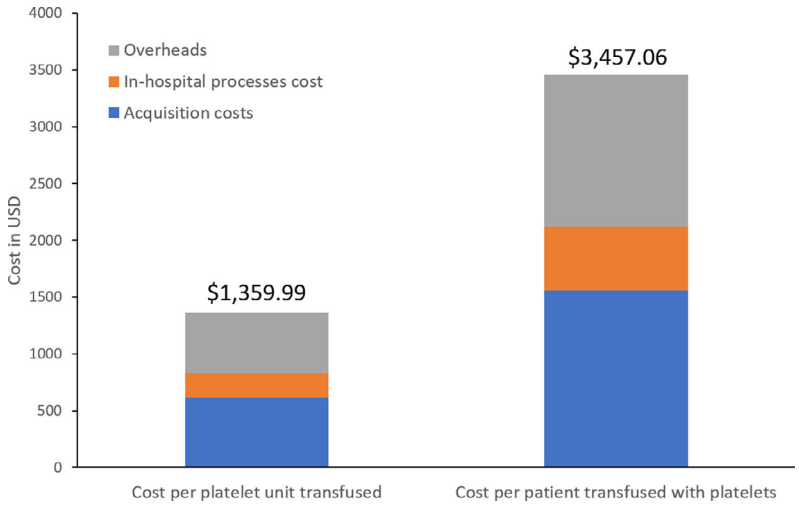


**Fig. 1** The mean cost of platelets per unit and per patient.

**Table 3** Platelet inpatient product acquisition cost and in-hospital processes.

| | Total hospital cost ($) | Percentage of total cost (%) |
|---|---|---|
| Total product acquisition cost | 204 115·25 | 45·1 |
| Per platelet unit transfused | 612·96 | - |
| Per patient transfused with platelets | 1558·13 | - |
| Total in-hospital process cost | 73 631·98 | 16·3 |
| Per platelet unit transfused | 221·12 | - |
| Per patient transfused with platelets | 562·08 | - |
| Total overhead cost | 175 128·13 | 38·7 |
| Per platelet unit transfused | 525·91 | - |
| Per patient transfused with platelets | 1336·86 | - |
| Total cost of platelets | 452 875·37 | 100·0 |
| Per platelet unit transfused | 1359·99 | - |
| Per patient transfused with platelets | 3457·06 | - |

adverse events and platelet refractoriness, it is limited to a specific clinical indication. The higher estimate in this case is most likely due to the incorporation of adverse event costs, with the model assuming 9·95% incidence of treatment-related adverse events and assuming all events would require additional care with events of grade 2 or higher leading to delays in elective procedures (which were also accounted for in the cost model).

Cost analyses have also been carried out to specifically identify areas where cost savings in plasma transfusion might be made. A multivariate logistic regression analysis model to identify and predict patients without excessive bleeding who would not need transfusions demonstrated cost savings in all blood products (RBCs, FFP and platelets) in patients undergoing coronary artery surgery. However, platelets represented the lowest cost saving (10·9%, 20 out of 183 doses) compared with RBCs (48·2%, 1712 out of 3554 doses) and FFP (38·9%, 432 out of 1111 doses) [16]. Due to the higher unit cost, it may be that platelets are used more sparingly and in cases where the need for transfusion is higher. A retrospective study of the use of prospective monitoring and triage in a Level 1 designated trauma centre and teaching hospital resulted in a 25% reduction in platelet transfusions over a 7-year period with savings of $100 per dose in platelet administration costs [17]. However, overhead costs were not included in this retrospective analysis. Costs may also be reduced by eliminating inappropriate platelet transfusions, with results from an inpatient hospital setting suggesting that implementing best practice protocol alerts into an electronic medical record resulted in a 20% reduction in transfusion corresponding to estimated annual savings of around $70 000 USD [18]. More specific methods for reducing platelet costs may also be calculated by considering specific indications, rather than overall hospital use, and considering how treatment for these indications may be streamlined and improved.

These results highlight the importance of mapping all activities associated with platelet use, which can best be done by comparing the ABC modelling costs. The results of the ABC analysis allow the full cost-effectiveness of therapy to be explored, along with the potential for reducing cost per transfusion either by reducing the cost of each transfusion or the overall number of transfusions carried out. It also allows cost comparisons between different treatments and different transfusion options to be made, to potentially identify further cost savings in clinical practice. The new FDA industry guidelines for bacterial risk control strategies for blood collection recommend pathogen reduction of apheresis platelets, or the use of a

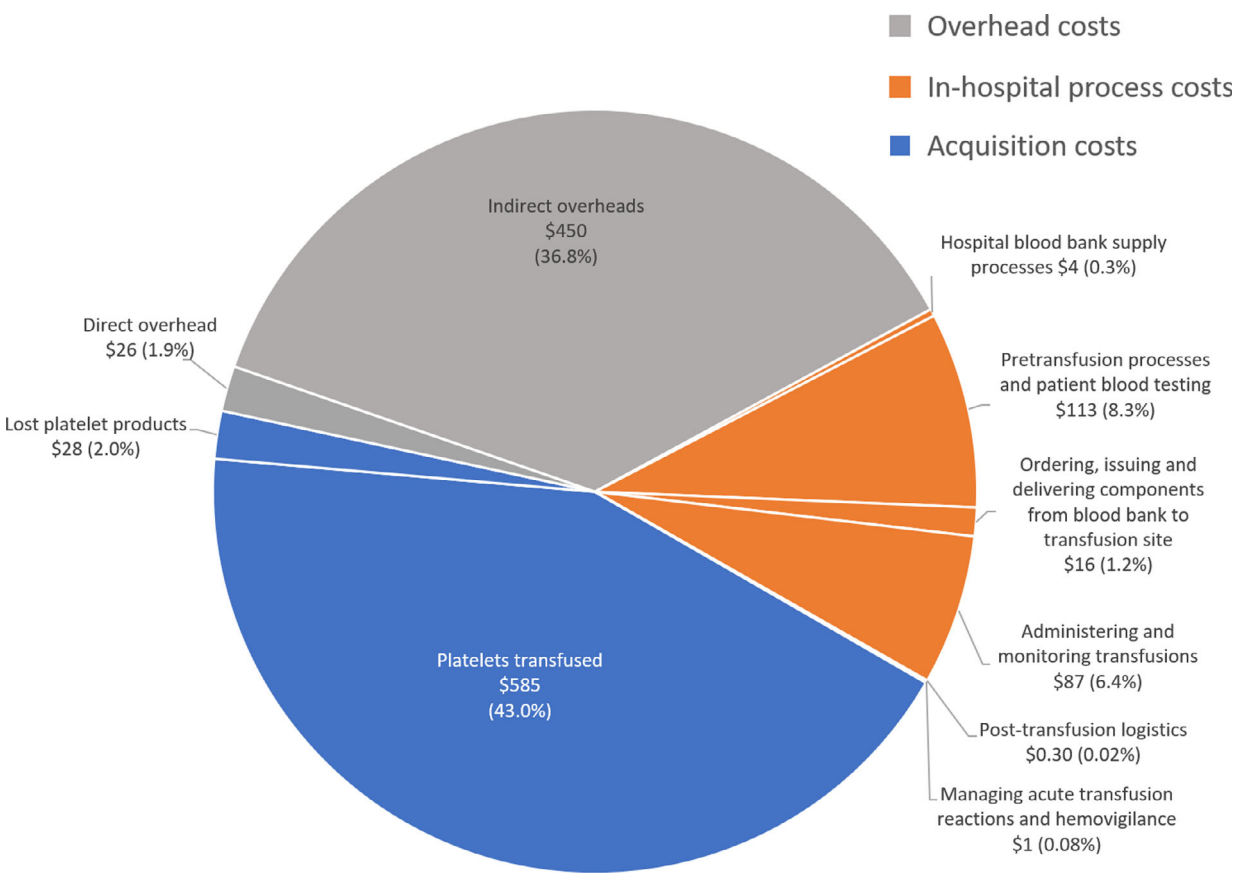


**Fig. 2** Costs per unit platelet transfusion, split by overhead costs, in-hospital process costs and acquisition costs.

secondary culture to test for bacterial contamination prior to transfusion [19], which may lead to increases in overall platelet costs. As this guidance was released after the data for this analysis were collected, the platelets in this study did not undergo pathogen reduction and the institution did not carry out secondary cultures to test for contamination; however, it will be interesting to see how the implementation of this guidance affects overall platelet cost in future analyses.

