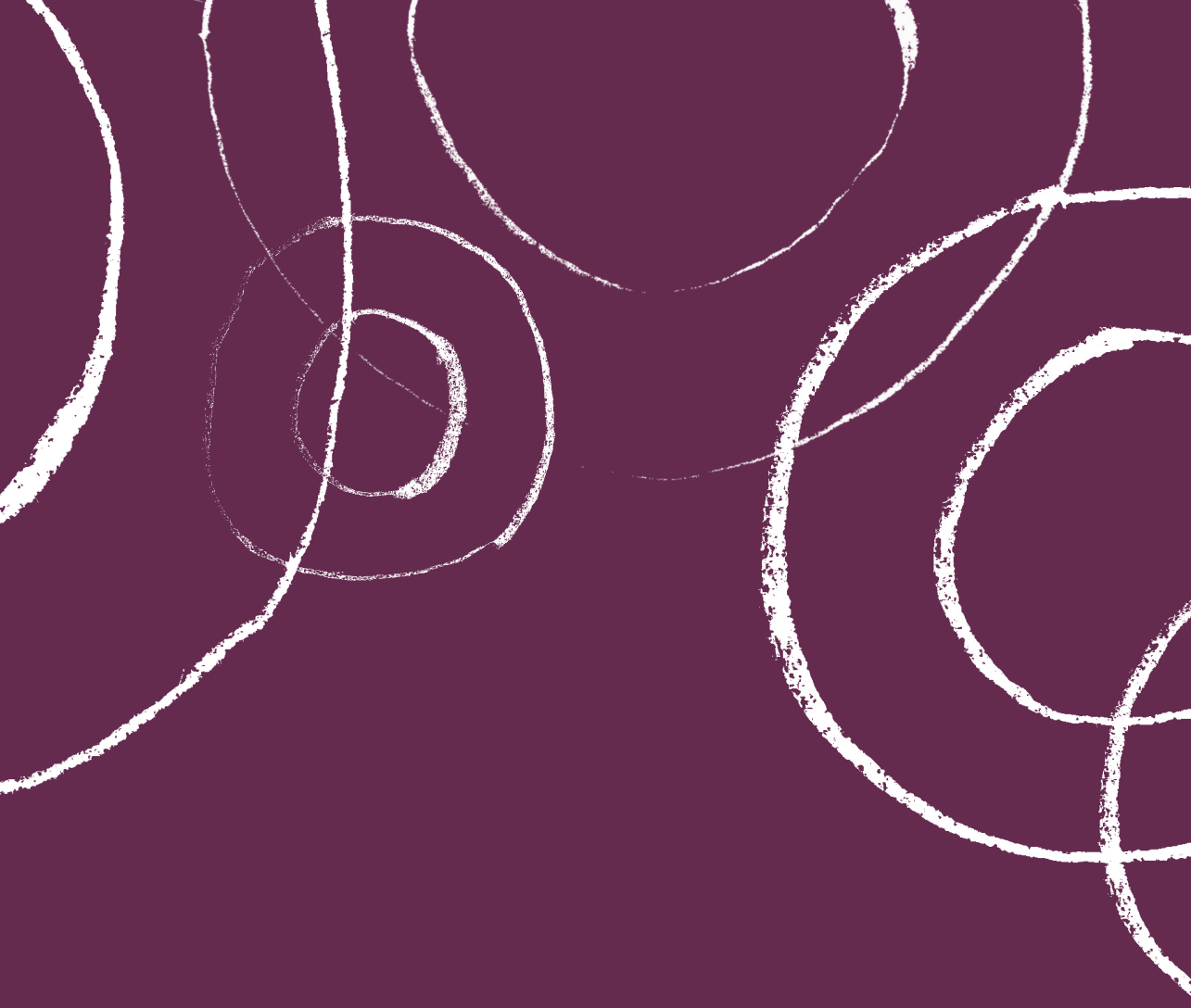

Transfusion practises and novel blood products

Pre-clinical and clinical studies

Sanne de Bruin

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Contents

CHAPTER 1

General introduction and scope of the thesis

Background

In the critically ill blood transfusion is regarded as a common used lifesaving therapeutic intervention. Oxygen delivery is dependent on several factors e.g. haemoglobin levels, cardiac output and arterial oxygen content to transport oxygen to the target organs. In the setting of compromised cardiac output or lower arterial oxygen content which is very common among anaemic critically ill patients, it is thought that increasing haemoglobin levels may compensate for this.

However, in the past decades it has become clear that transfusion, with either RBCs, platelets or plasma, is associated with different adverse effects, including transfusion transmissible infections, allergic reactions, transfusion related acute lung injury (TRALI) and transfusion associated cardiac overload (TACO)[1–3]. Insight in the mechanisms of onset of these complications has led to the design of preventive strategies to reduce the chance of adverse effects after blood transfusion including leukodepletion, solvent/detergent treatment of plasma, the use of male only plasma products or pooled plasma products and the use of novel additive solutions.

Critically ill patients are a population of extra interest within the transfusion research field. These patients can have, due to their underlying disease, a high need for blood components but are also more prone for developing blood transfusion related morbidity and mortality[4]. Therefore, the decision whether to transfuse a critically ill patient or not can be very complicated balancing the benefits and risks. While not transfusing anaemic patient with RBCs is associated with risk of dying[5,6], multiple large randomized controlled trials (RCTs) showed that applying a restrictive RBC transfusion strategy is equally safe or in some studies resulted in a higher survival than a liberal transfusion strategy[7–13]. Therefore, transfusion of RBCs should only be considered when the benefits of transfusions outweigh the potential adverse effects.

To summarize, blood transfusion is in concept a good therapy in specific settings but there seems to be room to improve efficiency and safety of blood components or the use of blood components. One of the potential improvements could be the use of other transfusion triggers or in the storage conditions of the different blood components.

In this introduction several blood components, including RBCs, platelets and plasma are discussed together with their applications in critically ill patients and storage conditions.

Blood components

Blood consists of a cellular and a fluid component. More than 99% of the cells in blood are RBCs. Furthermore, blood contains platelets, and white blood cells. White blood cells, although sometimes used as a transfusion product (i.e. granulocyte transfusions) are beyond the scope of this thesis and therefore will not be discussed any further in this introduction. Plasma is the liquid component of blood and makes up approximately 55% of the blood volume. It contains numerous proteins, including several coagulation factors.

The key function of RBCs is oxygen (O_2) and carbon dioxide (CO_2) exchange between lungs and peripheral tissues. RBC production, erythropoiesis, is located in the bone marrow. During the process of proliferation and maturation, RBCs lose their nucleus, mitochondria and most other cell organelles to optimize oxygen binding to haemoglobin (Hb). Under normal physiological circumstances RBCs have a lifespan of circa 120 days and are removed by the red pulp in the spleen and the liver. Anaemia is defined as a low number of circulating RBCs. The World Health Organisation (WHO) defined anaemia in non-pregnant women as Hb level < 12 g/dL and in men < 13 g/dL. Anaemia can have different causes: a decreased erythropoiesis, increased destruction of RBCs and/or loss of RBCs due to bleeding. Treatment includes iron supplementation, administration of erythropoiesis stimulating agents (ESAs) or RBC transfusion and is determined by the severity and the cause of the anaemia.

Platelets and coagulation factors in plasma play together with RBCs an essential role in haemostasis. To establish efficient coagulation, platelets and coagulation factors in the plasma are both required. Platelets are cell fragments derived from megakaryocytes, that lack a nucleus, but contain other cell organelles including mitochondria. The lifespan of platelets *in vivo* is approximately ten to twelve days. The main function of platelets is to form together with RBCs a blood clot after vessel injury. But platelets also play an important role in several immunological processes[14]. Coagulation factors are proteins, which are mostly produced by the liver. Multiple coagulations factors are described. The half-life differs per factor and ranges between 4 and 300 hours.

The coagulation system is activated in response to endothelial damage (Figure 1). During primary haemostasis a platelet and RBC plug forms at the site of the vessel injury. Simultaneously the secondary haemostasis, also known as the coagulation cascade, takes place. The coagulation cascade starts with release of tissue factor (extrinsic pathway) or the release of collagen (intrinsic pathway). In the end, in both pathways factor X is produced, which converts prothrombin to active thrombin. Thrombin con-

verts fibrinogen into fibrin monomers. Factor XIII crosslinks the fibrin monomers which stabilizes the blood clot.

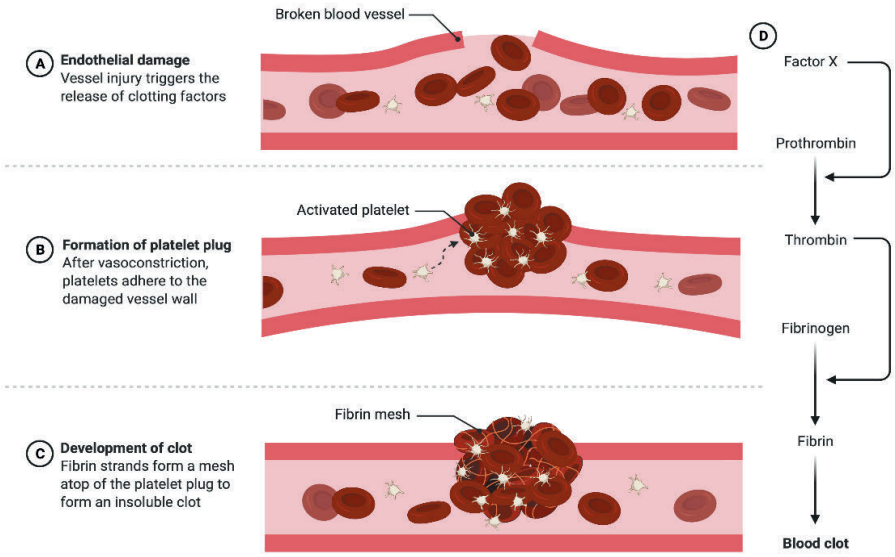

Figure 1. Clot formation after vessel injury

An impaired ability to form clots is called coagulopathy and includes platelets and coagulation factors deficiencies. Important parameters in the evaluation of coagulopathy are the prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen levels and platelet count.

The most common cause for coagulopathy is iatrogenic coagulopathy. In order to prevent thrombo-embolic events or cardiovascular events large numbers of patients receive anticoagulants such as direct oral anticoagulants (e.g. dabigatran), vitamin K antagonists (e.g. acenocoumarol) or anti-platelet therapy (e.g. dipyridamole). Other causes for coagulopathy include bone marrow depression and liver failure, characterized by reduced platelet count and reduced coagulation factor levels, respectively. Numerous treatment options are available, including transfusion of platelets, plasma or specific coagulation factors.

Transfusion in critically ill patients

A substantial number of critically ill patients develops anaemia and/ or coagulopathy on the ICU[15–17], which are independently associated with increased mortality and morbidity[16,18–20]. At the moment of admittance about two third of the patients have a Hb level < 12 g/dL[21] and about a quarter have a Hb level < 10 g/dL[15,22]. Furthermore, incidences of thrombocytopenia (platelet count <150 x10^9 cells/L) of 40% are reported[19] and a prolonged PT is reported in 25% in critically ill patients[17].

During the last decades a substantial decrease in RBC transfusion is observed. In 2008 approximately 25% of all patients received one or more red blood cell transfusions during ICU stay while a decade earlier about 40% of all ICU patients were transfused[21,22]. This shift towards a more restrictive transfusion strategy is the result of multiple large RCTs, which are performed during the last two decades, in which restrictive (transfusion at Hb levels < 7 g/dL) and liberal (transfusion at Hb levels < 9-10 g/dL) transfusion strategies were compared. In 1999 the first trial showed the safety of a restrictive transfusion strategy in critically ill patients and a beneficial effect in the patients who are less acutely ill[23]. This result was repeatedly confirmed in other large RCTs in and outside the ICU[8,13,24–26]. However, uncertainty still exists for different subpopulations, especially for patients with acute coronary syndrome, since the oxygen delivery from the coronary arteries is already compromised. In addition, patients with brain injury which might be more sensitive to anaemia induced cerebral hypoxia, and therefore it may be dangerous for this patient group as well to apply a more restrictive transfusion regime.

Even though the number of RBC transfusions on the ICU are decreasing, still a large proportion of patients is receiving RBC transfusions at Hb levels higher than 7 g/dL, with large differences between world regions[15]. It is unclear why physicians are tempted to transfuse patients at Hb levels higher than 7 g/dl. One of the explanations is the ongoing debate on transfusion triggers as they are not a read out of impaired oxygen delivery. In line with the definition of anaemia, which is defined by decreased Hb levels, treatment for anaemia is in guidelines also based on Hb levels[27,28]. Nevertheless, the aim of blood transfusion is not increasing a Hb value, but ensuring a sufficient oxygen supply. Therefore, several other triggers have been mentioned in literature which are based on global or regional oxygenation status. These triggers include lactate levels[29], central venous oxygen saturation[29] and regional tissue oxygenation[30,31]. These triggers might reflect the impaired oxygen delivery better than Hb levels. One small RCT compared haematocrit (Ht) levels with a personalized algorithm based on brain oxygenation during cardiac surgery. The use of this algorithm did not result in decreased number of

transfusions and less organ damage[31]. Hence, further research in personalized transfusion protocols is warranted.

Treatment of coagulopathy in ICU patients is even more complex. Coagulopathy is treated in order to stop or to prevent a bleeding. Treatment can include administration of specific coagulation factors, plasma transfusions and platelet transfusions. Optimal transfusion triggers to prevent or treat coagulopathy remain unclear. Especially transfusion of plasma and platelets in patients with coagulopathy in the absence of a bleeding or an upcoming procedure is controversial[32]. Several studies showed that more than 50% of the plasma transfusions did not result in an International Normalized Ratio (INR) <1.5[32,33] and evidence supporting the benefit of plasma transfusion on (post) procedural bleedings is lacking[34,35]. In addition, in adult patients with a spontaneous cerebral haemorrhage and in neonates, a more liberal platelet transfusion strategy was even associated with an increased mortality[36,37]. Even though these are completely different patient populations, these studies suggest that platelet transfusions might have potential harmful effects.

Current transfusion strategies for coagulation disorders are platelet count, PT and aPTT guided. Alternatively, the coagulation state of the blood of a particular patient can be evaluated using viscoelastic tests in which the transition from liquid to a gel state is measured. The most widely used techniques are rotational thromboelastometry (ROTEM) and thromboelastography (TEG). These techniques were initially only used in the operating room to guide transfusion strategy, but use of these techniques are also studied in the emergency room and on the ICU in the past years[38,39].

Quality of blood components

Several factors are associated with an impaired quality of blood components including donor characteristics and storage time. To mitigate donor related risks several measures are taken such as solvent/detergent treatment of pooled plasma to inactivate lipid-enveloped pathogens. Also, transfusion of plasma, pooled male- only plasma is used to minimize the amount human leukocyte antigen (HLA) and human neutrophil antigen (HNA) antibodies that women more often develop due to their ability of being pregnant. To reduce HLA and HNA antibody exposure after platelet transfusion either plasma from male donors or female plasma negative for HLA antibodies is used, or platelets are stored in a mixture of additive solution and plasma.

The changes RBCs and platelets undergo during storage are collectively called the storage lesion. Several changes are part of the storage lesion, including morphological,

biochemical and immunological changes. Several methods and techniques have been assessed in order to minimize this storage lesion including different storage temperatures, different additive solutions and different type of storage bags.

Table 1. Overview of different additive solutions used for RBC storage and their content. *PAGGGM is an experimental additive solution, currently under research

Ingredients (mmol/L)	Additive solution						
	SAGM	**AS-1**	**AS-3**	**AS-5**	**AS-7**	**PAGGGM***	**PAGGSM**
NaCl	150	154	70	150	-	-	72
NaHCO3	-	-	-	-	26	-	-
NaH2PO4	-	-	23	-	-	8	8
Na2HPO4	-	-	-	-	12	8	16
Na-gluconate	-	-	-	-	-	40	-
Citric acid	-	-	2	-	-	-	-
Na-citrate	-	-	23	-	-	-	-
Adenine	1.25	2	3	2.2	2	1.4	1.4
Guanosine	-	-	-	-	-	1.4	1.4
Glucose(dextrose)	50	111	55	45	80	47	47
Mannitol	29	41	-	45.5	55	55	55
pH	6.2	5.5	5.8	5.5	8.5	8.2	6.0

The storage lesion of RBCs

To preserve the quality of RBCs during storage, an additive solution is used. In Europe, the current standard additive solution is SAGM (saline-adenine-glucose-mannitol). In the Netherlands, RBCs can be stored for a maximum of 35 days at 4 °C. The storage lesion in RBCs includes several morphological, immunological and metabolic changes. Additive solutions have been developed to preserve the metabolic homeostasis of RBCs as well as possible. Worldwide several different additive solutions are used for RBCs (table 1).