Whilst this study provides a full and robust costing model for platelet transfusions, there are some limitations. In examining costs associated with platelet transfusions, the study did not address whether the improvement in patient outcomes caused by platelet transfusion led to long-term reduced costs in patient treatment. Studies have shown that platelet transfusion can lead to decreased mortality in trauma patients (although no effect was seen on hospital-free or ICU-free days) [1], and while platelet transfusions are more costly than other blood products, they are vital for patients with thrombocytopenia or platelet function disorders [5]. Costs relating to donor recruitment and blood collection for platelets were also not included. The study was carried out at one hospital and while this allows a full estimate of all in-hospital practices to be collected, it does not account for internal variation in hospital spending practices, screening protocols or overhead costs between hospitals. Further research could expand the study to more than one institution, using the ABC model to compare costs between them and potentially identify areas where cost savings could be made.

In conclusion, this is the first study which uses an ABC model to capture the costs of all necessary activities required for transfusion of platelet units. This method can provide a realistic cost assessment that includes all the hidden costs associated with the management of blood components in an inpatient setting. This information should help to guide clinicians and transfusion healthcare professionals to understand the full cost of platelet transfusion, and considerations for reducing costs in clinical practice.

## Acknowledgements

Editorial assistance with manuscript preparation was provided by Meridian HealthComms (Plumley, UK), funded by The Society for the Advancement of Blood

Management (SABM). The Society for the Advancement of Blood Management, Inc., funded this research with a research grant from Accriva Diagnostics, Inc., which is now part of Instrumentation Laboratory.

## Conflicts of interest

Axel Hofmann has received consultancy or speaker fees from Celgene, Belgium; Instrumentation Laboratories, Werfen; and Vifor Pharma, Switzerland. Sherri Ozawa reports no relevant conflicts of interest. Aryeh Shander has received speaker or consultant fees from Merck, AMAG, Baxter, CSL Behring, Daiichi Sankyo, Grifols, HbO2 Therapeutics LLC, Masimo, Octapharma, Pharmacosmos and Vifor Pharma; and reports grants/research support from CSL Behring, HbO2 Therapeutics LLC, Masimo and Instrumentation Laboratory.

## Funding

The Society for the Advancement of Blood Management, Inc., funded this activity with a research grant from Accriva Diagnostics, Inc., which is now part of Instrumentation Laboratory.

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## Supporting Information

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

Table S1 Full list of costs used in the ABC model: direct overhead costs and in-patient processing costs.
Table S2 Full list of indirect overhead costs, added to the model as a percentage of the overall hospital process costs.
Table S3 Top 10 recorded DRG codes for patients receiving platelet transfusion.

VoxSanguinis
The International Journal of Transfusion Medicine

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*Vox Sanguinis* (2021) **116**, 1005–1011


DOI: 10.1111/vox.13092

ORIGINAL PAPER

# Altered strategy of prophylactic anti-D administration in pregnancy to cover term and post-term – a pilot study

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Vox Sanguinis

**Background and objective** Routine antenatal anti-D prophylaxis (RAADP) to RhD-negative women is most often administered in gestational age (GA) 28–30 weeks with the next anti-D dose administered postpartum. The aim of this study was to analyse the proportion of RhD-negative women where RAADP is not detectable at term and in a pilot study to investigate whether RAADP administered in GA 28 and 38 results in detectable levels at term, post-term and post-delivery.

**Materials and methods** In a retrospective analysis, 4280 RhD-negative women carrying an *RHD* positive fetus were included and the proportion with a negative antibody screen at delivery was determined. In the second part, 39 pregnancies were included prospectively, a second dose of RAADP was administered in GA 38 weeks, and anti-D was quantified before the second dose and then weekly for 5 weeks.

**Results** In the retrospective analysis, 20·5% (856/4280) with RAADP administered in GA 28 were negative in routine antibody screening at delivery. In the small prospective study, 18% (7/39) had a negative antibody screen and 26% (10/39) had levels below 0·005 IU/ml, in the quantification assay, in GA 38. Anti-D prophylaxis administered in GA 38 showed detectable levels of anti-D up to 30 days post-delivery, with concentration at delivery 0·060 ± 0·034 IU/ml (mean ± SD).

**Conclusion** Approximately 20% of the RhD-negative women show non-detectable levels of anti-D at term. A second dose of RAADP at GA 38 results in stable concentrations of anti-D at term, post-term and post-delivery, but with large interindividual variation.

*Received: 9 October 2020, revised 10 January 2021, accepted 15 February 2021, published online 27 March 2021*

**Key words:** anti-D immunization, anti-D prophylaxis, pregnancy.

## Introduction

Despite that anti-D prophylaxis regimens to prevent alloimmunization during pregnancy are used for 50 years and are shown effective, anti-D is the most common red cell antibody causing severe haemolytic disease of the fetus and newborn (HDFN) [1]. Fetal anaemia needing treatment with intrauterine transfusions is in the majority of cases due to anti-D immunization [2]. Postnatal anti-D immunoglobulin prophylaxis to RhD-negative women with a newborn blood typed RhD positive, introduced in the late 1960s, reduced the risk of immunization of RhD-negative women from approximately 15% to 1–1·5%

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[3,4]. In the 1990s, many countries added routine antenatal anti-D prophylaxis (RAADP) in the third trimester to all RhD-negative women, further minimizing the risk of immunization to an incidence of 0·2–0·4% [5]. During the last decade, fetal *RHD* genotyping combined with targeted RAADP only to those carrying an *RHD* positive fetus has been proven effective and is implemented in many countries [6]. The strategies of doses and timing of administration of RAADP vary between countries, one single dose of anti-D; 1000–1500 IU in gestational age (GA) 28–30 or two doses of 500–625 IU at 28 and 34 weeks are the most common routines [7–9]. A second or third dose anti-D is given post-delivery, usually a dose of 1000–1500 IU. In some programmes, testing of fetomaternal haemorrhage (FMH) is recommended at delivery, to determine whether additional doses of anti-D are required [10].

It is known that one single dose anti-D in GA 28 is not detectable at delivery in a proportion of women, varying from 39 to 56% and up to 78% for those who deliver after 40 weeks of gestation [7,8] thus with unclear protection at the end of the third trimester, when the risk of immunization is increased [9,11,12]. The two-dose strategy gives a higher rate of detectable anti-D at term, 85%, but is associated with higher costs and lower compliance of receiving the two doses [13,14].

In Sweden, the recommendations are to perform fetal *RHD* screening in the first trimester and to administer targeted RAADP, 1500 IU in GA 28–29 and a second dose of anti-D 1500 IU post-delivery and at situations carrying an increased risk of fetomaternal haemorrhage during pregnancy [15,16]. The current information of the used product anti-D; Rhophylac® (CSL Behring) is that a dose of 50 IU (10 μg) protects from sensitization of one ml fetal blood in maternal circulation and 1500 IU (300 μg) protects from 30 ml fetal blood [17]. Using an estimated maternal plasma volume of 3750 ml and an extracellular volume of 4500 ml at term [11], a dose of 1500 IU should give a theoretical concentration of 0·180 IU/ml (36 μg/l). An anti-D concentration of 0.012 IU/ml (2.4 μg/l) is thus equivalent to cover FMH of 2 ml whole blood.