During storage in SAGM the intracellular pH declines to <6.5[40]. The intracellular pH has a large effect on the glucose metabolism during storage. Since RBCs do not contain mitochondria, they are completely dependent on the glycolysis for ATP production. In RBCs glucose is mainly catabolised in the glycolysis, Luebering Rapoport shunt and the pentose phosphate pathway (Figure 2). These pathways play a vital role in fulfilling the energy requirement of RBCs, the oxygen delivery capacity of RBCs and the detoxification of reactive oxygen species, respectively.

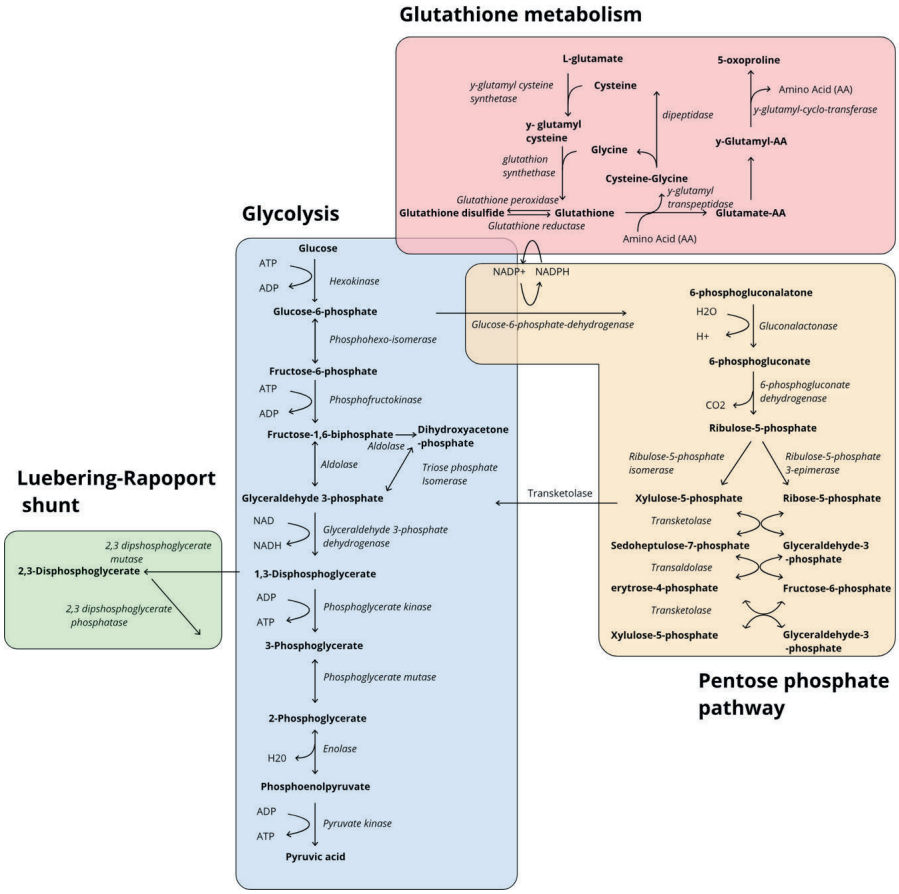

Figure 2 Overview glucose and glutathione metabolism in RBCs

During storage, glucose is catabolized to lactate to generate ATP. Consequently, the pH declines gradually throughout storage. The optimal pH of the rate limiting enzyme of the glycolysis, phosphofructokinase (PFK), is 8.1[41]. As RBCs are completely dependent on the glycolysis for their energy supply, this results in decreased ATP levels. Furthermore, the glycolysis provides metabolites to the Luebering Rapoport shunt which are converted to 2,3-DPG. The 2,3-DPG levels are completely depleted within 2 weeks after storage[40]. 2,3-DPG is a metabolite which decreases the oxygen affinity of haemoglobin and thereby maximizes the oxygen release to the peripheral tissues.

In the oxidative PPP, glucose is converted to ribose. In this process NADPH is produced, which is important to maintain reduced glutathione (GSH) levels. GSH detoxifies reac-

tive oxygen species. The optimal pH of glucose-6-phosphate dehydrogenase (G6PD), the rate limiting enzyme of the PPP is 7.8[42]. After 35 days of storage, G6PD activity was found to be decreased in SAGM stored RBCs[43]. These RBCs might be more vulnerable to oxidative stress.

The reversibility of the metabolic changes after transfusion into the recipient is not complete known. A few metabolites have been assessed after transfusion, including ATP and 2,3-DPG levels. They showed recovery in approximately 72 hours after transfusion[44]. Assessing the metabolic recovery over time after transfusion, could give more insight in how storage of RBCs could be further optimized.

To reduce the storage lesion, several additive solutions have been developed. The mechanism of action is that chloride-free additive solutions induce a shift of the intracellular chloride into the extracellular department (Figure 3). This results in an influx of hydroxyl ions into the cell and thereby increases the intracellular pH and this effect is enhanced by using alkaline additive solutions. Examples of chloride-free alkaline additive solutions are AS-7 and PAGGGM. These additive solutions have been shown to preserve the redox metabolism and glycolysis activity during storage[40,45]. In addition, RBCs stored in AS-7 showed a higher post transfusion recovery than AS-5 stored RBCs[45]. PAGGGM stored RBCs, have not been tested *in vivo* yet.

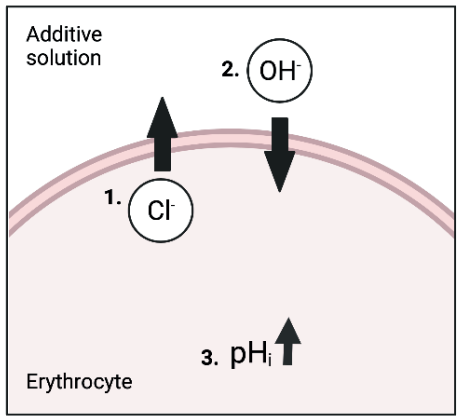

Figure 3. Mechanism of action of chloride-free alkaline additive solutions.

Storage lesion of platelets

Platelet concentrates can be obtained via two different methods: 1) They are obtained from a single donor via apheresis or 2) four to five buffy coats derived from whole blood donations are pooled and processed into one product. The preparation process of platelet concentrates includes several procedural steps such as centrifugation and resuspension of the platelets in an additive solution or plasma unit, depending applied method. These procedural steps are together with the storage of platelets the largest contributor to the platelet storage lesion.

Platelets are, in line with their function, easily activated. To minimize platelet activation, platelets are stored at 20-24˚C. The storage temperature of 20-24˚C has several disadvantages, it increases the risk of transfusion-transmitted infection and glucose metabolism is higher at this temperature. Due to the storage at this temperature platelet concentrates are at a higher risk for bacterial growth. The shelf life is, depending on local regulations, usually limited to 5-7 days. The increased glucose metabolism results in lactate accumulation which negatively effects the function of platelets.

Using an additive solution to store buffy coat and apheresis-derived platelet concentrates reduces the storage lesion, reduces the incidence of transfusion reactions[46], and possibly extends shelf-life. The composition of the additive solutions is based on maintaining an adequate energy source and minimize platelet activation. Several platelet additive solutions have been developed.

Monitoring quality of blood components

An important parameter of quality of blood components is the post transfusion recovery (PTR) *in vivo*. Hb level increment or platelet count increment, after RBC and platelet transfusion respectively is not a precise method as this is largely affected by characteristics of the recipient, especially the circulating volume.

It is possible to use human leukocyte antigen (HLA) mismatching to distinguish transfused platelets from non-transfused platelets in the circulation. RBCs can be distinguished by minor antigen mismatched between the donor and the recipient. The main advantage of these methods is that the transfused RBCs and platelets are not manipulated before transfusion. However, it has also several downsides, including that this method can only be applied after allogenic transfusion while testing of new products is most often conducted in healthy volunteers using autologous transfusions. Furthermore, when a patient receives multiple transfusions, it complicates the HLA or minor antigen mismatch measurement.

Currently, the gold standard for calculating the PTR, is using labelled cells. The most applied method of labelling is radioactive labelling with ^{111}Indium or ^{51}Chromium. This is both used in platelets and RBCs but has several disadvantages. Recipients are exposed to harmful Ionizing radiation and so this method is not applicable in vulnerable patient categories. Furthermore, radioactively labelled cells cannot be separately isolated from the circulation after transfusion and it is also not possible to trace different platelet populations simultaneously after transfusion.

Recently, biotin labelling is used as an alternative for radioactive labelling. Biotin, also called vitamin B7, has several advantages, including the possibility to isolate the transfused cells from the already circulating cells. This method has been validated extensively for RBCs. On a smaller scale biotinylation is also tested in platelets, but it is not yet validated on a larger scale. For platelets, the biotinylation process is more complicated as platelets are easily activated when manipulated. Currently, a standardized method for biotin labelling on a large scale would be desirable.

Aims of this thesis

This thesis focusses on three topics. First, current clinical transfusion practice is assessed in critically ill patients. Second, the application of a new additive solution is tested in healthy volunteers, assessing the RBC survival after transfusion and the metabolic recovery from the storage lesion after transfusion. And lastly, a new method for platelet labelling on a large scale is developed.

Short outline of the thesis

In **Chapter 2** and **Chapter 3** clinical transfusion practice is assessed with an online questionnaire regarding non-bleeding and bleeding patients, respectively.

In **Chapter 4** we performed a pilot study, assessing clinical practice in an academic intensive care unit and testing this study protocol prior to enrolling this worldwide.

In **Chapter 5** we reviewed the metabolic changes in erythrocytes in vivo, during storage and after transfusion.

In **Chapter 6** we assessed the effect of storage time and additive solution on post transfusion recovery and metabolic recovery of RBCs after transfusion.

In **Chapter 7** we validated a new method to label platelets, using biotin as an alternative for radioactive labelling.

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CHAPTER 2

Transfusion practice in the non-bleeding critically ill; an international online survey – The TRACE Survey

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Abstract

Background: Over the last decade multiple large randomized controlled trials have studied alternative transfusion strategies in critically ill patients, demonstrating the safety of restrictive transfusion strategies. Due to the lack of international guidelines specific for the intensive care unit (ICU), we hypothesized that a large heterogeneity in transfusion practice in this patient population exists. The aim of this study was to describe current transfusion practices and identify knowledge gaps.

Methods: An online, anonymous, worldwide survey among ICU physicians was performed evaluating red blood cell, platelet and plasma transfusion practices. Furthermore, the presence of a hospital or ICU specific transfusion guideline was asked. Only completed surveys were analysed.

Results: 947 respondents filled in the survey of which 725 could be analysed. Hospital transfusion protocol available in their ICU was reported by 53% of the respondents. Only 29% of respondents used an ICU specific transfusion guideline. The reported haemoglobin (Hb) threshold for the general ICU population was 7 g/dL (7-7). The highest reported variation in transfusion threshold was in patients on extracorporeal membrane oxygenation or with brain injury (8 g/dL (7.0-9.0)). Platelets were transfused at a median count of $20x10^9$ cells/L IQR (10-25) in asymptomatic patients, but at a higher count prior to invasive procedures ($p<0.001$). In patients with an international normalized ratio (INR) >3, 43% and 57% of the respondents would consider plasma transfusion without any upcoming procedures or prior to a planned invasive procedure, respectively. Finally, doctors with base specialty anaesthesiology transfused critically ill patients more liberally compared to internal medicine physicians.

Conclusion: Red blood cell transfusion practice for the general ICU population is restrictive, while for different subpopulations higher Hb thresholds are applied. Furthermore, practice in plasma and platelet transfusion is heterogeneous and local transfusion guidelines are lacking in the majority of the ICUs.

Introduction

As critically ill patients frequently develop anaemia, thrombocytopenia or coagulopathy[1–3], transfusion of blood components is a frequent intervention in the intensive care unit (ICU). About 12.5% of all transfused red cell concentrates (RCCs), 13% of all platelet concentrates (PC) and 30% of all plasma units in the hospital are transfused in the ICU[4]. However these products are associated with life threatening adverse events including transfusion related acute lung injury (TRALI), transfusion associated cardiac overload (TACO), and transfusion related immunomodulation(TRIM)[5–7].

Since the Transfusion Requirements in Critical Care (TRICC) trial, 20 years ago, it has been increasingly recognized that a restrictive RCC transfusion strategy may be as safe as a liberal strategy and even reduce patient mortality in specific patient subpopulations[8]. Consequently, ICU transfusion practice has shifted towards more restrictive strategies. From 2002 to 2012, the incidence of RCC transfusion in critically ill patients has dropped from 37%[9] to 26%[1] during ICU admission. This reduction coincided with the publication of multiple large international randomized controlled trials (RCTs) showing the safety of a restrictive transfusion strategy[8,10,11].

While multiple large RCTs have been performed to compare liberal versus restrictive strategies in red blood cell transfusions in ICUs, RCTs studying the optimal transfusion strategies in critically ill patients for plasma and platelets are limited or had a small sample size[12]. It is difficult to judge what "appropriate" transfusion triggers are for these blood products. This uncertainty is reflected in poor adherence to recommended best practices. It is estimated that hospital wide 37% of transfused plasma units and 33% of transfused platelets are administered outside guideline recommendations[13–15].

Of note there is no international ICU transfusion guideline. The aim of this survey was to evaluate the use of local transfusion guidelines in the ICU and the applied transfusion thresholds for RCC, PLT and plasma transfusion in ICU patients without an active haemorrhage.

Methods

Survey

An anonymous survey on transfusion practices in non-bleeding patients was conducted among intensivists, intensivist in training and non-intensivists specialists attending in

the intensive care medicine. This survey was initiated by the Cardiovascular Dynamics Section and endorsed by the European Society of Intensive Care Medicine (ESICM). In addition, multiple national intensive care societies distributed the survey to its members by newsletters and/or promoted it on their website (see Additional file 1 for contributors).

Study design

An online platform was used to set up the questionnaire (SurveyMonkey; Portland, OR, USA). After designing, the survey was tested by an international panel of intensivists to optimize the validity and accuracy of the questionnaire. The survey included 40 questions, divided into four sections, respondent demographics, transfusion practice regarding red blood cells (15-17 questions), platelets (5 questions) and plasma transfusions (8 questions, see Additional file 1 for static version). Multiple clinically relevant subpopulations (also non-bleeding) were addressed in each section. For red cell transfusion, first the preferred haemoglobin (Hb) threshold for the general ICU population was asked, followed by the preferred Hb level for each subpopulations. For platelet transfusion, a distinction was made between transfusion prophylactically and prior different invasive procedures. For plasma transfusions, a distinction was made between prophylactic transfusion without a planned procedure and prior to an invasive procedure in general.

Statistical analysis

Only completed surveys were analysed. Since some questions were not applicable for all doctors, respondents were allowed to leave specific questions about subgroups/specific interventions open. This missing data was not imputed.

Descriptive statistics were used to characterize the respondent demographics. Normal distributed and non-normal distributed data was reported as mean (standard deviation) or as median (first quartile-third quartile) respectively. Categorical data was presented as percentage. Participants were able to fill in Hb thresholds in g/dL, g/L, or mmol/L, and all answers were converted to g/dL for analysis.

Transfusion thresholds were not normally distributed, therefore the Kruskall-Wallis test was used to test whether the transfusion thresholds differed significantly subpopulations or between interventions. As post hoc test, the Dunn test with Bonferroni correction was used. In addition, transfusion thresholds were analysed using the Wilcoxon sum rank test or Kruskall-Wallis test to test the dependence of two grouping variables or more than 2 grouping variables respectively. Chi-square test with Yates's correction for continuity was used for categorical variables. For comparing different world regions,

only regions where at least ten respondents were working were taken into account because they may not accurately represent the transfusion practice across their region. All tests were two sided. A p-value <0.05 was considered to be statistically significant. Statistical tests were performed with R studio (2018, 3.5.1, Vienna).

Results

Demographics

The survey was open for 6 months (June 2018 to November 2018). Of the 947 received surveys, 769 were complete, of these 44 were excluded because the respondents did not fulfil study inclusion criteria (i.e. non-physician, or pediatric ICU physician). The remaining 725 completed surveys (representing 69 countries) were included in the study. The majority of the participants practiced ICU in Europe (76%) (Additional file 1, figure S1). Background specialties were mainly anaesthesiology (62%) and internal medicine (20%), other demographics are shown in table 1.

Table.1 Respondents demographics

Demographics	**No. of respondents (%)**
Certification level	
Intensivist	589 (81)
Resident, specialist in training	53 (7)
Specialist, non-intensivist practicing ICU	73 (10)
Other	10 (1)
Primary medical specialty	
Anaesthesiology	450 (62)
Cardiology	18 (2)
Internal medicine	144 (20)
Neurology	4 (1)
Pulmonology	25 (3)
Surgery	15 (2)
Other	67 (10)
Type of intensive care unit (ICU)	
Medical ICU	63 (9)
Surgical ICU	536 (74)
Mixed ICU	110 (15)
Other	16 (2)
Number of ICU beds	
<10	209 (29)

Table.1 Respondents demographics *(continued)*

Demographics	No. of respondents (%)
10-15	206 (28)
16-20	115 (16)
>20	193 (27)
Not specified	2 (0)
Annual number of patients treated in the ICU	
<500	178 (25)
501-1000	239 (33)
1001-1500	139 (19)
1501-2000	66 (9)
>2000	98 (14)
Not specified	5 (1)
Type of institution	
University hospital	326 (45)
University affiliated hospital	146 (20)
Non-university public hospital	183 (25)
Private hospital	64 (9)
Other	6 (1)
Which unit do you use to measure haemoglobin	
g/dL	499 (69)
g/L	171 (24)
mmol/L	55 (8)

Red cell transfusion

The median reported Hb threshold used in the general ICU population was 7 g/dL (7.0-7.5). Higher Hb transfusion thresholds were reported in patients with acute coronary syndrome (ACS), septic shock, acute brain injury, those receiving extracorporeal membrane oxygenation (ECMO), with acute respiratory distress syndrome (ARDS), age over 65 years, and with prolonged weaning were transfused at higher Hb thresholds ($p<0.001$ for all patient populations, see figure 1). Thresholds did not differ between oncological/hematologic patients and the general ICU population ($p=1$). The largest variation in transfusion thresholds was observed in patients on ECMO and in patients with traumatic brain injury. Respondents would transfuse these patient populations at a Hb threshold of 8.0 g/dL (7.0-9.0). The highest Hb threshold was reported for patients with ACS median 9.0 g/dL (8.0-9.7).

Following the transfusion of the first RCC, Hb levels were routinely not re-evaluated before transfusing a second unit. Of the respondents 28% always re-evaluate the Hb level while 16% never re-evaluate.

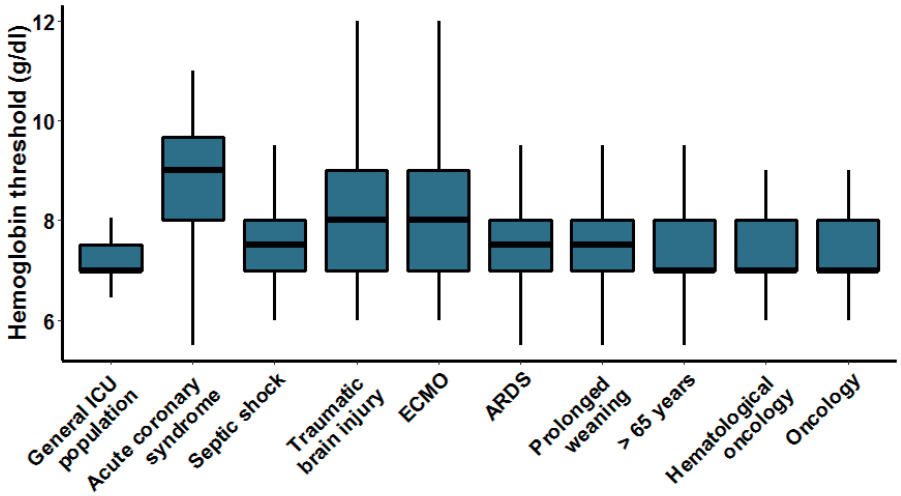

Figure 1. Respondents were asked which Hb threshold they used for RCC transfusion in the general ICU population and different subpopulations. Respondents used in the general population a Hb threshold of 7.0 g/dL (7.0-7.5). This is significantly lower ($p<0.001$) compared to patients with acute coronary syndrome (9.0 g/dL (8-9.7)), septic shock (7.5 g/dL (7.0-8.0)), acute brain injury (8.0 g/dL (7.0-9.0)), patients undergoing ECMO (8.0 (7.0-9.0) g/dL), issues of prolonged weaning (7.5 g/dL (7.0-8.0)), or patients with ARDS (7.5 g/dL (7.0-8.0)). No statistical differences were observed between the general ICU population and patients older than 65 years, patients with (haematological) oncology (all three groups were transfused at a Hb threshold of 7.0 g/dL (7.0-7.5)).

Transfusion triggers

The majority of the respondents used clinical markers such as hypotension and tachycardia along with Hb levels to guide transfusion. Among the respondents only 13% never uses other physiological triggers in addition to a Hb threshold. Of interest, 27% of the respondents would always use other physiological triggers (figure 2A).

Tachycardia (66%), hypotension (55%) and lactate levels >2 mmol/L (51%) were mentioned most often (figure 2B), while significant ECG changes were ranked as most important physiological trigger.

Prevention of RCC transfusion

Use of iron or iron in combination with erythropoietin (EPO) to improve erythropoiesis and prevent RCC transfusion was reported by for 41% and 17% of respondents, respectively. EPO was reported by 12% of the respondents as a monotherapy. A quarter of the respondents would never use these pharmacological agents for this purpose. Non-pharmacological blood conservation measures were less common in the ICU. Closed loop

blood sampling was the most common intervention (23%), followed by microtube sampling (13%). Computerized decision support was used by only 2% of the respondents

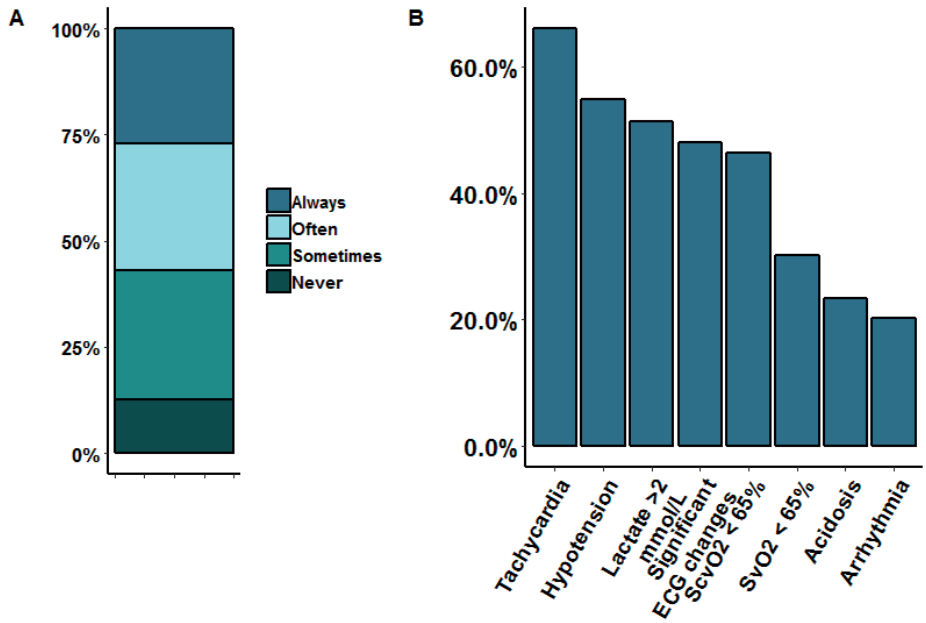

Figure 2. The use transfusion triggers in addition to a haemoglobin threshold.

Platelet transfusion

In non-bleeding patients undergoing an invasive procedure, respondents would transfuse patients at a platelet count of $20x10^9$ cells/L (10-25). Platelet concentrates were transfused at higher platelet counts prior an invasive procedure ($p<0.001$). Respondents would transfuse at a platelet count of $40x10^9$ cells/L (20-50) prior to central venous catheter (CVC) placement, 50 $x10^9$ cells/L (50-75) prior to tracheotomy, $50x10^9$ cells/L (50-80) prior to general surgery, and $100x10^9$ cells/L (70-100) prior to neurosurgery. When transfusing a PLT concentrate, 18% of the respondents never re-evaluate the platelet count before transfusing a second unit.

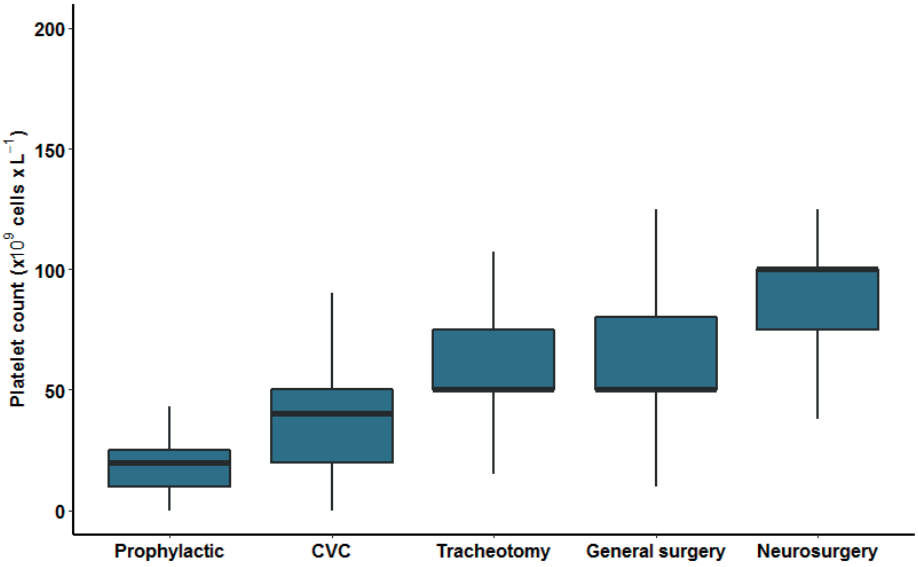

Figure 3. Platelet thresholds prophylactically without any planned invasive procedure and prophylactically prior to different procedures.

Coagulopathy

The majority (87%) of the respondents transfuse their patients with fresh frozen plasma, only 9.5% reported to use pooled plasma to correct coagulopathy.

In non-bleeding patients who will not undergo an invasive procedure, an international normalized ratio (INR) > 3 is infrequently corrected. Only 7% would always correct a prolonged INR (Figure 4A.). Vitamin K is the most commonly mentioned therapeutic agent to correct the INR in these patients (85%), followed by plasma (43%) and prothrombin complex (35%) (Figure 4B).

More physicians would correct a INR >3 prior to an invasive procedure compared to patients who are not undergoing an invasive procedure (p <0.0001). Among the respondents, 31% would always correct a prolonged INR in this setting (Figure 4A). Also, prior to an invasive procedure, the majority (70%) most of the respondents would use vitamin K as a therapeutic option, followed by prothrombin complex (58%) and plasma (57%) (Figure 4C).

To diagnose coagulopathy, INR/Prothrombin time (99%), activated partial thromboplastin time (APTT, 97%) and fibrinogen level (94%) are widely available tests in the

ICU. Visco-elastic tests are less common, only in the minority of the hospitals rotational thromboelastometry (ROTEM, 31%) or thromboelastography (TEG, 18%) are available as diagnostic tool.

Guideline

Among the respondents, 29% have an ICU specific and 53% a (not ICU specific) transfusion guideline in their ICU. The availability of a guideline has limited effect on the transfusion practice. Only for the general ICU population the presence of a (not ICU) specific transfusion guideline was associated with a lower transfusion threshold (p=0.028, Additional file 1 table S3). For other ICU subpopulations, this association with RCC transfusion practice was not present (Additional file 1 table S2 and S3). Also, for platelet and plasma transfusion no association was found between the presence of a guideline and transfusion practice (Additional file 1 table S5 and S6).

Background specialty

To investigate whether the base specialty influences transfusion practices, only the groups anaesthesiology (69% of the respondents) and internal medicine (20% of the respondents) were sufficiently present to perform additional testing. For RCC, PC and plasma transfusion, an association was found between base specialty and transfusion practice. Overall, the base specialty anaesthesiology was associated with a more liberal transfusion practice compared to internal medicine. With the exception of patients with ACS and patients on ECMO, physicians with the base specialty anaesthesiology said to transfuse all subpopulations at significantly higher Hb thresholds. Furthermore, anaesthesiologists more often report the use of physiological triggers in addition to Hb levels in their decision to transfuse than internal medicine physicians (p =0.02, see Additional file 1 table S4). Also, thrombocytopenic patients are transfused at higher platelet counts prior CVC placement (p=0.002) and prior to tracheotomy (p=0.007, Additional file 1 table S7) when treated by a physician with the base specialty anaesthesiology. For plasma transfusion, only in prophylactically transfusions a different practice was observed between these two base specialties. Physicians with base specialty anaesthesiology transfuse plasma prophylactically more frequently (Additional file 1, Figure S3).

Regional differences

In all world regions, a median Hb threshold of 7g/dL (7.7) was reported for the general ICU population. For different subpopulations, a greater variety was reported, especially in patients with ACS, traumatic brain injury, and patients receiving ECMO (Additional file 1al figure S2). In platelet transfusion some alignment exists for prophylactic platelet transfusion with a median applied platelet count prophylactically between 15 and 20

x10^9 cells/L for all world regions. However, prior to an invasive procedure more heterogeneity exist (Additional file 1 figure S4). Prior to general surgery the largest differences between regions was observed, in half of the regions the mean of the applied platelet threshold was 50 x10^9 cells/L while in Southern Asia and South-Eastern Asia a median platelet count of 72.5 and 100 x10^9 cells/L was applied, respectively. Lastly, also plasma transfusion practices differed between world regions (Additional file 1 figure S5). In Southern Europe only 17% would never correct a vitamin K induced INR >3 in the absence of an invasive procedure, whereas in South-Eastern Asia 50% would never correct this INR.

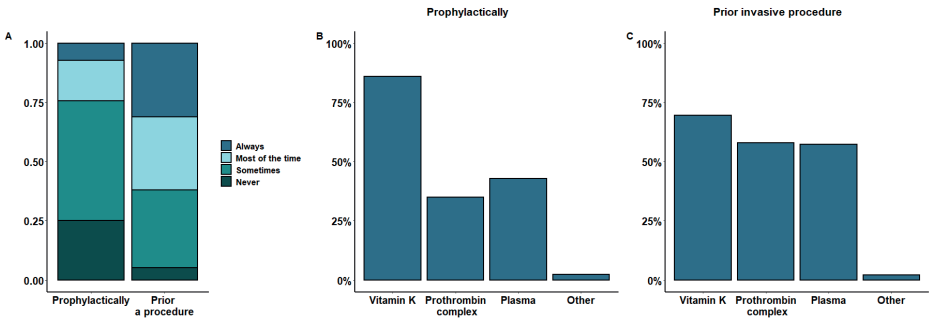

Figure 4. More respondents would correct an vitamin K induced INR >3 prior to an invasive procedure than in the absent of a procedure(4A). Both, in the absence of an invasive procedure (4B) and prior an invasive procedure (4C), the majority would correct this with vitamin K.

Discussion

This is the largest survey on transfusion practice in non-bleeding critically ill patients among ICU physicians to date. The main findings of this study are: 1) a high Hb threshold variation between ICU subpopulations, 2) the platelet transfusion threshold prior to invasive procedures differs greatly between and within the procedures, 3) plasma is considered by a large number of physicians in non-bleeding patients even in the absence of an invasive procedure, 4) base specialty of physicians is associated with variation in transfusion practices and 5) worldwide, institutions lack local ICU specific transfusion guidelines.

The reported Hb threshold for the general ICU population in this survey is in line with the finding of the TRICC study, which demonstrated the safety of a restrictive transfusion strategy in the ICU population [8]. However, when looking at different patient subpopu-

lations, a greater variety of applied Hb thresholds was found. For the septic patients respondents reported a significantly higher Hb threshold compared to the general population, what deviates from current evidence supporting a restrictive transfusion strategy also in septic patients[11]. For patients with ACS, the higher preferred Hb threshold of 9 g/dL (8-9.6) is in accordance with the transfusion guideline from the National Institute for Health and Care Excellence (NICE, 2018), in which a Hb threshold (8-10 g/dL) for patients with symptomatic coronary disease is advised. Also, patients with traumatic brain injury were transfused at higher Hb thresholds since these patients may be more sensitive to anaemia induced cerebral hypoxia. However, evidence to justify this more liberal transfusion practice is limited. Multiple large RCTs are currently studying whether these patients benefit from a liberal transfusion strategy (ClinicalTrials.gov, NCT02968654 and NCT02981407).

This survey also showed a high variety in preferred Hb thresholds for patients with ARDS and patients on ECMO. Since the evidence for these subpopulations is limited it is expected to observe a high heterogeneity in transfusion practice. For ARDS patients it is hypothesized that the hypoxaemia should be compensated by increasing the oxygen carrying capacity of the circulating blood by transfusing at higher Hb thresholds. However, there is no solid evidence to support this practice, and the downside of allogenic blood transfusion is not taken into account in this reasoning.