Today, when fetal *RHD* typing is widely implemented and the *RHD* type of the fetus is known with high accuracy [6], an alternative strategy could be to administer two RAADP doses, at GA 28 and 38, the latter to cover term and post-term of the pregnancy. In Sweden, 20% of the women delivered post-term at week 41 and 5% at week 42, 2019 [18].

The aim of this study was to retrospectively analyse the proportion of women with undetectable levels of prophylactic anti-D at the time of delivery after one dose of RAADP (1500 IU) at GA 28–29. Second, in a pilot study, to investigate whether a strategy with administration of the second dose of anti-D in GA 38, instead of post-delivery, would be safe and feasible and result in sufficient concentrations at term and post-delivery.

## Materials and methods

### Retrospective data analysis

The study population consisted of all consecutive cases of RhD-negative pregnant women with request from the delivery ward for a type-and-screen test, between October 2010 and October 2012 in the Stockholm region, Sweden. After this period, an RhD-negative test panel for type-and-screen requests in RhD-negative women was introduced, in order not to cause delay when blood transfusions were needed. According to clinical routine in the Stockholm region, RAADP used in this period was 1250 IU Rhesonativ® (Octapharma, AG, Austria) at GA 28–29. Pretransfusion tests from the delivery ward are requested for women with increased risk of postpartum haemorrhage, which includes approximately 25% of the women, for example before caesarean section. In the laboratory information system, the proportion of RhD-negative women, with an *RHD* positive fetus, and a negative screen test at delivery was identified retrospectively, that is those where the anti-D prophylaxis administered in GA 28–29, was not detectable at delivery.

#### *The prospective pilot study*

Between 2016 and 2018, RhD-negative women with a fetus typed *RHD* positive, were asked to participate in the study, at their appointment to receive RAADP, 1500 IU (Rhophylac®, CSL Behring GmbH, Marburg, Germany) in GA 28. Eligible participants were healthy women without previous history of pregnancy complications, with a singleton pregnancy and estimated due date confirmed by ultrasound examination in the second trimester. Exclusion criteria were administration of anti-D prophylaxis earlier in the current pregnancy, a multiple pregnancy, presence of maternal erythrocyte alloantibodies at time of inclusion or medication other than vitamins, folic acid and iron supplement. Before inclusion, a routine obstetric history was checked, as well as blood pressure. Information on body height and weight was received from the first antenatal visit in GA 10–14.

The women were given oral and written information from a research midwife, and if they agreed, they received a second dose of anti-D prophylaxis 1500 IU in GA 38. Anti-D concentration was analysed before the anti-D administration in GA 38 and then monitored weekly until the correspondent time of 43 weeks GA, including post-delivery. The quantitative anti-D analysis was done only daytime weekdays, so administration of anti-D prophylaxis after delivery was based on the type-

and-screen test. If the erythrocyte antibody screen was positive at delivery, the women received no more prophylaxis; if the anti-D level was low at delivery, defined as negative or a weak reaction in the routine antibody screen, it was decided to recommend anti-D prophylaxis for safety purposes. The anti-D concentration in the samples was analysed in a quantification assay afterwards. Determination of FMH was done at day one post-delivery. A total of 39 pregnant women gave informed consent and were included in the study. The demographic characteristics of the included women are shown in Table 1.

### Type-and-screen analysis

Screen test at delivery was performed in an automated system AutoVue Innova in BioVue Cassettes with anti-IgG (Ortho Clinical Diagnostics, Raritan, NJ, US) in the retrospective analysis, 2010–2012. During the prospective pilot study 2016–2018, the instrument was upgraded to an Ortho Vision, with the same reagents used.

Titration of the 2nd BRITISH STANDARD 1992 Anti-D (Rho) Antibodies (code: 73/515, NIBSC, Potters Bar, UK) in the BioVue Cassettes showed that the limit of detection for anti-D was 0·008 IU/ml (1·6 μg/l) quantified by flow cytometry. A weak reaction in the screening was defined as ≤1+ reaction and corresponded to anti-D less than 0·015 IU/ml (3 μg/l).

### Anti-D quantification

Anti-D quantification was performed using flow cytometry. To increase sensitivity erythrocytes were pretreated with the enzyme papain. All dilutions were made in ID-CellStab (Bio-Rad Laboratories Inc) with 0·5% BSA (A7906, Sigma-Aldrich) as additive. The calibration standard was the 2nd BRITISH STANDARD 1992 Anti-D (Rho) Antibodies (code: 73/515, NIBSC, Potters Bar, UK). In short, 50 μl 0·8% erythrocytes (R0r) and 100 μl standard or sample was incubated for 30 min at 37°C. Samples were diluted 10× before analysis. After washing with 3 × 200 μl PBS, 50 μl mouse anti-human IgG-PE (Southern Biotech) diluted 100-fold was added and incubated for 30 min at RT. After an additional wash as above, the erythrocytes were dispersed in 250 μl PBS and analysed on Cytomics FC500 MPL. The mean fluorescense intensity (MFI) on 10 000 gated cells were measured and imported to SoftMaxPro (Molecular Devices) which was used for constructing the calibration curve and calculation of anti-D concentration. QC samples at two levels were run in all assays, $n = 14$. The inter-assay variation for the low QC was 20% (mean 0·032 IU/ml) and 26% for the high QC (mean 0·57 IU/ml). LLOQ in samples was 0.005 IU/ml (1 μg/l).

**Table 1** Characteristics of included women $n = 39$

| | Median [min–max] |
|---|---|
| Age | 32 [19–49] |
| BMI at gw 10–12 | 23.9 [18.8–34.8] |
| GA at delivery (days) | 281 [266–295] |
| No of blood samples* | 4 [2–6] |
| Mode of delivery (%) | |
| Vaginal | 87% |
| Caesarean Section | 13% |

GA, gestational age.
*Number of samples analysed for anti-D concentration per woman.

### Analysis of fetomaternal haemorrhage

Determination of FMH was done by flow cytometry at day one, after delivery, using FITC-conjugated Anti-D reagent (9433FI, NHS Blood and Transplant) according to the Guidelines on the estimation of Fetomaternal Haemorrhage [19]. The LOD was 0·05% of fetal erythrocytes, corresponding to 1 ml FMH.

### Statistics

Statistics presented as median and IQ range or mean and SD as well as linear regression analysis, were made with GraphPad Prism 5.

### Ethical approval

The study was approved by Swedish Ethical Review Authority, D-nr 2016/2:4.

## Results

### Detectable levels of anti-D prophylaxis at delivery in a retrospective group

During the period Oct 2010–Oct 2012, type and screen were requested from the delivery ward in 4280 RhD-negative women carrying an *RHD* positive fetus. In 876 cases (20·5%), the antibody screen result was negative; that is, anti-D was not detectable at delivery, which corresponds to an anti-D concentration less than 0·008 IU/ml (1·6 μg/l). Individual information of GA at delivery was not available.

### Anti-D concentration before and after administration of anti-D prophylaxis at 38 weeks of pregnancy in the intervention group

The mean anti-D concentration at inclusion in the prospective pilot study was 0·014 ± 0·011 IU/ml

[mean ± SD, $n = 39$] in GA 38 ± 1. In 7/39 (18%) women, the anti-D was not detected in the antibody screening. In 10/39 (26%), the level was below LLOQ in the anti-D quantification assay, 0·005 IU/ml (1·0 μg/l), Fig. 1. The anti-D concentration measured one week after administration of prophylaxis was 0·075 ± 0·047 IU/ml [mean ± SD]. The increase of anti-D was 0·066 ± 0·045 IU/ml [mean ± SD] and showed a significant correlation ($P = 0·0118$) with body mass index, Fig. 2.