The applied platelet threshold differed between patients with and without an upcoming invasive procedure. The majority of the respondents (72%) would transfuse non-bleeding critically ill patients at a platelet count of $\leq 20x10^9$ cells/L. The potential harm of platelet transfusion is supported by two recent RCTs, in which it was shown that prophylactically platelet transfusion might be particularly harmful in neonates[16] and in patients with a cerebrovascular accident [17]. These studies cannot be directly translated to the non-bleeding critically ill adult patients, but they do show that platelet transfusion is not an intervention without risk. Prior to invasive procedures physicians transfuse platelets at higher platelet counts, while the evidence for this is limited. A meta-analysis has shown that complications prior to CVC placement in patients with coagulopathy, including thrombocytopenia and prolonged INR and APTT, are rare[18]. Thus, the need for any platelet transfusion prior to this procedure is questionable. A large RCT studying the need of platelet transfusion prior CVC placement in severe thrombocytopenic patients is now recruiting[19].

Multiple RCTs have failed to demonstrate beneficial effects of prophylactic plasma transfusion prior to an invasive procedure in critically ill patients with an prolonged INR [12,20,21].

The finding of this survey that 57% and 43% of physicians would consider to transfuse plasma to correct the prolonged INR in patients who used vitamin K antagonists prior an invasive procedure or without a planned invasive procedure, respectively, is striking in the absence of evidence for this practice.

To our knowledge, the influence of base specialty of intensivists on transfusion practice has not been studied before. Our survey showed that doctors with a base specialty anaesthesiology transfuse more liberal than those with internal medicine as base specialty. It might be that doctors with an internal medicine background are more aware of the harmful side effects of blood products, alternatively, anesthesiologists may tend to treat patients at higher risk of bleeding, and these practices spill over into the ICU.

The strength of this survey is the large number of respondents. However, both the anonymous character of this survey and the origin of the respondents might have introduced a selection bias and limits therefore the worldwide generalizability of our findings. Theoretically, it is possible that multiple respondents are employed in the same hospital, however also within hospitals heterogeneity in transfusion practice may exist. Furthermore, the number of respondents who did receive this survey but did not fill it in is unknown. We cannot exclude that non-responders transfuse differently than the responders of this survey. It may be possible that physicians with more interest in transfusion practice and thus with more awareness of the possible side effects of transfusion are over presented in this survey. In addition, due to the study design it was not appropriate to perform multivariable analysis. As result, it was not possible to exclude the presence of confounding variables on the observed significant associations. And finally, as with any clinical practice survey, the reported transfusion practices might differ from actual transfusion practices. Ideally, these results are confirmed in a prospective cohort study.

Conclusion

In conclusion, in the general non-bleeding ICU population, the reported RCC transfusion practice was rather restrictive, however, in certain subpopulations including the critically ill with septic shock, higher applied Hb thresholds were reported, which deviates from current evidence. For other subpopulations such as patients with ARDS and patients on ECMO well powered RCTs are needed. In addition, optimal platelet thresholds are currently controversial and more awareness is necessary for correct indications of plasma

transfusion in non-bleeding patients. Finally, a local transfusion guideline for critically ill patients is lacking in the majority of ICUs worldwide.

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Supplementary material

Supplemental Figures

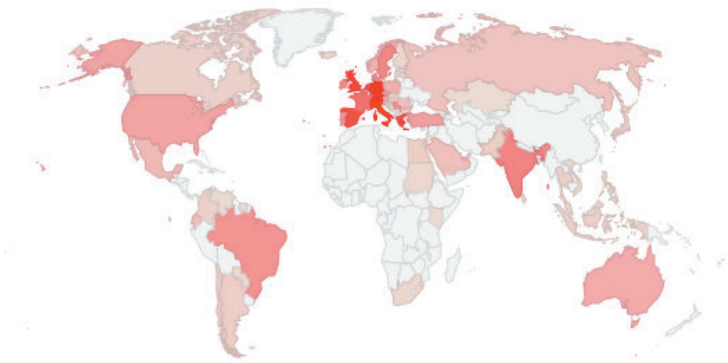

Figure S1 Origin of respondents

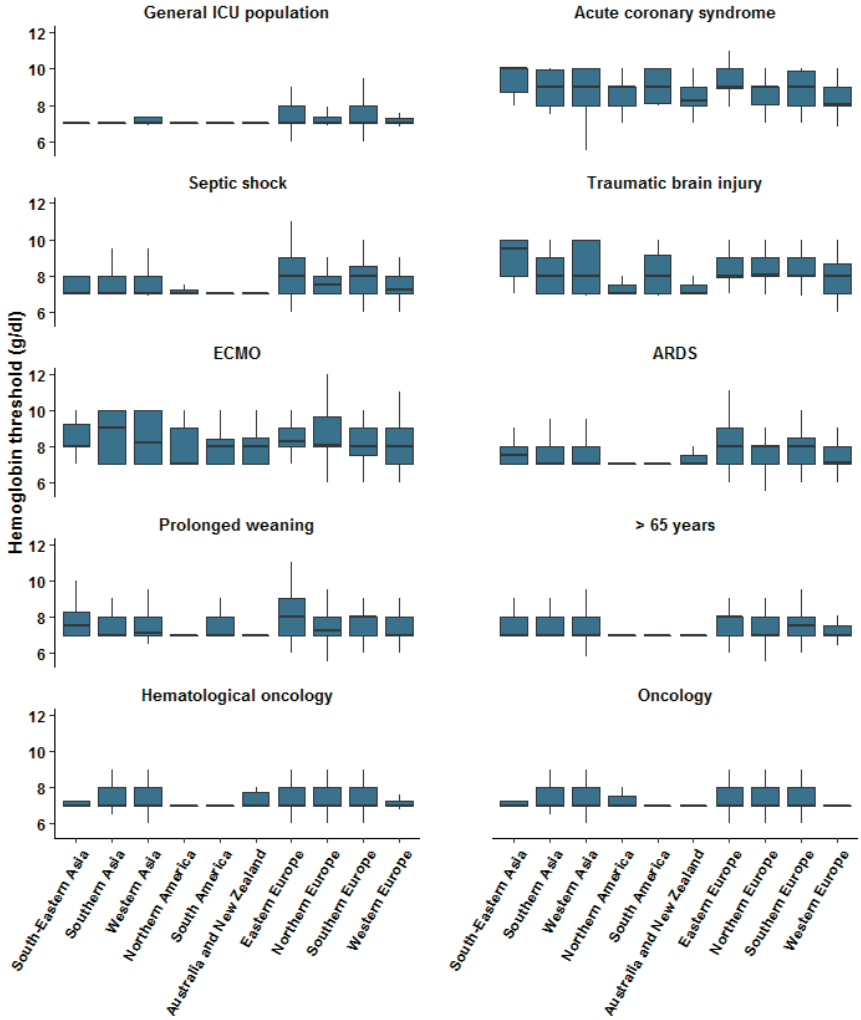

Figure S2. Patients with acute coronary syndrome were transfused at highest Hb levels in South Eastern Asia with a median Hb level of 10 g/dL (8.75-10) while in Western Europe the median reported transfusion threshold was 8.1 g/dL (8-8.1). For patients with traumatic brain injury also respondents from South Eastern Asia reported the highest Hb levels as transfusion thresholds of 9.5 g/dL (8-9.5) and lowest Hb levels were reported in Northern America with a median Hb of 7 g/dL (7-7). In Southern Asia the highest Hb thresholds for patients receiving ECMO were reported with 9 g/dL (7-9), while the lowest Hb levels were reported by respondents working in Northern America with 7 g/dL (7-7).

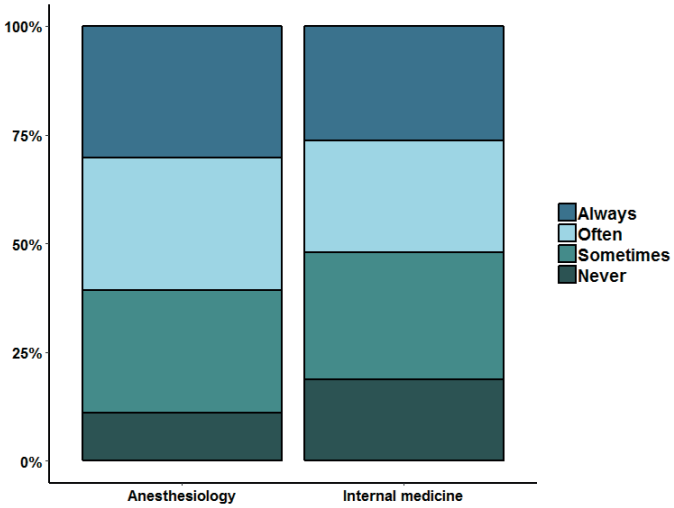

Figure S3. The use of a physiological transfusion trigger in addition to a Hb threshold. Significant differences were observed for anaesthesiology versus internal medicine (p = 0.02).

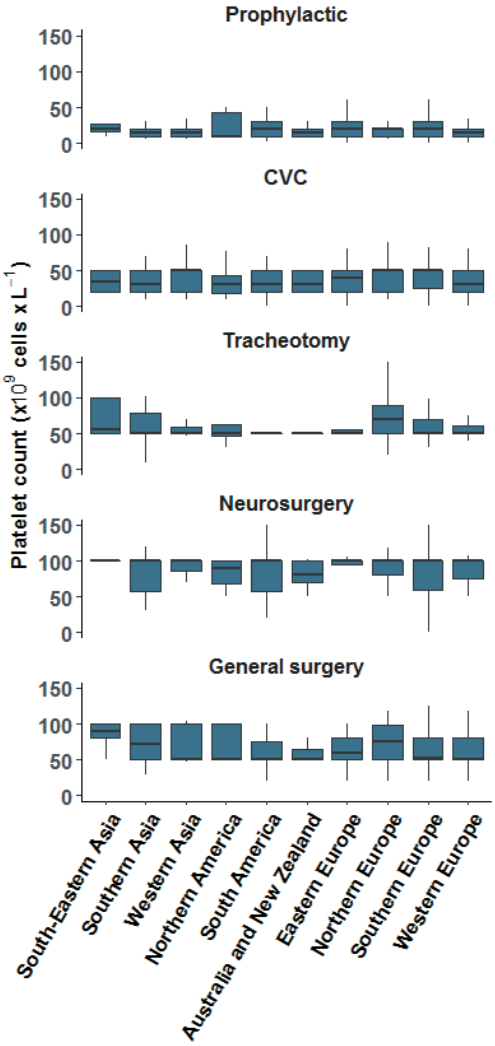

Figure S4. Regional differences of applied platelet thresholds prophylactically without any planned invasive procedure and prophylactically prior to different procedures

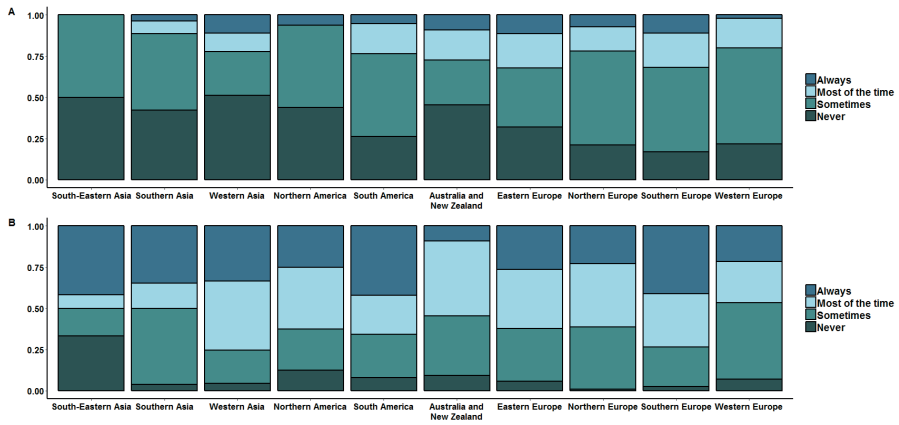

Figure S5. Regional differences of the correction of a vitamin K induced INR >3 (A) prophylactically in the absence of an invasive procedure and (B) prior to an invasive procedure

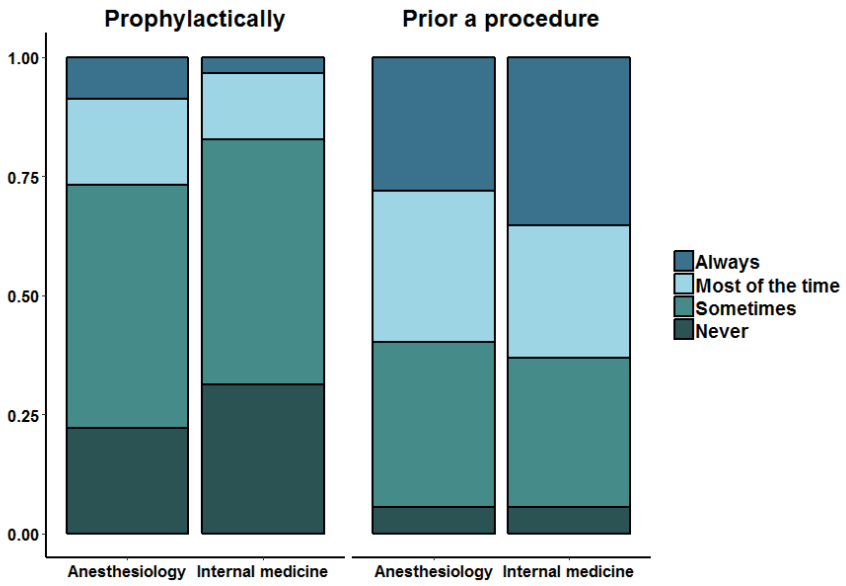

Figure S6. Effect of base specialty of ICU doctor on the correction of a vitamin K induced INR >3 (A) prophylactically in the absent of an invasive procedure (p= 0.0272) and (B) prior to an invasive procedure (p=0.03983).

Table S1

World region	n =
Australia and New Zealand	11
Caribbean	1
Central America	8
Central Asia	1
Eastern Africa	2
Eastern Asia	4
Eastern Europe	53
Northern Africa	6
Northern America	16
Northern Europe	109
South-Eastern Asia	12
South America	38
Southern Africa	2
Southern Asia	26
Southern Europe	197
Western Asia	45
Western Europe	184

Table S2

	ICU specific guideline		
	Available	**Not available**	**p value**
General ICU population	7(7-7.5)	7(7-7.5)	0.1341
Acute coronary syndrome	9(8-9.7)	9(8-9.6)	0.1009
Septic shock	7.3(7-8)	7.5(7-8)	0.389
Traumatic brain injury	8(7-9)	8(7-9)	0.9795
ECMO	8(7-9)	8(7.5-9)	0.2455
ARDS	7(7-8)	7.5(7-8)	0.0517
Prolonged weaning	7.5(7-8)	7.4(7-8)	0.9892
Age ≥65 years	7(7-8)	7(7-8)	0.6015
Haematological oncology	7(7-8)	7(7-8)	0.2184
Oncology	7(7-8)	7(7-8)	0.8695

Table S3

	(not-ICU specific) Guideline		
	Available	**Not available**	**p value**
General ICU population	7(7-7.5)	7(7-7.9)	0.0283
Acute coronary syndrome	9(8-9.7)	9(8-9)	0.8161
Septic shock	7.3(7-8)	7.5(7-8)	0.2662
Traumatic brain injury	8(7-9)	8(7-9)	0.783
ECMO	8(7-9)	8(7-9)	0.2794

ARDS	7(7-8)	7.5(7-8)	0.262
Prolonged weaning	7.1(7-8)	7.5(7-8)	0.1283
Age ≥65 years	7(7-8)	7(7-8)	0.0649
Haematological oncology	7(7-8)	7(7-8)	0.3209
Oncology	7(7-8)	7(7-8)	0.191

Table S4

	Main specialty		
	Anaesthesiology	**Internal medicine**	**p value**
General ICU population	7(7-8)	7(7-7)	0.0452
Acute coronary syndrome	9(8-9.7)	9(8-9.9)	0.3662
Septic shock	8(7-8)	7(7-8)	< 0.001
Traumatic brain injury	8(7.8-9)	8(7-9)	0.036
ECMO	8(7.7-9)	8(7-9.5)	0.5545
ARDS	8(7-8.2)	7(7-8)	< 0.001
Prolonged weaning	8(7-8)	7(7-8)	< 0.001
Age ≥65 years	7.2(7-8)	7(7-7.7)	0.0003
Haematological oncology	7(7-8)	7(7-7.4)	0.0224
Oncology	7(7-8)	7(7-7.2)	0.0459

Table S5

	ICU specific transfusion guideline		
	Available	**Not available**	**p value**
Prophylactic	20(10-20)	20(10-30)	0.2827
Prior to CVC placement	30(20-50)	45(20-50)	0.1299
Prior to tracheotomy	50(50-70)	50(50-71)	0.7226
Prior to general surgery	50.5(50-93)	50(50-80)	0.8616
Prior to neurosurgery	100(67.5-100)	100(75-100)	0.3684

Table S6

	(not-ICU specific) transfusion guideline		
	Available	**Not available**	**p value**
Prophylactic	20(10-25)	20(10-30)	0.8973
Prior to CVC placement	45(20-50)	40(20-50)	0.5185
Prior to tracheotomy	50(50-71)	50(50-70)	0.1976
Prior to general surgery	50(50-80)	53(50-90)	0.4122
Prior to neurosurgery	100(70-100)	100(75-100)	0.1442

Table S7

	Main specialty		
	Anaesthesiology	**Internal medicine**	**p value**
	Platelet count	**Platelet count**	
Prophylactic	20(10-30)	10(10-20)	0.0052
Prior to CVC placement	48.5(20-50)	30(20-50)	0.0023
Prior to tracheotomy	50(50-80)	50(50-51)	0.0069
Prior to general surgery	51(50-80)	50(50-81)	0.3439
Prior to neurosurgery	100(75-100)	100(80-100)	0.9409

Appendix 1: Overview collaborating societies

- Australian and New Zealand Intensive Care Society (ANZICS)
- Czech Society of Anaesthesiology, Resuscitation & Intensive Care (CSARIM)
- Hellenic Society of Intensive Care (HSIC)
- Societa Italiana di Anestesia Analgesia Rianimazione e Terapia Intensiva (SIAARTI)
- Intensive Care Society of Ireland (ICSI)
- Lebanese Critical Care Society (LCCS)
- Nederlandse Vereniging voor Intensive Care (NVIC)
- Serbian Association of Anaesthesiologists & Intensivits (SAAI)
- Slovenian Society of Intensive Medicine (SSIM)
- Swedish Society of Anaesthesiology & Intensive Care Medicine (SFAI)
- Swiss Society of Intensive Care Medicine (SGI-SSICM)
- Turkish Society of Anesthesiology and Reanimation (TARD)
- Intensive Care Society (ICS, United Kindom)

Appendix 2: Static version questionnaire

Demographics

1. **In which country do you work?**

2. **What is your intensive care certification level**
   a. Intensivist
   b. Resident, specialist in training
   c. Specialist non intensivist practising ICU
   d. Nurse
   e. Student
   f. Other, please specify

3. **What is your primary medical specialty**
   a. Anaesthesiology
   b. Cardiology
   c. Internal medicine
   d. Neurology
   e. Paediatrics
   f. Pulmonology
   g. Surgery
   h. Other, please specify

4. **Type of intensive care unit (ICU)**
   a. Medical ICU
   b. Surgical ICU
   c. Mixed ICU
   d. Other, please specify

5. **Number of ICU beds**
   a. <10
   b. 10-15
   c. 16-20
   d. >20

6. **Annual number of patients treated in the ICU**
   a. <500
   b. 500-1000
   c. 1001-1500
   d. 1501-2000
   e. >2000

7. **Type of institution**
   a. University hospital
   b. University affiliated hospital
   c. Non-university public hospital
   d. Private hospital
   e. Other, please specify

8. **Do you have a transfusion protocol in your hospital?**
   a. Yes
   b. No
   c. I don't know
   d. Other, please specify

9. **Do you have a transfusion protocol specific for the intensive care unit in your hospital?**
   a. Yes
   b. No
   c. I don't know
   d. Other, please specify

Red blood cell transfusion

10. **Which unit do you use to measure hemoglobin levels?**
    a. g/dL
    b. g/L (=mg/ml)
    c. mmol/L

11. **What is your overall threshold for blood transfusion in a general population of anemic critically ill patients?**

12. **What is your threshold for blood transfusion in anemic critically ill patients with acute coronary syndrome?**

13. **What is your threshold for blood transfusion in anemic critically ill patients with septic shock?**

14. **What is your threshold for blood transfusion in anemic critically ill patients with traumatic brain injury?**

15. **What is your threshold for blood transfusion in anemic critically ill patients receiving ECMO?**

16. **What is your threshold for blood transfusion in anemic critically ill patients with ARDS?**

17. **What is your threshold for blood transfusion in anemic critically ill patients with prolonged weaning from mechanical ventilation?**

18. **What is your threshold for blood transfusion in anemic critically ill patients >65 years?**

19. **What is your threshold for blood transfusion in anemic critically ill patients with a hematologic malignancy?**

20. **What is your threshold for blood transfusion in anemic critically ill oncology patients?**

21. **Do you check hemoglobin levels after transfusion of one unit red cell concentrates before transfusing a second unit in non-bleeding critically ill patients?**
    a. Always
    b. Most of the time
    c. Sometimes
    d. Never

22. **Do you also use physiological transfusion triggers (e.g. tachycardia) in addition to a hemoglobin threshold?**
    a. Always
    b. Often
    c. Sometimes
    d. Never

23. **Which physiological transfusion triggers do you use in non-bleeding patients? (multiple answers possible)**
    a. Tachycardia
    b. Hypotension
    c. Arrhythmia
    d. Significant ECG changes
    e. SvO_2 (mixed venous saturation of oxygen) < 65 %
    f. $ScvO_2$ (central venous oxygen saturation) < 65 %
    g. Lactate >2 mmol/L
    h. Acidosis
    i. Other, please specify

24. **How would you rank the following triggers of importance to you? (1 means most important)**
    a. All triggers ticked by respondent in previous question are mentioned.

25. **Which of the following treatment options do you use to prevent transfusion of red**

cell concentrates in anemic critically ill patients with iron deficiency? (multiple answers possible)

a. Iron suppletion
b. Erythropoietin (epo) suppletion
c. Iron suppletion in combination with erythropoietin (epo)
d. None of the above
e. Other, please specify

26. Which measures are available in your ICU to minimize the amount of red cell concentrate transfusion? (multiple answers possible)

a. Closed loop sampling
b. Computer decision making program
c. Microtube sampling
d. None
e. Other, please specify

Platelets

27. What is your threshold for prophylactic platelet transfusion in thrombocytopenic non-bleeding patients on the ICU (thus not prior to an invasive procedure)? (10^9 cells/L)

28. What is your threshold for platelet transfusion in non-bleeding thrombocytopenic patients prior to placing a central line on the ICU? (10^9 cells/L)

29. What is your threshold for platelet transfusion in critically ill non-bleeding thrombocytopenic patients prior to a tracheotomy? (10^9 cells/L)

30. What is your threshold for platelet transfusion in thrombocytopenic non-bleeding critically ill patients prior to neurosurgery? (10^9 cells/L)

31. What is your threshold for platelet transfusion in thrombocytopenic non-bleeding critically ill patients on the ICU prior to general surgery? (10^9 cells/L)

32. Do you check thrombocyte count after transfusion of one unit thrombocyte concentrate (1 unit is approx. 300 x 10^9 platelets) before transfusing a second unit in non-bleeding critically ill patients?

a. Always
b. Most of the time
c. Sometimes
d. Never

Plasma

33. Which coagulation tests are available as standard care on your ICU? (multiple answers possible)

a. PT/INR
b. aPTT
c. Fibrinogen
d. Rotational thromboelastometry (ROTEM)
e. Thromboelastography (TEG)
f. Other, please specify

34. Do you check the INR after transfusion of one unit of plasma before transfusing a second unit in a non-bleeding patient with plasmatic coagulopathy (INR>3.0) who used vitamin K antagonists?

a. Always
b. Most of the time
c. Sometimes
d. Never
e. Not applicable

35. **How do you correct a plasmatic coagulopathy (INR>3.0) prophylactically in non-bleeding patients who used vitamin K antagonists on the ICU? (multiple answers possible)**
    a. Vitamin K
    b. Cofact (prothrombin complex)
    c. Plasma
    d. Other, please specify

36. **Do you correct a plasmatic coagulopathy (INR >3) in non-bleeding critically ill patients on the ICU prior to an invasive procedure (e.g. placing a central line) who used vitamin K antagonists?**
    a. Always
    b. Most of the time
    c. Sometimes
    d. Never

37. **How do you treat plasmatic coagulopathy (INR >3.0) prior to an invasive procedure in non-bleeding patients who used vitamin K antagonists on the ICU? (multiple answers possible)**
    a. Vitamin K
    b. Cofact (prothrombin complex)
    c. Plasma
    d. Other, please specify

38. **Do you check the INR after transfusion of one unit of plasma before transfusing a second unit in a non-bleeding patient with plasmatic coagulopathy (INR >3.0) prior to an invasive procedure on the ICU who used vitamin K antagonists?**
    a. Always
    b. Most of the time
    c. Sometimes
    d. Never
    e. Not applicable

39. **If you decide to transfuse a non-bleeding critically ill patient, what type of plasma do you use? (multiple answers possible)**
    a. Pooled plasma (e.g. Omniplasma)
    b. FFP (fresh frozen plasma)
    c. Other (please specify)

CHAPTER 3

Transfusion practice in the bleeding critically ill; an international online survey – The TRACE-2 Survey

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Abstract

Background: Transfusion is very common in the intensive care unit (ICU), but practice is highly variable, as has recently been shown in non-bleeding critically ill patients practices survey. Bleeding patients in ICU require different blood products across a range of specific patient categories. We hypothesize that a large variety in transfusion practice exists in bleeding patients.

Study design and methods: An international online survey was performed among physicians working in the ICU. Transfusion practice in massively and non-massively bleeding patients was examined, including transfusion ratios, thresholds and the presence of transfusion guidelines.

Results: 611 respondents filled in the survey of which 401 could be analyzed, representing 64 countries. Among the respondents, 52% had a massive transfusion protocol (MTP) available at their ICU. In massively bleeding patients, 46% of the respondents used fixed transfusion component ratios. Of those who used fixed blood ratios, the 1:1:1 ratio (red blood cell (RBC) concentrates: plasma: platelet concentrates) was most commonly used (33%). The presence of an MTP was associated with a more frequent use of fixed ratio's ($p<0.001$).

For RBC transfusion in the general non-massively bleeding ICU population, a hemoglobin (Hb) threshold of 7.0[7.0-7.3] g/dL was reported. In the general ICU population, a platelet count threshold of 50[26-50] $x10^9$ /L was applied.

Discussion: Half of the centers had no massive transfusion protocol available. Transfusion practice in massively bleeding critically ill patients is highly variable and driven by the presence of an MTP. In the general non-massively bleeding ICU population restrictive transfusion triggers were chosen.

Keywords: Transfusion, critically ill, bleeding, massive transfusion, anemia, coagulation

Background

Transfusion is common practice in the intensive care unit (ICU), with about 40-50% of the critically ill being transfused during ICU admission [1]. While the transfusion of blood products can enhance the life expectancy of critically ill patients [2], there has been growing awareness about the possible side effects of transfusion [2,3]. Blood products contain inflammatory components including reactive oxygen species, foreign antigens and various pro-inflammatory micro-particles [4–7]. These inflammatory components may induce harmful transfusion reactions, such as allergic reactions, hemolysis and acute lung injury, especially in the critically ill [8,9]. This explains why restrictive transfusion strategies in the non-bleeding critically ill are safe and decrease exposure to RBC transfusion as compared to liberal transfusion practices[10–14].

There are no data available on transfusion practices specifically for bleeding critically ill patients. The majority of transfusion studies in bleeding patients were conducted in trauma patients. In general, trauma patients are a relatively healthy population with limited comorbidities. Therefore, this evidence might not be directly generalizable to bleeding, non-trauma, critically ill patients. Transfusion practice in bleeding patients is challenging, with multiple causes including coagulopathy, thrombocytopenia and can occur as a consequence of surgery. Coagulopathy can also be a consequence of bleeding. To control bleeding, patients often receive different types of blood products, many of which are delivered simultaneously.

This survey aims to assess the practice of caregivers towards transfusion practices in the bleeding critically ill patient, including transfusion thresholds, choices of blood products and diagnostic tests. We hypothesized that in this patient population a large heterogeneity exists between and within different subpopulations.

Methods

Survey

A questionnaire was distributed to physicians working in adult ICUs worldwide using an online platform (SurveyMonkey, Portland, OR, USA). This questionnaire was a follow-up of the first TRACE survey, which focused on non-bleeding critically ill patients [15]. This study was endorsed by the European Society of Intensive Care Medicine (ESICM) and by several national intensive care societies (Additional file 2).

Study design

During two focus group meetings with clinical experts on transfusion practices themes were identified and used to compile the questionnaire. The questionnaire was piloted with physicians working in different countries within Europe and Northern America.

In this survey, the use of different blood products including red blood cells (RBCs), platelet concentrates and plasma products in different subpopulations (e.g. trauma, obstetric, etc.) was explored. The survey included a maximum of 50 questions divided into 3 subsections: respondents' demographics (7 questions), transfusion practice in the massively bleeding patient (7-10 questions) and transfusion practices in the non-massively bleeding patient (33 questions, see Additional file 1 for static version). Massive bleeding was defined as having one or more of the following conditions: 1) a systolic blood pressure <90mmHg with bleeding + non-responsiveness to resuscitation therapy, 2) any case where a massive transfusion protocol (MTP) was initiated or 3) the administration of ≥4 blood products within 2 hours.

In non-massively bleeding patients, hemoglobin (Hb), platelet count and fibrinogen level thresholds were investigated for RBC transfusion, platelet transfusion and fibrinogen administration, respectively. The use of tranexamic acid (TXA) was examined in different subpopulations (i.e. trauma patients, obstetrics, gastroenterology).

Statistical analysis

Only completed surveys were included for analysis. A questionnaire was defined as complete when the respondents went through all question. Since, not all questions were applicable for all respondents, some questions were allowed to leave open.

Continuous data were assessed for distribution: normally distributed variables were described by mean (standard deviations) and non-parametric data by median [first quartiles- third quartile]. 10^{th} and 90^{th} percentiles were estimated by the largest observation less than or equal to Q3 + 1.5 x the interquartile range and the lowest observation or higher than Q1 – 1.5 x interquartile range, respectively.

Normal distributed variables were analyzed using Students t-test and analysis of variance (ANOVA). Non-parametric data were analyzed with Mann Whitney U-test or Kruskal-Wallis. The Dunn test with Bonferroni correction was used to assess the differences in applied transfusion thresholds between different subpopulations. Categorical variables were tested using the Chi-squared test with Yates correction for continuity and were described by frequencies and percentages. Data was analyzed using R statistics

(version 3.5.2) with the R Studio interface (The R Foundation, Lucent Technologies, Inc., Murray Hill, NJ, USA, www.r-project.org).

Results

Demographics

A total of 611 respondents participated in the survey of which 401 finished the complete survey and were thus included for analysis (Figure 1A). These respondents represented 64 countries of which the majority were high-income countries (72%, Figure 1B). The majority of the respondents were board-certified intensivists (84%) with a primary medical specialty in anesthesiology (61%) or internal medicine (19%). Participants worked in mixed ICUs (73%), surgical (16%) or medical ICUs (8%). Most participants worked at university hospitals (44%) or university affiliated hospitals (26%). An MTP was available in 52% of the respondents hospitals. The availability of a hospital-wide transfusion protocol and ICU-specific transfusion protocol was less common – 45% and 40% respectively. The demographics of survey respondents are displayed in Table 1.

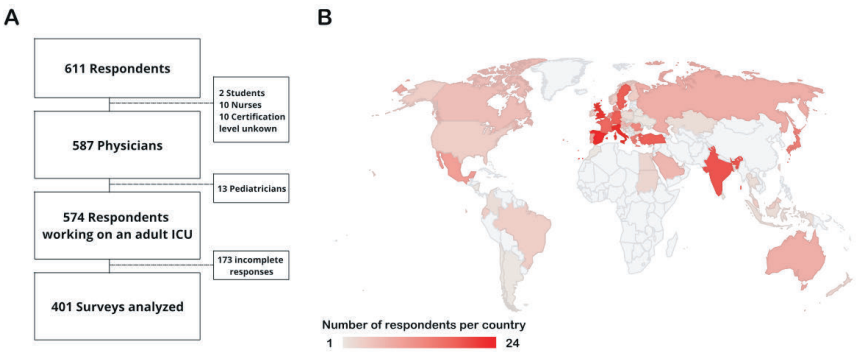

Figure 1. 611 respondents filled in the survey of which 401 were analyzed (panel A), representing 64 countries (panel B).

Table 1. Characteristics of the survey respondents

Demographic	No. of respondents(%)
Certification level	
Intensivist	337(84%)
Specialist non-intensivist practicing ICU	33(8%)
Resident, specialist in training	26(6%)
Other	5(1%)
Primary medical specialty	3(1%)
Anesthesiology	243(61%)
Internal medicine	78(19%)
Pulmonology	13(3%)
Surgery	9(2%)
Cardiology	7(2%)
Neurology	1(0%)
Other (please specify)	47(12%)
Type of ICU	
Medical ICU	33(8%)
Surgical ICU	64(16%)
Mixed ICU	294(73%)
Other	10(4%)
Number of ICU beds	
<10	95(24%)
10-15	124(31%)
16-20	64(16%)
>20	116(29%)
Type of institution	
University hospital	178(44%)
University affiliated hospital	104(26%)
Non-university public hospital	82(20%)
Private hospital	36(9%)
Other	1(0%)
Availability of transfusion guideline	
Hospital-wide transfusion protocol	180(45%)
ICU-specific transfusion protocol	159(40%)
Massive transfusion protocol	209(52%)
Unit used to measure hemoglobin	
g/dl	282(70%)
g/L (=mg/mL)	94(23%)
mmol/L	25(6%)
Economy	
High income	287(72%)
Lower middle income	33(8%)
Upper middle income	80(20%)

Massive bleeding

Product choice

Approximately half of the respondents (46%) used fixed blood product ratios (RBC: Plasma: PLT). Among these respondents, the 1:1:1 ratio was most often reported (33%) followed by 3:3:1 ratio (24%). During massive bleeding, the use of blood products was most often guided by viscoelastic testing (73%) and conventional laboratory-based testing (67%).