### *Anti-D concentration at delivery in the intervention group*

At delivery, the variation of anti-D concentration was large, between 0·014 and 0·184 IU/ml, with mean and SD of 0·060 ± 0·034 IU/l, Fig. 3. Median GA of delivery was 40 weeks (38–42 weeks). Five women were recommended additional prophylaxis due to a weak result in the screen test at delivery. The anti-D concentrations measured afterwards were between 0·014 and 0·030 IU/ml (3–6 μg/l). Two of the five women had levels below 0·015 IU/ml (0·0144 and 0·0145), theoretically protective of 2·5 ml fetal blood. One of the women had high BMI, 34, and both had low concentrations at all measurements. The other three women had concentrations of 0·027–0·030 IU/ml. In 25/39, FMH was analysed after delivery and the results were negative in all, that is below the LOD, 1 ml fetal blood in maternal circulation.

Samples for anti-D quantification were received from all women at delivery. In 16/39, no more samples were obtained, in 14 only one sample was obtained, and in 9, 2–4 samples were obtained after delivery. The levels showed stable levels up to 30 days postpartum, Fig. 3. The concentration of anti-D analysed in 1 - 5 weeks post-delivery samples ($n = 31$) were 0·058 ± 0·019 IU/l (mean ± SD) after administration of anti-D 1500 IU in GA 38 ± 1, excluding the five women who got a new dose anti-D at delivery.

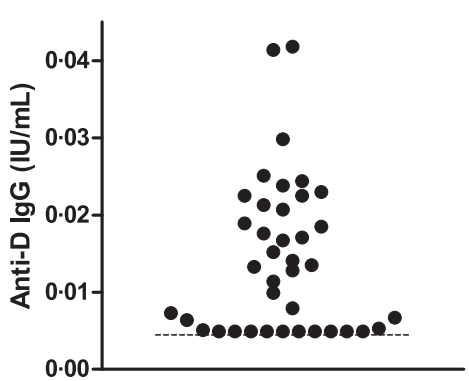


**Fig. 1** Anti-D concentration (IU/ml) at inclusion in the study at week 38 ± 1, $n = 39$, after administration of RAADP 1500 IU in gestational week 28. The dotted line represents limit of detection (LLOQ) for anti-D with the quantification assay, 0·005 IU/ml (1 μg/l), with 10/39 below LLOQ.

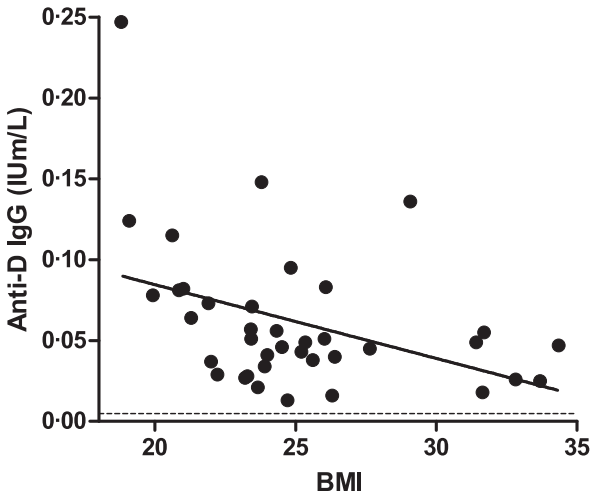


**Fig. 2** Increase of anti-D IgG after administration of RAADP 1500 IU in pregnancy week 38 was quantified by flow cytometry one week after administration. Linear regression analysis showed a significant correlation to body mass index, $P = 0·0118$, $n = 39$. The dotted line represents limit of detection (LLOQ) for anti-D with the quantification assay, 0·005 IU/ml (1 μg/l).

## Discussion

The retrospective data analysis of more than 4000 screen tests at delivery in RhD-negative women with a fetus typed *RHD* positive revealed that 20·5% in the cohort had

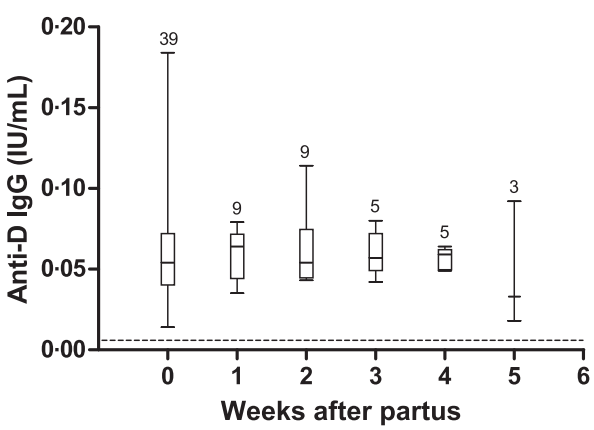


**Fig. 3** Concentrations of anti-D measured by flow cytometry at different time points after delivery. Data are shown as box plots with median values and quartiles in the box and the range as whiskers. $n$ = no of anti-D analyses performed at delivery and each week after delivery. Samples at delivery were obtained from all 39, post-delivery: no sample was obtained from 16, one sample from 14 and 2–4 samples from 9. The dotted line represents limit of detection (LLOQ) for anti-D with the quantification assay, 0·005 IU/ml (1 μg/l).

non-detectable anti-D levels after RAADP at GA 28–29. In the prospective pilot study including 39 women, 18% had a negative screening result, defined as no reactions in routine microcolumn screening, and 26% had levels below LLOQ (<0·005 IU/ml) with the quantification assay, at 38–39 weeks of gestation, after RAADP 1500 IU at 28–29 weeks of gestation. The findings support previous reports, where a large proportion, 44–78%, had non-detectable levels of anti-D in screening tests at delivery after one dose RAADP in GA 28 and 15–39% after two doses in GA 28 and 34 [7,8]. In pregnancies post 40 weeks, 78% is reported to lack detectable anti-D [8]. Administration of 1500 IU anti-D in GA 38 resulted in levels between 0·018 and 0·113 IU/ml 1–5 weeks post-delivery in the 31 analysed samples up to GA 43, but with reservation for a limited no at the final measurements. Two of 39 women had low levels at delivery, just below 0·015 IU/ml, estimated to cover a FMH of 2·5 ml, which is shown to occur in less than 1% of deliveries [20]. Small amounts of fetal blood pass into the maternal circulation, with increasing risk during the third trimester and the greatest risk at delivery [21]. In a study of more than 20 000 pregnancies, it was found that 74% of the women had less than 0·5 ml, 96% had less than 1 ml, and 98% had less than 2 ml fetal blood in the circulation after delivery [22]. In our study, with analysis in 25 pregnancies, no cases of FMH were found, but the sample size was small, and based on prevalence from publications, only 1/25 analysed samples were expected to have FMH above 1 ml.

The study showed a large interindividual variation of anti-D concentration at delivery. The variation of anti-D levels may depend on individual IgG clearance from plasma and consumption of anti-D, giving a variability in residual anti-D levels and in half-life. Uptake from muscular compartments and fat tissue may vary as well. As has been shown before, anti-D levels correlated to BMI [23,24]. In our region, 16·5% had BMI > 30 and 4·4% had BMI > 35 compared to 11·4% and 3·0%, respectively, nationally [18]. The weeks after delivery, the concentration anti-D seemed to be stable, but with the reservation that we had no FMH > 1 ml in the cohort.

With current anti-D prophylaxis regimens including RAADP in GA 28–30, the incidence of anti-D immunization is low, 0·2–0·4%. Despite this low incidence, anti-D immunization is still the most common cause of severe fetal anaemia requiring intrauterine blood transfusions to the fetus with its inherent risk of complications and perinatal mortality and morbidity [2,5]. If 10–20% of the RhD-negative women with unmeasurable levels of anti-D can be assumed to have 1% risk of immunization at term and post-term, that may cause a major proportion of the remaining anti-D immunizations.

Fetal *RHD* screening is now a routine in many countries, resulting in that the fetal *RHD* status is known with high accuracy prenatally, with a sensitivity of the analysis above 99·9% [6]. To administer anti-D prophylaxis in gestational week 38, to ensure adequate concentration of anti-D at term and post-term in RhD-negative women at risk of immunization, it seems relevant and logistically possible in most programmes. Much is unknown about the mechanisms for immunization, but that anti-D administration protects from immunization is convincingly and repeatedly shown [25]. Before RAADP was introduced in our region, we could show that the majority of immunization occurred in the third trimester [12]. After the introduction of RAADP, the incidence of anti-D immunizations was decreased to less than half, to 0·2%, and similar to other studies [26]. The cases still occurring was either detected at the screening test in GA 28, before RAADP or at the first screening in the subsequent pregnancy.

It has previously been concluded that adding a dose of anti-D in the third trimester cannot be justified due to high costs [22], but if the post-delivery anti-D dose could be replaced, this strategy might be cost effective. The potential of high compliance at 38 gestational weeks, which is crucial, must be assessed in different antenatal programs, but most women have a visit planned in gestational week 37–38. For women non-compliant to RAADP at gestational week 38 or delivered preterm, anti-D prophylaxis may be administered within 72 h post-delivery.

## Limitations

The study population of the intervention study was small and had dropouts. There was an obvious difficulty to include subjects with repeated measurements close to term and post-delivery. There is a limitation by the relatively high incidence of protocol violations regarding the analysis of anti-D concentrations postpartum. Only 9/39 was monitored more than two weeks post-delivery, in all of these stable concentrations were maintained up to 30 days post-delivery, but with large inter- individual variations. In addition, analyses of FMH at delivery were missing in 14/39 (36%) of the cases and none of the 25 analysed samples FMH was detected. FMH above the detection level of 1 ml fetal blood is reported to occur in 4% of pregnancies [22].

## Conclusion

Approximately 20% of RhD-negative women have very low to non-detectable levels of anti-D (<0·005 IU/ml) at term and post-term, leaving them at risk of immunization at the end of pregnancy. In addition to RAADP in GA 28

the feasibility of a second dose of RAADP in GA 38, replacing the post-delivery dose, was evaluated in this study. The anti-D dose at 38 weeks GA showed detectable anti-D concentrations up to 30 days postpartum, in the analysed samples. If this strategy may lower the incidence of D-immunization further must be addressed in a large prospective trial.

## Acknowledgements

The authors want to thank Maryan Ismail Adan and Daniel Wessman for technical assistance and to the women who agreed to participate in the study for their time and donation of extra samples. Warm thanks to the staff at departments of Transfusion Medicine and Obstetrics for help and support with the study, around the clock.

## Conflict of interests

The authors have no conflicts of interests.

## Funding

This study was supported by Stockholms Läns Landsting FOU 2018-2019.

## Authors contribution

AW, ET and GA contributed to study design. EJ, AK and AM contributed to laboratory analyses. YJ, EJ, AK and GA contributed to study inclusion of pregnant women. AW and AM contributed to analysis of data. AW, AM, ET and GA contributed to interpretation of results. AW and AM contributed to drafting of the manuscript. All authors contributed to writing and final approval of manuscript.

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VoxSanguinis
The International Journal of Transfusion Medicine
ISBT International Society of Blood Transfusion
*Vox Sanguinis* (2021)

ORIGINAL PAPER


DOI: 10.1111/vox.13099

# Optimization of diagnostic strategy for non-invasive cell-free foetal RHD determination from maternal plasma

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Vox Sanguinis

**Background and objectives** The aim of the study was to optimize routine non-invasive prenatal detection of fetal *RHD* gene from plasma of RhD-negative pregnant women (the median of gestational age was 25 weeks, range 10–38) to detect RhD materno-fetal incompatibility and to avoid the redundant immunoprophylaxis.

**Materials and methods** Initially only one exon of *RHD* gene (exon 10) was investigated in 281 plasma samples (144 verified after delivery), in the second phase three *RHD* exons (5, 7, 10) were analyzed in 246 samples of plasma and maternal genomic DNA (204 verified) by real-time PCR method. Detection of Y-chromosomal sequence *DYS-14* and five X-chromosomal insertion/deletion polymorphisms was used to confirm the fetal cfDNA detectability in plasma. Specific polymorphisms in *RHD* gene were detected by sequence-specific primer PCR in nine samples.

**Results** When only the *RHD* exon 10 was tested, 2·8% of verified samples were false positive and 3·5% false negative. With three *RHD* exons (5, 7, 10) and maternal genomic DNA testing, only one case was false negative (0·5%). Nine samples were inconclusive due to *RHD*-positive results in maternal genomic DNA. These samples were analyzed for specific mutations in *RHD* gene. Combination of both methods for fetal cfDNA verification succeeded in 75% of tested group.

**Conclusion** Implementation of analysis of three *RHD* exons and maternal genomic DNA to routine practice lowers dramatically the ratio of false positive and negative results. This method enables more accurate determination of fetal *RHD* status with the reduction of unnecessary medical care and RhD immunoprophylaxis.

*Received: 25 May 2020,*
*revised 20 November 2020,*
*accepted 1 March 2021*

**Key words:** fetal testing, genetics, haemolytic disease of the fetus and newborn.

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## Introduction

Haemolytic disease of foetus and newborn (HDFN) is caused by the transplacental transfer of antibodies from an alloimmunized woman to the circulation of foetus and

the subsequent destruction of foetal erythrocytes [1]. The most frequent form of HDFN is associated with the anti-D IgG antibodies from an RhD-negative woman transmitted to the blood of an RhD-positive foetus [2, 3]. In a Caucasian population, the prevalence of *RHD*-negative homozygotes is 15%–17% [4, 5], even reaching up to 20% [6, 7]. Regarding the previous alloimmunization, HDFN is very rarely seen in primigravida [8]. To prevent RhD sensitization, initially the postpartum anti-D Ig had been used which was then replaced by routine antenatal anti-D prophylaxis. The immunization risk in developed countries is minimized to 0·2%–0·4% due to the combination of antenatal and postnatal prophylaxis [1, 9–12].

Screening of all pregnant women for irregular anti-erythrocyte antibodies before the 14th gestational week belongs to the standard recommended approaches. Antibodies in screen-positive samples are specified and quantified. The screening positivity in our population is 5%, representing approximately 5000 pregnancies per year. However, only 1·5% of these antibodies have clinical relevance (approx. 1500 pregnancies). In reality, only pregnant women bearing foetuses with specific surface antigens are immunized; this occurs in about 0·5% of all pregnancies, representing 500 cases per year in our population [13]. These foetuses are potentially threatened by anaemia in utero and postpartum hyperbilirubinemia. In utero, ultrasound monitoring should be able to recognize anemization, allowing for appropriate treatment (intraumbilical transfusion or pregnancy termination when >35 weeks) [14]. The disadvantage of the imunohematologic screening of RhD-negative pregnancies is the relatively high false positivity that leads to unnecessary management of pregnancies with an RhD-negative foetus. Immunoglobulin anti-D, as a product of human origin, has limited resource [15], and its administration involves a certain risk of infection [16]; thus, it should be used strictly on a proper indication. Therefore, there is a need for reliable diagnostics for those who are not threatened by alloimmunization and do not need any immunoprophylaxis [17].

A feasible clinical non-invasive method for the determination of foetal *RHD* status has been available since the discovery of foetal cell-free DNA (cffDNA) in maternal plasma by Lo in 1997 [18]. It became a part of routine screening diagnostics in some European countries, for example in Denmark (2010), the Netherlands (2011), Finland (2014) [19] and others [20], replacing the invasive methods, such as amniocentesis and chorionic villi sampling [21].

The cffDNA originates mainly from placental trophoblast [22]. It can be detected as early as in the 5th week of gestation and varies dependently on gestational week [23]. After delivery of the foetus, the cffDNA elimination is fast and occurs within hours [24]. Although there is the evidence that foetal cells persist in the woman's tissues for decades after pregnancy [25], it was demonstrated that there is no risk of interference for cffDNA diagnostics [26].

The *RHD* gene consists of ten exons and is very polymorphic [5]. The first cffDNA studies focused on single-exon genotyping only [2]. Subsequent studies analysed rather two or even more exons, mostly some of combinations of exons 5, 7 and 10 [17]. On the other hand, some studies presented that single-exon testing should be sufficient for reliable diagnostics [27, 28].