The use of fibrinogen and prothrombin complex concentrate (PCC) during massive bleeding was highly variable: fibrinogen was most often (36%) administered based on conventional laboratory-based tests or empirically followed by laboratory test guided additional fibrinogen administration (30%). Viscoelastic testing was used by 19% of the respondents and 11% administered fibrinogen only empirically.

Prothrombin complex concentrate administration was most often guided by conventional laboratory-based testing (39%) followed by viscoelastic testing (23%) and 21% stated they initially administered PCC empirically but titrated the following doses based on conventional laboratory results.

The majority (93%) of the respondents used TXA during massive bleeding. Among those respondents, it was usually administered empirically (89%), but 9% used viscoelastic tests to guide the administration of TXA. Large differences were observed between different subpopulations (Figure 2A). The subpopulations where most respondents would always administer TXA were

trauma patients (59%), followed by massively bleeding obstetric patients (40%). Few respondents would always administer TXA to septic patients (11%). In patients on extracorporeal membrane oxygenation (ECMO), respondents most often stated they would never use TXA for this patient population (35%). More data regarding massive bleeding is displayed in table 2.

Correcting iatrogenic coagulopathy during massive bleeding

The strategy to correct iatrogenic coagulopathy was dependent on the class of anticoagulant medication that was used. In patients with a vitamin K antagonist (VKA) induced coagulopathy (defined as an INR >1.5x reference value), most respondents would treat this by administering vitamin K (68%), PCC (78%) and plasma (61%). When the coagulopathy was direct oral anticoagulant (DOAC)-induced, respondents would

use PCC (68%), plasma (64%), Idarucizumab for dabigatran (48%), vitamin K (23%) or andexanet alpha for rivaroxaban or apixaban (21%).

Table 2.

	No. of respondents(%)
What kind of plasma do you use during massive transfusion?	
Pooled plasma (e.g. Omniplasma)	29(7%)
Fresh frozen plasma	370(92%)
Lyophilized plasma	12(3%)
What guides the choice of type of blood products prescribed to patients requiring massive transfusion?	
I use fixed ratios of blood products	184(46%)
Conventional lab based testing (e.g. International Normalized Ratio (INR), platelet count , fibrinogen, hemoglobin)	268(67%)
Point of care viscoelastic testing (Thromboelastography (TEG) or Thromboelastometry (ROTEM))	163(41%)
What ratio of blood products do you use during massive transfusion (one platelet concentrate = pooled product from 5 donors)	
1 : 1 : 1 (red blood cells : plasma : platelets concentrate)	60(15%)
3 : 3 : 1 (red blood cells : plasma : platelets concentrate)	45(11%)
6 : 6 : 1 (red blood cells : plasma : platelets concentrate)	19(5%)
6 : 3 : 1 (red blood cells : plasma : platelets concentrate)	23(6%)
Whole blood	2(0%)
Other	38(9%)
How do you correct a plasmatic coagulopathy (INRx1.5 reference value or prolonged clotting time with TEG or ROTEM) in critically ill patients with massive blood loss who used vitamin K antagonists?	
Vitamin K	274(68%)
Prothrombin complex (Cofact/Octoplex/Beriplex)	314(78%)
Plasma	246(61%)
Other	7(2%)
Nothing	3(1%)

Table 2. *(continued)*

	No. of respondents(%)
How do you correct a plasmatic coagulopathy in critically ill patients with massive blood loss who used direct oral anti-coagulants(DOACs)?	
Vitamin K	92(23%)
Prothrombin complex (Cofact/Octoplex/Beriplex)	273(68%)
Plasma	256(64%)
Recombinant factor VIIa (Novoseven/Eptacog alfa)	68(17%)
Idarucizumab (for dabigatran)	194(48%)
Andexanet (for rivaroxaban or apixaban)	84(21%)
Nothing	6(1%)
Other	28(7%)
What guides your use of fibrinogen in critically ill patients with massive bleeding?	
I administer fibrinogen after lab testing (fibrinogen level)	146(36%)
I administer fibrinogen after viscoelastic testing (TEG/ROTEM)	78(19%)
I empirically administer fibrinogen	43(11%)
I empirically administer fibrinogen, but start titrating when first lab results are available	121(30%)
Other	12(3%)
What guides your use of prothrombin complex (Cofact,Octoplex,Beriplex) in critically ill patients with massive bleeding.	
I administer prothrombin complex after lab testing (PT/INR)	157(39%)
I administer prothrombin complex after viscoelastic testing (TEG/ROTEM)	91(23%)
I empirically administer prothrombin complex	24(6%)
I empirically administer prothrombin complex, but start titrating when first lab results are available	85(21%)
Other (please specify)	40(10%)
Do you use tranexamic acid in critically ill patients with massive bleeding?	
Yes	374(93%)
No	26(6%)
What guides your use of tranexamic acid in critically ill patients with massive bleeding?	
I administer tranexamic acid after viscoelastic testing (TEG/ROTEM)	33(8%)
I empirically administer tranexamic acid	332(83%)
Other	9(2%)

The effect of an MTP on transfusion practice

Several differences were observed between respondents with and without an MTP available in their ICU (Additional file 3 table S1). The respondents with an MTP available were more often working in high income countries (162(80%) versus 119(62%), p<0.001). When an MTP was available, more often fixed ratios were used (120(57%)) than when no MTP was available (64(33%), p<0.001). In addition, when fixed ratios were used, most often the 1:1:1 ratio was used, while in the absence of the MTP, a wide range of ratios employed were reported. Tranexamic acid was more often used if an MTP was available (96% versus 90%, p=0.019).

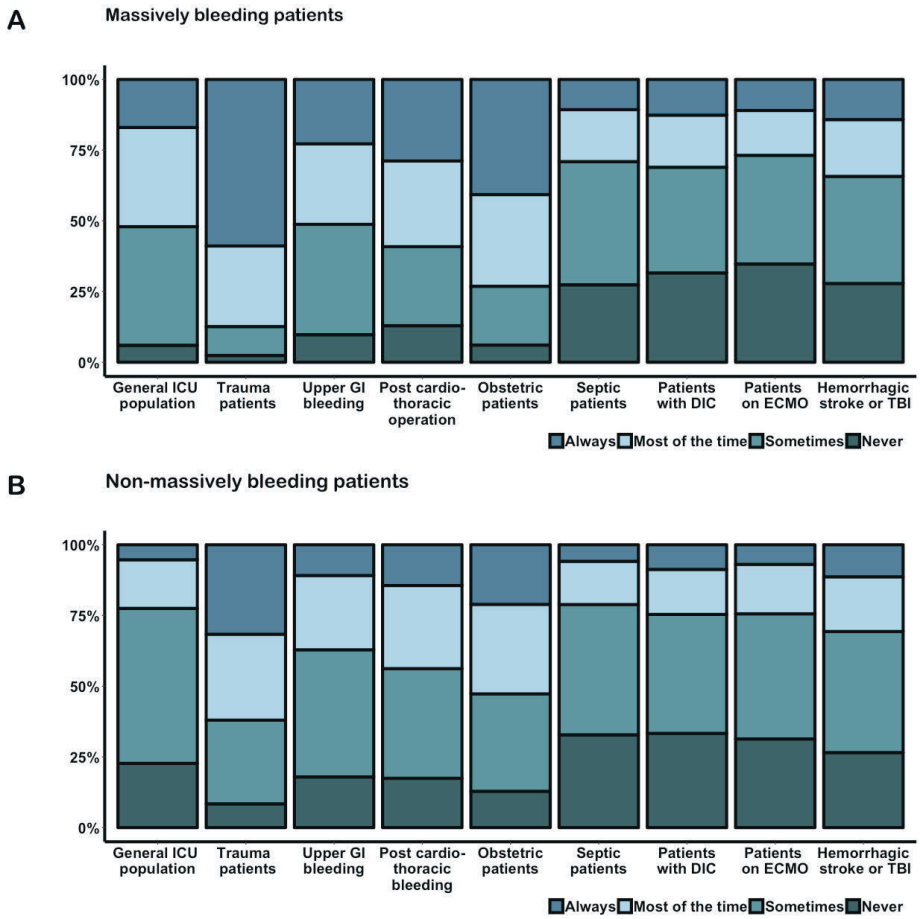

Figure 2. The use of tranexamic acid (TXA) in massively (panel A) and non-massively (panel B) bleeding patients on the ICU.

Non-massively bleeding patients

Red cell transfusion

Respondents used different thresholds in different non-massively bleeding subpopulations (Figure 3). For the general ICU population, a Hb threshold of 7.0[7 – 7.3] g/dL was used. This was significantly lower than for all other specified subpopulations (Figure 3A). For patients admitted with upper gastrointestinal (GI) bleeding, obstetric complications and sepsis, the reported RBC threshold was 7[7 – 8] g/dL. The highest RBC thresholds were reported for post-cardiothoracic surgery patients 8[7.9-9] g/dL. The highest variability was observed for patients on ECMO and

patients with stroke and/or TBI: 7[7-9] g/dL. In patients with TBI and those post-cardiothoracic surgery, 32% of the respondents would transfuse at a Hb level of 9 g/dL or higher. In the general population, 3.5% would transfuse at a Hb level of 9 g/dL or higher. No consistent differences were observed between world regions (Additional fileal Figure S2-S7).

34% and 40% of respondents respectively reported always or most of the time checking the Hb level before administering additional RBC units. This was never checked by 8% of the respondents and sometimes by 18%. Whether the respondents would check the Hb in between transfusions did not correlate with the transfusion thresholds in any of the subpopulations (Additional file 4 figure S1).

Platelet transfusion

The applied platelet threshold for the general non-massively bleeding ICU population was 50 [20-50] x 10^9/L (Figure 3B). This was similar in septic patients and patients with disseminated intravascular coagulation (DIC, p=1). Significantly higher thresholds ($p<0.001$) were reported in several other bleeding subpopulations including patients with upper gastrointestinal bleeding (50 [50-62] x 10^9/L), obstetric complications (50 [50-70] x 10^9/L), after cardio-thoracic surgery (50 [50-80] x 10^9/L), ECMO (50 [48-80] x 10^9/L) and with a hemorrhagic stroke or traumatic brain injury (75 [50-100] x 10^9/L, Figure 3B). Patients with hemorrhagic stroke or traumatic brain injury were transfused at the highest platelet count, and 31.2% of the respondents would transfuse this population to platelet levels of 100 x 10^9/L or higher. Also, in patients receiving anti-platelet therapy, a high variance in the platelet threshold utilized was observed (50[50-100] x 10^9/L). In these patients, 27% of the respondents would transfuse to platelet levels of 100 x 10^9/L or higher. No consistent differences were observed between world regions (Additional file 4 Figure S2-S7).

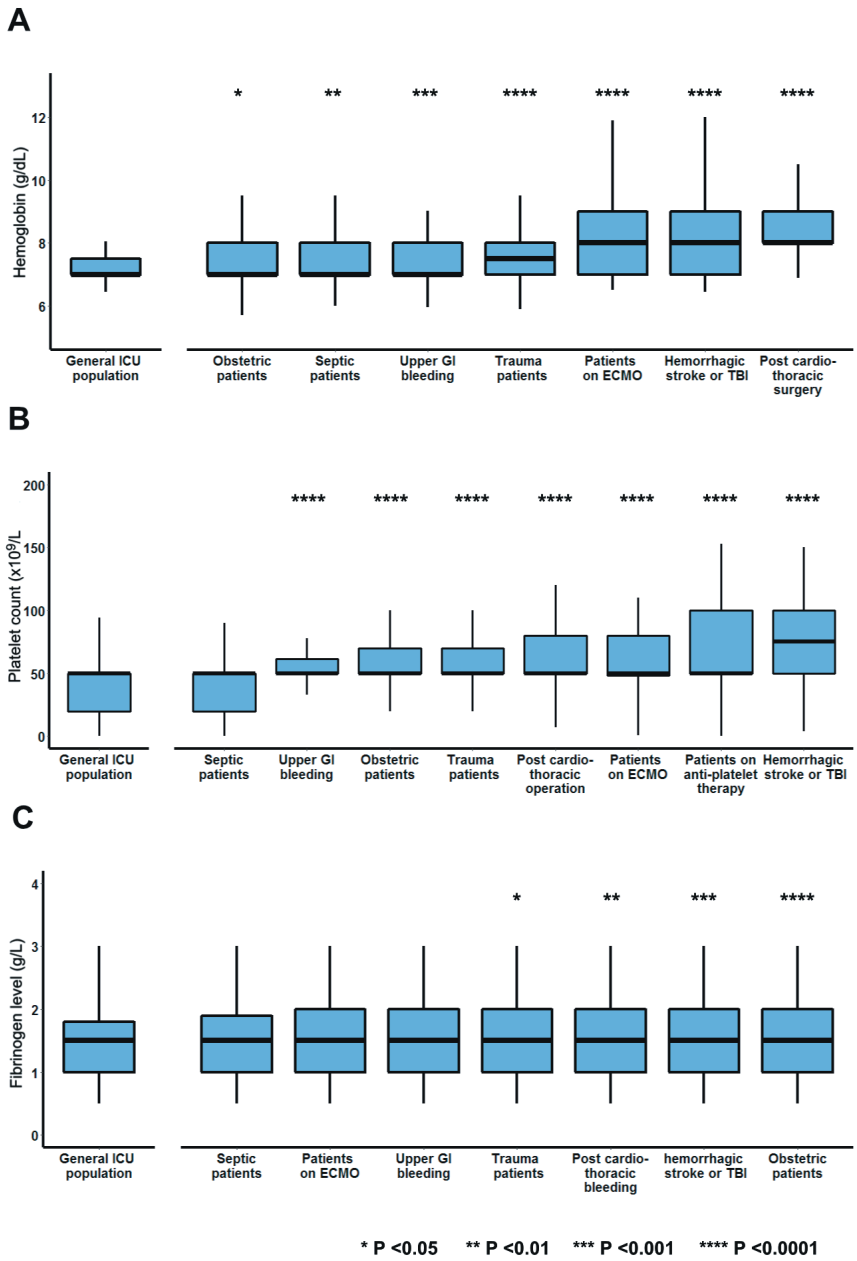

Figure 3. Respondents were asked to report for the general bleeding ICU population and several subpopulations their Hb threshold (panel A), platelet count threshold (panel B) and fibrin threshold (panel C) for RBC transfusion, platelet transfusion and fibrin administration respectively. Subpopulations were compared with the general ICU population using the Dunn test with Bonferroni correction.

Of the respondents, 67% reported that they always checked the platelet count before transfusing a second unit of platelets. Furthermore, 13% reported doing this most of the time, and 13% only sometimes. Respondents who only sometimes or never checked the platelet count transfused at higher platelet counts in patients after cardiothoracic surgery (p=0.044, Additional file 4 Figure S1).

Coagulation supportive therapy

Fibrinogen administration was triggered at a level of 1.5[1-1.8] g/L in the general ICU population for non-massive bleeding. The differences in fibrinogen thresholds used in other subpopulations were small, but statistically significant. Trauma patients, obstetric patients, patients on ECMO, upper GI bleeding cases, post-cardiothoracic surgery patients and patients with traumatic brain injury would receive fibrinogen at a threshold of 1.5[1-2] g/L and in patients with sepsis, fibrinogen would be administered at a fibrinogen level of 1.5[1-1.9] g/L (Figure 3C).

The use of TXA differed between subpopulations. It was most often considered in trauma followed by obstetric patients (Figure 2B). TXA was mostly administered empirically in non-massively bleeding patients (68%), whereas some respondents (24.4%) performed viscoelastic testing before administering TXA.

Most respondents reported that they use the INR or PT to decide whether a non-massively bleeding patient could benefit from a plasma transfusion (88%), followed by activated partial thromboplastin time (aPTT, 59%), fibrinogen level (48%) and viscoelastic testing (42%). An INR of 2 (IQR=1.6 – 2.5) was used as the threshold for plasma transfusion. Of the respondents, 24% and 31.9% respectively reported that they always or most of the time checked the INR, PT or the viscoelastic test again before transfusing a second unit of plasma. 23% and 20% checked these tests sometimes or never.