The high polymorphism of the *RHD* gene contributes to difficulties in cffDNA diagnostics of the foetal *RHD* type. Nowadays, more than 300 alleles of this gene have been identified [29–31] and their expression leads to a number of variants of RhD epitopes [32, 33]. Women carrying these variants could be identified as serologically positive, weak positive or negative, and they may or may not be at risk of alloimmunization [34]. A weak RhD type is a variant with a reduced amount of antigen D [35]. Anti-D immunization is rare for D weak types 1, 2 and 3 [36], so there is no need for anti-D prophylaxis; however, other variants are potentially at risk of alloimmunization [37]. Carriers of the DEL form express very small amounts of antigen D, and they are at potential risk of alloimmunization and should be treated as RhD negative with a possible indication of anti-D prophylaxis. A group of partial RhD types includes variants with some amino acid substitutions in the extracellular loop. Alloimmunization is possible; the probability differs according to the specific type [36]. Consequently, it is very important to thoroughly investigate the *RHD* status of the woman because it may affect the result of foetal *RHD* testing. Cell-free DNA of maternal origin is also present in plasma, and in the case of maternal genomic *RHD* positivity, its detection can lead to a false-positive result of foetal *RHD* [38].

Another complication associated with this diagnostic is the absence of a universal-positive foetal DNA marker for the cffDNA fraction verification. Currently, there is no marker found to be optimal for this prenatal testing [38, 39].

In this study, we present the results of our consecutive optimization of the routine prenatal *RHD* diagnostic methodology and its advantages. We deal with its complications and demonstrate our arrangement of the diagnostics that we have achieved through the last decade.

## Materials and methods

From January 2008 to December 2019, 527 plasma samples of RhD-negative pregnant women with detectable

levels of specific RhD antibodies were tested for cffDNA *RHD*, firstly 281 samples as a pilot study (2008–2012) and, afterwards, 246 samples as a part of routine diagnostics (2013–2019). The median of gestational age was 25 weeks (range 10–38 weeks). All women were serologically predicted as RhD negative. The RhD status of newborns was serologically verified after delivery in 144 cases (51·2%) in the pilot study and in 204 cases (82·9%) in the routine diagnostics part. In the diagnostics period, buccal swabs were collected together with blood samples from all women.

In the period 2014–2019, 152 samples (72% of the total 210 samples from this period) were analysed for the *DYS-14* sequence specific for the Y chromosome for the verification of presence of cffDNA. In 2019, 41 samples (negative both for *RHD* and *DYS-14*) were retrospectively analysed for the panel of five insertion-deletion polymorphisms (INDELs) localized on the X chromosome to confirm the foetal fraction in female foetus bearing pregnancies using digital droplet PCR (ddPCR) as a part of our research project [40].

## Samples

Samples of peripheral blood were collected into Vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) to prevent coagulation. The tubes were centrifuged immediately at 4°C for 10 min/2600 g, the supernatant was transferred to a new tube and centrifuged at room temperature for 10 min/14 000 g. Obtained plasma samples were stored at −20°C until utilization.

Cell-free DNA including the cffDNA fraction was isolated manually from 1 ml of maternal plasma by QIAamp Circulating Nucleic Acid Kit (Qiagen, Germany) (in the period 2010–2015) and by MagNA Pure Compact NA Isolation Kit I – Large Volume by MagNA Pure Compact Instrument (Roche Molecular Systems, Switzerland) (2016–2019), respectively, according to the manufacturer's instructions. Both isolation methods were tested to give convenient and comparable amounts of cfDNA in sufficient quality for subsequent analysis (data not shown). Cell-free DNA was finally eluted in 50 μl of the supplied elution buffer.

Maternal genomic DNA from buccal swabs was isolated by QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions and eluted in 150 μl of supplied elution buffer.

## Real-time PCR

The *RHD* status was determined via a real-time PCR method on the ABI HT7900 Real-time PCR instrument (Applied Biosystems, USA).

In the first pilot phase (2008–2012), only one exon of the *RHD* gene (exon 10) was investigated; in the second phase (2013–2019), three *RHD* exons (5, 7 and 10) were tested simultaneously. Gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was used as a reference in both phases. Detection of the *DYS-14* sequence was used for the foetal fraction presence verification.

The TaqMan Gene Expression Assay was used for detection of the *RHD* exon 10 (FAM labelled), and the Custom MGB TaqMan Gene Expression Assays were used for detection of the *RHD* exons 5 and 7, the *DYS-14* sequence and the *GAPDH* gene. The primers and MGB probe for *RHD* exon 5 and *DYS-14* sequence (both FAM labelled) were ordered according to the published sequences [41, 42], and for *RHD* exon 7 and *GAPDH* (both VIC labelled) as published by Svobodova et al. [43] (all assays Life Technologies, USA). For further details, see Table S1.

The duplex reactions were performed as follows: for *GAPDH* + *RHD* exon 10 in septuplicates in the first part of the study and in triplicates in the second part, for *RHD* exon 5 + *RHD* exon 7 in quadruplicates. The *DYS-14* reaction was performed as a single reaction. Each PCR reaction was performed in 20 μl of total reaction volume containing 5 μl DNA. The maximum CT (cycle threshold) value for the positive reaction was set to 37.

Results were finally processed using SDS Software version 2.4 (Applied Biosystems, USA). Foetal *RHD* was considered as positive when the amplification was determined in more than five out of seven *RHD* (exon 10) replicates in the first part of the study and in more than seven out of 11 *RHD* replicates (four replicates for each of exons 5 and 7, and three replicates for exon 10) in the second part of the study. The sample was considered as *RHD* negative when the amplification was detected in no more than in one replicate (in both parts of the study). In the case of the detection of two to six *RHD* replicates, the analysis was repeated.

## Digital droplet PCR

The INDELs rs2307932; rs16397; rs16637; rs3048996; and rs16680 were genotyped using ddPCR. Two Custom assays (Primers + TaqMan hydrolysis probe) for each INDEL specific for ddPCR were designed by Bio-Rad (USA). For each INDEL, one of the variants (insertion or deletion) was FAM labelled and the opposite variant was HEX labelled. Each reaction contained 5 μl DNA. The samples were analysed on QX100 Droplet Reader, and the evaluation was performed by QuantaSoft Software version 1.6.6.0320. All chemicals, instruments and software were provided by Bio-Rad (USA). The methodology is described in detail in our article [40].

### Sequence-specific primer PCR (PCR-SSP)

In inconclusive cases (mother serologically defined as RhD negative with *RHD* gene positivity in the genomic DNA obtained from the buccal swab), the specific polymorphisms in the *RHD* gene were detected by the CE-certified PCR-SSP technique combined with a fluorescence endpoint detection based on a modified TaqMan probe system (FluoGene, Inno-train). The FluoGene kits (RBC-FluoGene CDE and RBC-FluoGene D weak/variant) detect the most common weak D types, exons 1–7, 9, 10 of the *RHD* gene and the most common partial D types. The reactions were performed with FluoGene system instrument and FluoVista Analyzer using FluoGene software.

## Results

In all plasma samples and all genomic DNA samples, sufficient levels of isolated cfDNA were detected. The median CT of reference *GAPDH* gene was 32 (range 29–35) for plasma samples and 26·3 (range 23–29) for genomic DNA samples. The median CT of all three *RHD* exons for plasma samples (when detected) was 35 (range 31–37).

In the first pilot study (2008–2012), a total of 281 samples were tested only for one *RHD* exon (exon 10). Of these, 62·3% were positive for the *RHD* gene, 34·5% were negative, and 3·2% could not be analysed (Table 1). The RhD blood group of newborns was serologically confirmed in 144 (51·2%) of 281 cases. Out of these, 93·8% were analysed correctly (66% *RHD* positive, 27·8% *RHD* negative), but four samples (2·8%) were determined as false positive for *RHD*, and five samples (3·5%) were false negative. The sensitivity of the test in this phase was 95% (95% confidence interval 88·7%–98·4%), specificity 90·9% (78·3%–97·5%) and accuracy 94% (88·5%–97·1%).

Regarding the false-positive results, two cases of the proven false positivity represented the same woman in her two subsequent pregnancies. Comparing the CT values of *RHD* exon 10 and *GAPDH* in her samples yielded CT value differences of 0·5 (tested in the 30$^{th}$ gestational week in her first gravidity) and 2·2 (tested in the 19th gestational week in her second gravidity). In the other two cases of false-positive results, the CT values difference was very low in one case (0·5) and relatively high (5·8) in the other case. Regarding the false-negative results, the *GAPDH* CT values were around 32 in all these samples, indicating sufficient cfDNA extraction. In two out of five samples, there were no detected amplifications of *RHD* (exon 10) in any of the replicates; in the remaining three samples, we detected amplification in one replicate out of seven.

In the second period (2013–2019), the samples were analysed for three exons of the *RHD* gene (exons 5, 7 and 10), and the maternal genomic DNA obtained from buccal swabs was tested in the same way. From all 246 plasma samples included in this part of the study, 63% were determined as positive for *RHD*, 32·9% as negative and 4·1% (10 samples representing nine women, one of whom was tested in two following pregnancies) could not be analysed due to the *RHD*-positive results of maternal genomic DNA (Table 2). These nine undetermined samples were afterwards analysed for specific mutations in the *RHD* gene. Four of them were determined as D weak (1·6% of all 246 samples), three cases were DEL type (1·2% of 246 samples), and one partial D carrying *RHD DCS-1* allele (0·4% of 246 samples). In one sample, no specific mutation was found by Inno-train analysis. For more detailed information, see Table 3.

In the second period, the RhD blood group of 82·9% newborns of 246 tested samples was postnatally verified (data from the last year are not yet available). There was only one false-negative case (0·5%) in the year 2013 (*GAPDH* CT 30, no *RHD* amplification in any of the replicates). The sensitivity of the test increased to 99% (95% confidence interval 95·5%–100%), specificity to 100% (95·1%–100%) and accuracy to 99% (97·2%–100%).

Since the subsequent year (2014), the presence of foetal fraction of cfDNA was tested using *DYS-14* sequence detection. Of the 152 tested samples, 92 (60·5%) were determined as being *DYS-14* positive, bearing male foetus and thus informative for cffDNA presence validation. In the remaining 60 cases (39·5%), the sequence was not

**Table 1** Results of the foetal *RHD* status testing from maternal plasma using the one-exon method (exon 10) without maternal genomic DNA analysis in the first (pilot) phase of the study in 2008–2012

| Year | Total | *RHD* positive | *RHD* negative | Undetermined | Confirmed | Confirmed *RHD* positive | False positive | Confirmed *RHD* negative | False negative |
|---|---|---|---|---|---|---|---|---|---|
| 2008 | 65 | 40 | 23 | 2 | 35 | 21 | 2 | 10 | 2 |
| 2009 | 61 | 37 | 21 | 3 | 32 | 20 | | 11 | 1 |
| 2010 | 50 | 29 | 17 | 4 | 27 | 20 | 1 | 6 | |
| 2011 | 52 | 34 | 18 | | 25 | 16 | | 7 | 2 |
| 2012 | 53 | 35 | 18 | | 25 | 18 | 1 | 6 | |
| Total | 281 | 175 (62·3%) | 97 (34·5%) | 9 (3·2%) | 144 (51·2%) | 95 (66%) | 4 (2·8%) | 40 (27·8%) | 5 (3·5%) |

**Table 2** Results of the foetal *RHD* status testing from maternal plasma using three-exon method (exons 5, 7 and 10) including maternal genomic DNA analysis in the second phase of the study in 2013–2019

| Year | Total | *RHD* positive | *RHD* negative | Undetermined | Confirmed | Confirmed *RHD* positive | False positive | Confirmed *RHD* negative | False negative | Mother *RHD* positive |
|---|---|---|---|---|---|---|---|---|---|---|
| 2013 | 36 | 25 | 11 | | 33 | 22 | | 10 | 1 | |
| 2014 | 40 | 23 | 17 | | 38 | 22 | | 16 | | |
| 2015 | 44 | 25 | 16 | 3 | 40 | 22 | | 15 | | 3 |
| 2016 | 31 | 22 | 7 | 2 | 30 | 22 | | 7 | | 1 |
| 2017 | 40 | 23 | 14 | 3 | 38 | 21 | | 14 | | 3 |
| 2018 | 34 | 18 | 14 | 2 | 25 | 11 | | 12 | | 2 |
| 2019 | 21 | 19 | 2 | | | | | | | |
| Total | 246 | 155 (63%) | 81 (32·9%) | 10 (4·1%) | 204 (82·9%) | 120 (58·8%) | 0 (0%) | 74 (36·3%) | 1 (0·5%) | 9 (4·4%) |

**Table 3** Overview of the *RHD* variants detected in maternal genomic DNA from buccal swabs in serologically RhD-negative mothers

| Sample | Allele name | Nucleotide change(s) | Exon/Intron | Amino acid change(s) | Allele name detail |
|---|---|---|---|---|---|
| 1 | RHD*weak D type 1 | c.809T > G | exon 6 | p.Val270Gly | RHD*809G |
| 2 | RHD*weak D type 1 | c.809T > G | exon 6 | p.Val270Gly | RHD*809G |
| 3 | RHD*weak D type 1 | c.809T > G | exon 6 | p.Val270Gly | RHD*809G |
| 4 | RHD*weak D type 3 | c.8C > G | exon 1 | p.Ser3Cys | RHD*8G |
| 5 | RHD*DEL8 | c.486 + 1G>A | intron 3 | splice site mutation | RHD*486 + 1A |
| 6 | RHD*DEL8 | c.486 + 1G>A | intron 3 | splice site mutation | RHD*486 + 1A |
| 7 | RHD*DEL4 | c.147delA | exon 1 | p.Val50Leufs*5 | RHD*147delA |
| 8 | RHD*DCS1 | 667G > T, 676G > C | exon 5 | Phe223Val, Ala226Pro | RHD*667T, 676C |

detected, and the samples were not informative. Afterwards, during another part of our research (in 2019), 41 of these samples were genotyped for the panel of five INDEL variants localized on chromosome X. The proportion of informative samples with successful detection of paternal X chromosome allele was 58·5% (24 cases) in this small group. Since the start of cffDNA fraction verification, there were neither false-positive nor false-negative results for the *RHD* gene.

## Discussion

The aim of the study was to optimize routine non-invasive prenatal detection of the foetal *RHD* gene from plasma of RhD-negative pregnant women. In the first (pilot) part of the study, only one exon of the *RHD* gene (exon 10) was tested, and high levels of false-negative (3·5%) and false-positive (2·8%) results were reported. In addition, this method cannot distinguish between the *RHD* status of the mother and the foetus, meaning that it is strictly applicable only for women with negative results of the *RHD* gene detection. In this period, maternal genomic DNA was unobtainable, and the *RHD* status of the mother could not be verified, which consequently contributed to the false-positive ratio. Another factor that could contribute to false-positive and false-negative results is the accuracy of the postnatal confirmation of the RhD type of the children based on the serological cord blood typing, which could yield false-positive or false-negative results.

In the second (diagnostic) period, the DNA isolation method was changed, and the gene detection analysis was upgraded in two different ways. Regarding the DNA isolation, we switched from the manual extraction system to an automated system, which has smaller yields of cfDNA (mean CT value differentiation 0·6) but on the other hand lowers the risk of cross-contamination. Concerning the gene detection, the use of more than one *RHD* exon is recommended as it may reduce the error rate, so we increased the number of analysed exons to three (exon 5, 7 and 10) [44]. The *RHD* exons 7 and 10 contain several specific sequences distinguishing *RHD* and *RHCE* genes, and exon 5 is included for the *RHDψ* variant detection [41, 45].