Effect of respondents' primary specialty on transfusion practices during non-massive bleeding

The primary specialties of anesthesiology and internal medicine were sufficiently powered to test the effect of specialty on transfusion practice. For RBC transfusion, only for patients with traumatic brain injury or a hemorrhagic stroke was a small difference seen. Anesthesiologists would transfuse at a higher Hb level of 8[7.4-9] g/dL versus internists 8[7-9] g/dL (p=0.044). (See Additional file 3 table S2). For platelet transfusion, significant differences were observed in more patient categories (Additional file 3 table S2). In cardiothoracic surgery, obstetric complications, septic patients and those who recently used anti-platelet drugs, anesthesiologists would transfuse at higher platelet

levels. For fibrinogen administration, no association was found between the primary specialty and the reported fibrinogen threshold.

Effect of having transfusion guidelines during non-massive bleeding

The effect of a hospital-wide and an ICU-specific transfusion protocol was assessed for bleeding critically ill patients. When a hospital-wide transfusion protocol was available, lower platelet transfusion thresholds were applied to patients with upper GI bleeding and in post-cardiothoracic surgery patients. A hospital-wide transfusion protocol did not affect the thresholds for RBC transfusion and fibrinogen administration (Additional file 3 table S3). The availability of an ICU-specific transfusion protocol only showed an effect on the RBC transfusion threshold in ECMO patients (Additional file 3 table S4). When this protocol was available, ECMO patients were transfused at lower Hb levels (p=0.026).

Each boxplot represents the medians with first and third quartile. The upper and lower whiskers are estimates of the 10th and 90 the percentile, respectively.

Viscoelastic tests

The majority of the respondents reported the use of viscoelastic tests to guide the blood product choice (RBC, plasma and platelet concentrates) during massive hemorrhage (73%). However, only 23% reported using viscoelastic tests to guide the use of PCC and 19% to guide the use of fibrinogen. In the decision-making process for the administration of TXA during massive bleeding, 8% reported using viscoelastic tests to guide its use. This is significantly lower ($p<0.001$) than in non-massively bleeding patients, where 24% reported using viscoelastic tests prior to TXA administration. When deciding to transfuse non-massively bleeding ICU patients with plasma, 42% reported using viscoelastic tests. The use of viscoelastic tests during non-massive bleeding for administration of other blood products was not studied in this survey.

Discussion

This is the first international survey among ICU physicians assessing transfusion practices in bleeding critically ill patients. The main findings of this study were: (1) half of the respondents did not have an ICU specific transfusion protocol available at their ICU; (2) the presence of an MTP was correlated with the use of fixed transfusion ratios during massive bleeding; (3) a high variation in practice in the use of diagnostic tests, transfusion ratios, fibrinogen, TXA and PCC in the setting of hemorrhage; (4) during non-massive bleeding, a high variability in platelet and RBC transfusion thresholds within and

between different subpopulations; and (5) plasma was still often administered for VKA induced coagulopathy during massive bleeding.

In general, this survey showed that the majority of the respondents did not use fixed transfusion ratios in the ICU – only 46% would consider this during massive bleeding. The 1:1:1 ratio was most commonly reported (33%). The use of this ratio is controversial as no beneficial effect on mortality in trauma patients was observed in a large RCT[16]. In addition, the potential harm of a high FFP ratio in an ICU setting was reported in a retrospective study, where a high plasma:RBC ratio was associated with increased mortality in patients in general surgery and medicine[17].

Tranexamic acid use in the ICU differed significantly across all subpopulations. Overall, trauma and obstetric patients most often received TXA in the ICU during bleeding as compared to the general ICU population. We speculate that the rationale behind this is that both obstetric and trauma patients have relatively fewer comorbidities compared to the other subpopulations and the benefit of early TXA administration was proven in these patients in a non-ICU setting: the CRASH-2 Trial[18] showed reduced mortality in trauma patients in the emergency room and in the WOMAN-trial, early TXA administration in women with post-partum hemorrhage decreased mortality due to bleeding[19]. In contrast, a recent study showed that in patients with upper GI bleeding, the use of high dose TXA did not result in a reduction in mortality[20]. In this survey, half of the respondents reported that they would administer TXA always or most of the time during massive upper GI bleeding. However, it should be mentioned that the abovementioned study was published after closing this survey. Therefore, the results on the use of TXA in this specific patient group may already be obsolete.

In the general ICU populations and several subpopulations, including septic, obstetric, trauma and patients with upper GI bleeding, a relatively restrictive RBC transfusion strategy was reported, with a median Hb threshold of 7-7.5 g/dL. This is in accordance with several large RCT's comparing liberal and restrictive transfusion strategies [12,13]. The highest Hb thresholds in this survey were reported for bleeding patients after cardiothoracic surgery 8[7.9-9] g/dL and bleeding patients supported with ECMO 8[7-9] g/dL. This is in contrast to multiple RCTs showing that a liberal transfusion strategy was not superior to a restrictive transfusion strategy after cardiothoracic surgery[11,22,23]. In our previous survey, there were also significantly higher Hb thresholds reported in patients with acute coronary syndrome compared to the general ICU population (9[8-9.7] g/dL versus 7[7-7.5] g/dL) [15]. Physicians might associate cardiothoracic surgery with an increased risk of coronary syndrome, which is an indication to consider higher Hb

thresholds in several guidelines [24,25]. The high variety in Hb thresholds in ECMO patients is not surprising, as this was reported earlier[15,26]. As long as no randomized studies are performed in patients receiving ECMO, the optimal Hb trigger in ECMO patients will remain a matter of debate, thus explaining the heterogeneity in the Hb thresholds applied to transfuse these patients.

Despite limited evidence in the ICU, a large proportion of respondents were using viscoelastic tests to guide the choice of blood products during massive bleeding (73.3%). But when deciding to administer fibrinogen or PCC, the number of respondents who use viscoelastic tests was lower: 19% and 23% respectively. In this survey, the use of viscoelastic tests during non-massive bleeding to guide platelet and plasma transfusion was not assessed. However, viscoelastic testing did play a role in the use of TXA during non-massive bleeding, as 24% of respondents used viscoelastic tests to assess whether a patient would benefit from TXA administration. None of these indications have been studied yet in the ICU setting, but there may be potential to reduce the amount of transfusion and thereby the exposure to the potential harmful side effects of blood products[27].

In this survey, 78% would correct a VKA induced coagulopathy with PCC in massively bleeding patients. However, 61% of the respondents also reported that they considered using plasma for this indication, although no evidence is available to support this practice. Multiple RCTs have shown the superiority of PCC versus plasma for VKA reversal in patients with major bleeding or for patients prior to urgent surgical procedures[28,29]. Since plasma transfusion has several disadvantages including slower infusion rate, risk of transfusion reactions and risk of fluid overload[30], we expected a smaller number of respondents administering plasma for iatrogenic coagulopathy. Therefore, we conclude that the use of plasma could be safely reduced by evidence-based transfusion guidelines.

This study has several limitations. First, due to the nature of the design of the study, the survey reflects the perceived practice of respondents. Actual practice may still differ from the responses given in the survey. Second, as it is unknown who the non-responders were, we cannot estimate the effect of this participation bias. Physicians with more interest in blood transfusion might be more likely to fill in this survey, and this group of physicians is likely to be more aware of the latest literature on transfusion practices. Third, to avoid a too long survey, we did not question the use of cryoprecipitate. Since the majority of the respondents works in countries where fibrinogen is used, we believe this did not influence our results. Fourth, the majority of our respondents are working in

high income countries, therefore are our findings mainly generalizable to high resource settings. Finally, the definition of massive bleeding is currently still under debate. We used a broad definition of massive bleeding, however respondents may have used their own personal definitions for this term.

Conclusion

In conclusion, we observed a high variety in transfusion practice among intensive care physicians and a lack of guidelines for the management of bleeding critically ill patients. The presence of a massive transfusion protocol influenced transfusion practices. Current transfusion practice was influenced by large transfusion studies in trauma patients. However, since these studies might not be completely applicable to all critically ill patients, more research specifically into the management of bleeding critically ill patients is warranted.

List of abbreviations

ANOVA	analysis of variance
DOAC	direct oral anticoagulant
ECMO	extracorporeal membrane oxygenation
GI	gastro-intestinal
Hb	hemoglobin
ICU	intensive care unit
MTP	massive transfusion protocol
PCC	prothrombin complex concentrate
RBC(s)	red blood cell(s)
tbi	traumatic brain injury
TXA	tranexamic acid
VKA	vitamin K antagonist

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Supplement 1: Static version online survey

Demographics:

1. **In which country do you work?**

2. **What is your intensive care certification level?**
   a. Intensivist
   b. Resident, specialist in training
   c. Specialist non intensivist practicing ICU
   d. Nurse
   e. Student
   f. Other, please specify

3. **What is your primary medical speciality?**
   a. Anesthesiology
   b. Cardiology
   c. Internal medicine
   d. Neurology
   e. Pediatrics
   f. Pulmonology
   g. Surgery
   h. Other, please specify

4. **Type of intensive care unit (ICU)**
   a. Medical ICU
   b. Surgical ICU
   c. Mixed ICU
   d. Other, please specify

5. **Number of ICU beds**
   a. <10
   b. 10-15
   c. 16-20
   d. >20

6. **Type of institution**
   a. University hospital
   b. University affiliated hospital
   c. Non-university public hospital
   d. Private hospital
   e. Other, please specify

7. **What kind of transfusion protocol do you have in your hospital? (multiple answers possible)**
   a. Hospital-wide transfusion protocol
   b. ICU-specific transfusion protocol
   c. Massive transfusion protocol
   d. Other, please specify

Massive transfusion

The following questions concern ICU patients that have massive blood loss.

Massive blood loss is defined as:
Systolic blood pressure <90mmHg with bleeding + non-responsive to resuscitation therapy
OR ≥4 products within 2 hours
OR When a massive transfusion protocol (MTP) is initiated

8. **What kind of plasma do you use during massive transfusion? (multiple answers possible)**
   a. Pooled plasma (e.g. Omniplasma)
   b. Fresh frozen plasma
   c. Lyophilized plasma

9. **What guides the choice of type of blood products prescribed to patients with requiring massive transfusion? (multiple answers possible)**
   a. I use fixed ratios of blood products

b. Conventional lab based testing (e.g. INR, platelet count, fibrinogen, hemoglobin)
c. Point of care viscoelastic testing (TEG/ROTEM)

10. What ratio of blood products do you use in your massive transfusion protocol? (one platelet concentrate = pooled product from 5 donors)
a. 1 : 1 : 1 (red blood cells : plasma : platelets concentrate)
b. 3 : 3 : 1 (red blood cells : plasma : platelets concentrate)
c. 6 : 6 : 1 (red blood cells : plasma : platelets concentrate)
d. 6 : 3 : 1 (red blood cells : plasma : platelets concentrate)
e. Whole blood
f. Other, please specify

11. How do you correct a plasmatic coagulopathy (International Normalized Ratio > x1.5 reference value or prolonged cloth time with TEG/ROTEM) in critically ill patients with massive blood loss in the ICU who used vitamin K antagonists? (multiple answers possible)
a. Vitamin K
b. Prothrombin complex (Cofact/Octoplex/Beriplex)
c. Plasma
d. Nothing
e. Other, please specify

12. How do you correct a plasmatic coagulopathy in critically ill patients with massive blood loss in the ICU who used direct oral anti-coagulants (DOAC's)? (multiple answers possible)
a. Vitamin K
b. Prothrombin complex (Cofact/Octoplex/Beriplex)
c. Plasma
d. Recombinant factor VIIa
e. Idarucizumab (for dabigatran)
f. Andexanet (for rivaroxaban or apixaban)
g. Nothing
h. Other, please specify

13. What guides your use of fibrinogen in critically ill patients with massive bleeding? (multiple answers possible)
a. I empirically administer fibrinogen
b. I empirically administer fibrinogen, but start titrating when first lab results are available
c. I administer fibrinogen after lab testing (fibrinogen level)
d. I administer fibrinogen after viscoelastic testing (TEG/ROTEM)
e. Other, please specify

14. What guides your use of prothrombin complex (Cofact/Octoplex/Beriplex) in critically ill patients with massive bleeding? (multiple answers possible)
a. I empirically administer prothrombin complex
b. I empirically administer prothrombin complex, but start titrating when first lab results are available
c. I administer prothrombin complex after lab testing (INR/PT)
d. I administer prothrombin complex after viscoelastic testing (TEG/ROTEM)
e. Other, please specify

15. Do you use tranexamic acid in critically ill patients with massive bleeding?
a. Yes
b. No

16. What guides your use of tranexamic acid in critically ill patients with massive bleeding? (multiple answers possible)
a. I empirically administer tranexamic acid
b. I administer tranexamic acid after viscoelastic testing (TEG/ROTEM)
c. Other, please specify

17. Do you give tranexamic-acid in massive bleeding critically ill patients who are considered...

	Always	Most of the time	Sometimes	Never
A general bleeding ICU population				
Traumatic bleeding				
Upper gastrointestinal tract bleeding				
Post-cardiothoracic surgery bleeding				
Obstetric bleeding				
Sepsis + bleeding				
Disseminated intravascular coagulation + bleeding				
Extracorporeal membrane oxygenation + bleeding				
Hemorrhagic stroke and/or traumatic brain injury + bleeding				

Red blood cell transfusion

The following questions concern ICU patients that have non-massive blood loss.

18. Which unit do you use to measure hemoglobin levels?
- a. g/dl
- b. g/L (=mg/ml)
- c. mmol/L

What is your threshold for red blood cell concentrate (RBC) transfusion in...

19. ... a general population of non-massively bleeding critically ill patients?
20. ... traumatic non-massively bleeding critically ill patients?
21. ... upper gastrointestinal-tract non-massively bleeding critically ill patients?
22. ... post-cardiothoracic surgery non-massively bleeding critically ill patients?
23. ... obstetric non-massively bleeding critically ill patients?
24. ... septic non-massively bleeding critically ill patients?
25. ... non-massively bleeding critically ill patients on extracorporeal membrane oxygenation?
26. ... non-massively bleeding critically ill patients suffering from a hemorrhagic stroke and/or traumatic brain injury?

27. Do you check hemoglobin levels before transfusing a second unit of RBC in non-massively bleeding critically ill patients in the ICU?
- a. Always
- b. Most of the time
- c. Sometimes
- d. Never

Platelets

What is your threshold for platelet transfusion in…

28. **… a general population of non-massively bleeding critically ill patients? (10^9 cells/L)**
29. **… traumatic non-massively bleeding critically ill patients? (10^9 cells/L)**
30. **… upper gastrointestinal-tract non-massively bleeding critically ill patients? (10^9 cells/L)**
31. **… post-cardiothoracic surgery non-massively bleeding critically ill patients? (10^9 cells/L)**
32. **… obstetric non-massively bleeding critically ill patients? (10^9 cells/L)**
33. **… non-massively bleeding critically ill patients suffering from sepsis and/or disseminated intravascular coagulation? (10^9 cells/L)**
34. **… non-massively bleeding critically ill patients on extracorporeal membrane oxygenation? (10^9 cells/L)**
35. **… non-massively bleeding critically ill patients suffering from a hemorrhagic stroke and/or traumatic brain injury? (10^9 cells/L)**
36. **… non-massively bleeding critically ill patients receiving anti-platelet therapy (e.g. acetylsalicylic acid, dipyridamol, clopicogrel, ticagrelor)? (10^9 cells/L)**

37. **Do you check platelet count after transfusion of one unit thrombocyte concentrate before transfusing a second unit in non-massive bleeding critically ill patients in the ICU?**
    a. Always
    b. Most of the time
    c. Sometimes
    d. Never

Plasma

38. **Which coagulation test do you use in order to decide whether a non-massively bleeding critically ill patient could benefit from a plasma transfusion? (multiple answers possible)**
    a. PT/INR
    b. aPTT
    c. Fibrinogen
    d. Rotational thromboelastometry (ROTEM)
    e. Thromboelastography (TEG)
    f. Other, please specify

39. **From which INR-value would you consider transfusing plasma to a general population of non-massively bleeding critically ill patients?**

40. **Do you check the PT/INR or perform TEG/ROTEM before transfusing a second unit of plasma in a non-massively bleeding critically ill patient with plasmatic coagulopathy?**
    a. Always
    b. Most of the time
    c. Sometimes
    d. Never

Coagulation products

What is your threshold for administering fibrinogen in…

41. **… a general population of non-massively bleeding critically ill patients? (10^9 cells/L)**
42. **… traumatic non-massively bleeding critically ill patients? (10^9 cells/L)**
43. **… upper gastrointestinal-tract non-massively bleeding critically ill patients? (10^9 cells/L)**
44. **… post-cardiothoracic surgery non-massively bleeding critically ill patients? (10^9 cells/L)**
45. **… obstetric non-massively bleeding critically ill patients? (10^9 cells/L)**
46. **… non-massively bleeding critically ill patients suffering from sepsis and/or disseminated intravascular coagulation? (10^9 cells/L)**
47. **… non-massively bleeding critically ill patients on extracorporeal membrane oxygenation? (10^9 cells/L)**
48. **… non-massively bleeding critically ill patients suffering from a hemorrhagic stroke and/or traumatic brain injury? (10^9 cells/L)**