The other innovation was the verification of maternal *RHD* status via the analysis of maternal genomic DNA obtained from buccal swabs. The same analysis could be done from another source of maternal genomic DNA, such as a buffy coat. Currently, the national screening programme for the detection of RhD-negative foetuses is still based on the serologic detection of antibodies, and some serologically determined RhD-negative women are, in fact, genetically *RHD* positive. Consequently, this leads to discrepancies in molecular and serologic results and

contributes to the false-positive ratio in this type of diagnostics [46]. A different approach was used in the study by Sørensen et al. [38], who compared CT values of the *RHD* gene (anticipated foetal origin) and the *GAPDH* gene (both maternal and foetal origin). When the CT value of the *RHD* gene was lower or the same as the *GAPDH* one, the mother was determined as being positive for the *RHD* gene, and the sample was evaluated as inconclusive. In general, these findings are in agreement with our results. But looking at the CT values differences at our false-positive samples, the result for these four samples are ambiguous. Two times the CT difference was low (0·5) but two times high (2·2 and 5·8). Our method is slightly more demanding but more accurate because three exons (5, 7 and 10) of the *RHD* gene are tested not only in plasma samples (where the positive signals are supposed to be of the foetal origin) but also in samples from maternal buccal swabs, which provides information about the maternal genotype. This approach avoids the possible false-positive results, as the one reported in Sørensen's study (wherein the maternal *RHD* variant gene provided a weak signal and resulted in the CT value corresponding to the amplification of cffDNA) [38].

As a result of the verification of maternal genotypes, we found nine women with discrepancies between their serological and genotyping results. Four of them (1·6%) were determined as carriers of weak D, three had a DEL form (1·2%), and one was a carrier of the *RHD-DCS1* variant (0·4%) (Table 3). The percentage of detected weak D is slightly higher than reported in the literature (0·2%–1%) [35, 36] probably due to the fact that our cohort did not reflect the whole population but only one part of it – serologically determined RhD-negative pregnant women with detectable levels of antibodies. For example, in Wang's prenatal *RHD* variant genotyping study of the US population [47], the percentage of the *RHD* variant was 2·2%. The analysed *RHD* weak variants were types 1 and 3, which is in agreement with the most frequently referenced variants [36]. The detection of the *RHD-DCS1* variant corresponds with the previous findings of the greater prevalence of this allele in the Czech Republic [48].

Concerning the literature, no universal foetal DNA marker was found to verify the cffDNA fraction size. Due to the low false-negative rate during *RHD* testing, the lack of this marker is considered to be acceptable [39]. In our study, we tried to use detection of the *DYS-14* sequence, which should be theoretically reliable in about 50% of samples and was sufficient in 60% of the samples analysed (male foetus). It is necessary to consider the higher sensitivity (approximately 10×) of *DYS-14* assay in comparison with all *RHD* assays. *DYS-14* detects the repeated DNA target, but *RHD* assays focus on unique sequences occurring in the hemizygous state in foetal cfDNA. This could lead (by *RHD*-positive foetus samples) to false security in a situation where the *DYS-14* sequence will be detected, and the *RHD* assay will have a false-negative result due to its lower sensitivity. From a statistical point of view, this fact could be partly compensated with the number of tested replicates (we test 11 replicates of *RHD* gene and one replicate of *DYS-14*). We also have the experience that the CT value of *DYS-14* is approximately 32 (the average CT values difference between *DYS-14* and *RHD* exons is about 3). Therefore, significantly higher *DYS-14* CT would signal potential issues with cffDNA processing.

In 2019, we tested the genotyping of X-chromosomal INDEL variants to detect the paternal X chromosome in the female foetus bearing pregnancies on a small group of samples (41 out of the uninformative cases from the previous part, negative both for the *RHD* gene and the *DYS-14* sequence) via ddPCR. Almost 60% of these samples (24 cases) were informative in at least one of the five determined polymorphisms. The technology used is different, but we compared the parameters of quantitative PCR and digital droplet PCR (ddPCR) in the process of *RHD* genotyping in our previous study [43]. We reported no striking differences between the two techniques. The next step in the methodology development could be represented by the preconcentration of cfDNA samples isolated from plasma to achieve more positive signals for correct quantification, and addition of other INDEL polymorphisms to enlarge the current panel with the goal of reaching the informativity in more pregnancies.

Using these two steps, the confirmation of the cffDNA presence could be performed in about 75% of the samples. Regarding the workflow, costs and outcomes, we prefer the one-step cffDNA detection by *DYS-14* sequence. If both the *RHD* and *DYS-14* tests provide negative results prior to the 25th gestational week, additional testing after this term will be performed. A recent study [49] demonstrated that *RHD* testing performed in the 25th gestational week and later is both reliable and accurate and it is not influenced by maternal BMI.

## Conclusion

In our study, we tried to clearly demonstrate the benefit of *RHD* testing innovations based on three-exon determination (5, 7 and 10), including maternal genomic DNA analysis connected with a subsequent cffDNA presence proving step. In the first part of the study, we tested only one exon of the *RHD* gene (exon 10) with a relatively high ratio of false-negative (3·5%) and false-positive (2·8%) results. In the second part, we improved the methodology and tested three exons of the *RHD* gene (exons 5, 7 and 10) with a simultaneous analysis of the maternal genomic DNA. In this part of the study, we had

only one false-negative result in the first year of this phase (2013). The sensitivity of the test increased from 95% to 99%, the specificity from 90·9% to 100% and the accuracy from 94% to 99%. Afterwards, an analysis of the *DYS-14* sequence was implemented as a marker of cffDNA presence and alternatively complemented with the genotyping of X-chromosomal INDEL polymorphisms. Since applying all these innovations, we had no false-positive and no false-negative results, dramatically improving the reliability of the method in correspondence with previously published results [38, 50]. This methodology enables more accurate administration of anti-D prophylaxis and avoids an unnecessary management of the pregnancies with *RHD*-negative foetuses.

## Author contributions

EP and AH designed the study. EP performed the experiments and drafted the manuscript. IZ and MK performed the INDEL part of the study. JK and MP performed the PCR-SSP part of the study. MN and PC interpreted the patients' data. All authors contributed with data analysis, data interpretation and manuscript revision and approved the final version.

## Conflict of interest

The authors declare no competing interests.

## Funding

This study was supported by the Ministry of Health, Czech Republic – conceptual development of research organization RVO-VFN 64165, and by the Ministry of Education, Youth and Sports, Czech Republic – project of Charles University Progres Q25/LF1 and grant no. LTACH19005.

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## Supporting Information

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

Table S1 Detailed information about the used real-time PCR primers and probes

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The International Journal of Transfusion Medicine

ISBT International Society of Blood Transfusion

*Vox Sanguinis* (2021) **116**, 932


DOI: 10.1111/vox.12962

# DIARY OF EVENTS

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See also http://www.isbtweb.org/congresses/

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|---|---|
| 4.5.2021 | IPFA/PEI – The International Workshop on Surveillance and Screening of Blood-borne Pathogens |
| 13–15.5.2021 | The Canadian Society for Transfusion Medicine (CSTM) are holding their annual scientific conference virtually in 2021. |
| 26–27.05.21 | 21st Congress of the European Society for Hemapheresis |
| 5–9.6.2021 | ISBT In Focus, the 31st regional congress of the ISBT, will be a virtual event in 2021 |
| 17.9.2021 | 11th BIC International Conference – Advances in Haemostasis and Bleeding Disorders |
| 22–24.9.2021 | Deutsche Gesellschaft für Transfusionsmedizin und Immunhämatologie e.V. |
| 23–26.9.2021 | 16th International Congress on Myelodysplastic Syndromes (MDS 2021) |
| 13–16.11.2021 | 32nd Regional congress of ISBT, Brisbane, Australia |