49. **What guides your use of tranexamic acid (TXA) in critically ill patients with non-massive bleeding?**
    a. I empirically administer TXA
    b. I administer TXA after viscoelastic testing (TEG/ROTEM)
    c. Other (please specify)

Tranexamic-acid

50. **Do you give tranexamic-acid in non-massively bleeding critically ill patients who are considered…**

	Always	Most of the time	Sometimes	Never
A general bleeding ICU population				
Traumatic bleeding				
Upper gastrointestinal tract bleeding				
Post-cardiothoracic surgery bleeding				
Obstetric bleeding				
Sepsis + bleeding				
Disseminated intravascular coagulation + bleeding				
Extracorporeal membrane oxygenation + bleeding				
Hemorrhagic stroke and/or traumatic brain injury + bleeding				

Supplement 2: Overview of national intensive care societies endorsing the TRACE2-survey

Canada	Canadian Critical Care Society (CCCS)
France	Société Française d'Anesthésie et de Réanimation (SFAR)
Greece	Hellenic Society of Intensive Care (HSIC)
Japan	Japanese Society of Intensive Care Medicine (JSICM)
Lebanon	Lebanese Critical Care Society (LCCS)
Taiwan	Society of Emergency & Critical Care Medicine, Taiwan, R.O.C.(SECCM)
Turkey	Turkish Society of Medical and Surgical Intensive Care Medicine (TSMSICM)
United Kingdom	Intensive Care Society (ICS)

Additional file 3: Supplemental tables

Table S1. Difference between respondents with and without a massive transfusion protocol

	Massive transfusion protocol (N= 209)	No massive transfusion protocol (N= 192)	P-value
What kind of plasma do you use during massive transfusion?			
Pooled plasma (e.g. Omniplasma)	19(9%)	10(5%)	0.191
Fresh frozen plasma	196(94%)	174(91%)	0.32
Lyophilized plasma	7(3%)	5(3%)	0.885
What guides the choice of type of blood products prescribed to patients requiring massive transfusion?			
I use fixed ratios of blood products	120(57%)	64(33%)	p<0.001
Conventional lab based testing (e.g. International Normalized Ratio (INR), platelet count , fibrinogen, hemoglobin)	133(64%)	135(70%)	0.189
Point of care viscoelastic testing (Thromboelastography (TEG) or Thromboelastometry (ROTEM))	101(48%)	62(32%)	0.002
What ratio of blood products do you use during massive transfusion (one platelet concentrate = pooled product from 5 donors)			
1 : 1 : 1 (red blood cells : plasma : platelets concentrate)	43(21%)	17(9%)	0.023
3 : 3 : 1 (red blood cells : plasma : platelets concentrate)	29(14%)	16(8%)	
6 : 6 : 1 (red blood cells : plasma : platelets concentrate)	11(5%)	8(4%)	

Table S1. Difference between respondents with and without a massive transfusion protocol *(continued)*

	Massive transfusion protocol (N= 209)	No massive transfusion protocol (N= 192)	P-value
6 : 3 : 1 (red blood cells : plasma : platelets concentrate)	10(5%)	13(7%)	
Other (please specify)	29(14%)	9(5%)	
Whole blood	0(0%)	2(1%)	
How do you correct a plasmatic coagulopathy (INRx1.5 reference value or prolonged clotting time with TEG or ROTEM) in critically ill patients with massive blood loss who used vitamin K antagonists?			
Vitamin K	144(69%)	130(68%)	0.882
Prothrombin complex (Cofact/Octoplex/Beriplex)	178(85%)	136(71%)	0.001
Plasma	114(55%)	132(69%)	0.005
Nothing	1(0%)	2(1%)	0.941
How do you correct a plasmatic coagulopathy in critically ill patients with massive blood loss who used direct oral anti-coagulants(DOACs)?			
Vitamin K	45(22%)	47(24%)	0.56
Prothrombin complex (Cofact/Octoplex/Beriplex)	155(74%)	118(61%)	0.009
Plasma	119(57%)	137(71%)	0.004
Recombinant factor VIIa (Novoseven/Eptacog Œ±)	32(15%)	36(19%)	0.433
Idarucizumab (for dabigatran)	119(57%)	75(39%)	0.001
Andexanet (for rivaroxaban or apixaban)	51(24%)	33(17%)	0.099

Table S1. Difference between respondents with and without a massive transfusion protocol *(continued)*

	Massive transfusion protocol (N= 209)	No massive transfusion protocol (N= 192)	P-value
Nothing	3(1%)	3(2%)	1
What guides your use of fibrinogen in critically ill patients with massive bleeding?			
I administer fibrinogen after lab testing (fibrinogen level)	67(32%)	79(41%)	0.331
I administer fibrinogen after viscoelastic testing (TEG/ROTEM)	41(20%)	37(19%)	
I empirically administer fibrinogen	26(12%)	17(9%)	
I empirically administer fibrinogen, but start titrating when first lab results are available	69(33%)	52(27%)	
Other	6(3%)	6(3%)	
What guides your use of prothrombin complex (Cofact, Octoplex,Beriplex) in critically ill patients with massive bleeding.			
I administer prothrombin complex after lab testing (PT/INR)	77(37%)	80(42%)	0.494
I administer prothrombin complex after viscoelastic testing (TEG/ROTEM)	54(26%)	37(19%)	
I empirically administer prothrombin complex	14(7%)	10(5%)	
I empirically administer prothrombin complex, but start titrating when first lab results are available	44(21%)	41(21%)	

Table S1. Difference between respondents with and without a massive transfusion protocol *(continued)*

	Massive transfusion protocol (N= 209)	No massive transfusion protocol (N= 192)	P-value
Other	19(9%)	21(11%)	
Do you use tranexamic acid in critically ill patients with massive bleeding?			
Yes	201(96%)	173(90%)	0.019
No	7(3%)	19(10%)	
What guides your use of tranexamic acid in critically ill patients with massive bleeding?			
I administer tranexamic acid after viscoelastic testing (TEG/ROTEM)	18(9%)	15(8%)	0.040
I empirically administer TXA	180(86%)	152(79%)	
Other	4(2%)	5(3%)	
Primary medical specialty			
Anesthesiology	127(61%)	116(60%)	0.025
Cardiology	1(0%)	6(3%)	
Internal medicine	41(20%)	37(19%)	
Neurology	1(0%)	0(0%)	
Other (please specify)	31(15%)	16(8%)	
Pulmology	2(1%)	11(6%)	

Table S1. Difference between respondents with and without a massive transfusion protocol *(continued)*

	Massive transfusion protocol (N= 209)	No massive transfusion protocol (N= 192)	P-value
Surgery	5(2%)	4(2%)	
Certification level			
Intensivist	176(84%)	161(84%)	0.451
Specialist non-intensivist practising ICU	18(9%)	15(8%)	
Resident, specialist in training	11(5%)	15(8%)	
Other	4(2%)	1(1%)	
Type of ICU			
Mixed ICU	159(76%)	135(70%)	0.011
Medical ICU	8(4%)	25(13%)	
Surgical ICU	36(17%)	28(15%)	
Other (please specify)	4(2%)	4(2%)	
Number of ICU beds			
<10	51(24%)	44(23%)	0.143
10-15	53(25%)	71(37%)	
16-20	36(17%)	28(15%)	
>20	68(33%)	48(25%)	

Table S1. Difference between respondents with and without a massive transfusion protocol *(continued)*

	Massive transfusion protocol (N= 209)	No massive transfusion protocol (N= 192)	P-value
Type of institution			
University hospital	100(48%)	78(41%)	0.231
University affiliated hospital	56(27%)	48(25%)	
Non-university public hospital	39(19%)	43(22%)	
Private hospital	14(7%)	22(11%)	
Other (please specify)	0(0%)	1(1%)	
Availability of transfusion guideline			
Hospital-wide transfusion protocol	89(43%)	91(47%)	0.386
ICU-specific transfusion protocol	73(35%)	86(45%)	0.056

Table S2. Difference between respondents with anesthesiology and internal medicine as base specialty

	Anesthesiology (N= 243)	Internal medicine (N= 78)	P-value
Hemoglobin threshold in different subpopulations			
General ICU population	7[7-7.5]	7[7-7]	0.17
Trauma patients	7.6[7-8]	7.2[7-8]	0.619
Upper gastro-intestinal bleeding	7[7-8]	7[7-8]	0.2
Post cardiothoracic surgery	8[8-9]	8[7.4-9]	0.217
Obstetric patients	7[7-8]	7[7-8]	0.941
Patients with Sepsis	7[7-8]	7[7-7.5]	0.076
Patients on ECMO	8[7-9]	8[7-9]	0.842

Table S2. Difference between respondents with anesthesiology and internal medicine as base specialty *(continued)*

	Anesthesiology (N= 243)	Internal medicine (N= 78)	P-value
Patients with TBI or stroke	8[7.4-9]	8[7-9]	0.044
Platelet count thresholds in different subpopulations			
General ICU population	50[20-50]	50[20-50]	0.099
Trauma patients	50[50-79]	50[50-51]	0.087
Upper gastro-intestinal bleeding	50[50-72]	50[50-50]	0.136
Post cardiothoracic surgery	60[50-90]	50[50-70]	0.008
Obstetric patients	50[50-80]	50[50-50]	0.016
Patients with Sepsis	50[21-50]	50[20-50]	0.042
Patients on ECMO	50[49-80]	50[43-77]	0.854
Patients with TBI or stroke	80[50-100]	70[50-100]	0.06
value.anti_platelet	55[50-100]	50[50-76.25]	0.034
Fibrin thresholds in different subpopulations			
General ICU population	1.5[1-1.8]	1.5[1-1.5]	0.685
Trauma patients	1.5[1-2]	1.5[1-2]	0.111
Upper gastro-intestinal bleeding	1.5[1-2]	1.5[1-2]	0.326
Post cardiothoracic surgery	1.5[1-2]	1.5[1-1.7]	0.155
Obstetric patients	1.5[1.2-2]	1.5[1-1.5]	0.005
Patients with Sepsis	1.5[1-2]	1.5[1-1.5]	0.167
Patients on ECMO	1.5[1-2]	1.5[1-1.5]	0.051
Patients with TBI or stroke	1.5[1.2-2]	1.5[1-2]	0.277
Certification level			0.372
Intensivist	199(82%)	70(90%)	
Specialist non-intensivist practising ICU	25(10%)	4(5%)	
Resident, specialist in training	17(7%)	4(5%)	

Table S2. Difference between respondents with anesthesiology and internal medicine as base specialty *(continued)*

	Anesthesiology (N= 243)	Internal medicine (N= 78)	P-value
Other	2(1%)	0(0%)	
Type of ICU			p<0.001
Medical ICU	7(3%)	18(23%)	
Surgical ICU	58(24%)	3(4%)	
Mixed ICU	171(70%)	56(72%)	
Other	5(2%)	1(1%)	
Number of ICU beds			0.906
<10	59(24%)	17(22%)	
10-15	70(29%)	24(31%)	
16-20	43(18%)	13(17%)	
>20	69(28%)	24(31%)	
Type of institution			0.081
University hospital	110(45%)	29(37%)	
University affiliated hospital	64(26%)	26(33%)	
Non-university public hospital	50(21%)	11(14%)	
Private hospital	19(8%)	12(15%)	
Availability of transfusion guideline			
Hospital-wide transfusion protocol	110(45%)	32(41%)	0.514
ICU-specific transfusion protocol	89(37%)	37(47%)	0.107
Massive transfusion protocol	127(52%)	41(53%)	1

Table S3. Difference between respondents with and without a hospital wide transfusion protocol

	No hospital-wide transfusion protocol (N=221)	Hospital-wide transfusion protocol (N=180)	P-value
Hemoglobin threshold in different subpopulations			
General ICU population	7[7-7.5]	7[7-7]	0.246
Trauma patients	7.5[7-8]	7.1[7-8]	0.563
Upper gastro-intestinal bleeding	7[7-8]	7[7-8]	0.326
Post cardiothoracic surgery	8[8-9]	8[7.8-9]	0.057
Obstetric patients	7[7-8]	7[7-8]	0.918
Patients with Sepsis	7[7-8]	7[7-8]	0.654
Patients on ECMO	8[7-9]	8[7.1-9]	0.141
Patients with TBI or stroke	8[7-9]	8[7-9]	0.581
Platelet count thresholds in different subpopulations			
General ICU population	50[20-50]	49.5[20-50]	0.103
Trauma patients	50[50-70]	50[50-70]	0.326
Upper gastro-intestinal bleeding	50[50-70]	50[49-53]	0.013
Post cardiothoracic surgery	50[50-89]	50[50-80]	0.049
Obstetric patients	50[50-71]	50[49-70]	0.133
Patients with Sepsis	50[20-50]	50[20-50]	0.791
Patients on ECMO	50[49-80]	50[48-80]	0.93
Patients with TBI or stroke	75[50-100]	70[50-100]	0.195
value.anti_platelet	51[50-100]	50[40-100]	0.12
Fibrin thresholds in different subpopulations			
General ICU population	1.5[1-1.5]	1.5[1-2]	0.753
Trauma patients	1.5[1-2]	1.5[1-2]	0.124

Table S3. Difference between respondents with and without a hospital wide transfusion protocol *(continued)*

	No hospital-wide transfusion protocol (N=221)	Hospital-wide transfusion protocol (N=180)	P-value
Upper gastro-intestinal bleeding	1.5[1-2]	1.5[1-2]	0.252
Post cardiothoracic surgery	1.5[1-2]	1.5[1-2]	0.895
Obstetric patients	1.5[1.1-2]	1.5[1-2]	0.679
Patients with Sepsis	1.5[1-1.8]	1.5[1-2]	0.967
Patients on ECMO	1.5[1-2]	1.5[1-2]	0.58
Patients with TBI or stroke	1.5[1.5-2]	1.5[1-2]	0.563
Type of ICU			**0.192**
Medical ICU	18(8%)	15(8%)	
Surgical ICU	40(18%)	24(13%)	
Mixed ICU	159(72%)	135(75%)	
Other	2(1%)	6(3%)	
Number of ICU beds			**0.125**
<10	63(29%)	32(18%)	
10-15	68(31%)	56(31%)	
16-20	31(14%)	33(18%)	
>20	58(26%)	58(32%)	
Type of institution			**0.689**
University hospital	95(43%)	83(46%)	
University affiliated hospital	56(25%)	48(27%)	
Non-university public hospital	46(21%)	36(20%)	
Private hospital	23(10%)	13(7%)	
Other	1(0%)	0(0%)	
Availability of transfusion guideline			

Table S3. Difference between respondents with and without a hospital wide transfusion protocol *(continued)*

	No hospital-wide transfusion protocol (N=221)	Hospital-wide transfusion protocol (N=180)	P-value
ICU-specific transfusion protocol	120(54%)	39(22%)	p<0.001
Massive transfusion protocol	120(54%)	89(49%)	0.354

Table S4. Difference between respondents with and without an ICU-specific transfusion protocol

	No ICU-specific transfusion protocol (N= 242)	ICU-specific transfusion protocol (N= 159)	P-value
Hemoglobin threshold in different subpopulations			
General ICU population	7[7-7]	7[7-7.5]	0.937
Trauma patients	7.05[7-8]	7.9[7-8]	0.315
Upper gastro-intestinal bleeding	7[7-8]	7[7-8]	0.542
Post cardiothoracic surgery	8[7.7-9]	8[8-9]	0.723
Obstetric patients	7[7-8]	7[7-8]	0.946
Patients with Sepsis	7[7-8]	7[7-8]	0.573
Patients on ECMO	8[7.2-9]	8[7-9]	0.026
Patients with TBI or stroke	8[7-9]	8[7-9]	0.305
Platelet count thresholds in different subpopulations			
General ICU population	50[20-50]	50[20-50]	0.861
Trauma patients	50[50-70]	50[50-75]	0.868
Upper gastro-intestinal bleeding	50[50-51.5]	50[50-70]	0.321
Post cardiothoracic surgery	50[50-80]	50[50-80.5]	0.901
Obstetric patients	50[50-70]	50[50-70]	0.673

Table S4. Difference between respondents with and without an ICU-specific transfusion protocol *(continued)*

	No ICU-specific transfusion protocol (N= 242)	**ICU-specific transfusion protocol (N= 159)**	**P-value**
Patients with Sepsis	50[20-50]	50[20-50]	0.258
Patients on ECMO	50[49.75-80]	50[34.75-80]	0.769
Patients with TBI or stroke	71[50-100]	75[50-100]	0.759
Patients using antiplatelet therapy	50[50-100]	50[49.75-100]	0.344
Fibrin thresholds in different subpopulations			
General ICU population	1.5[1-1.5]	1.5[1-1.92]	0.424
Trauma patients	1.5[1-2]	1.5[1-2]	0.911
Upper gastro-intestinal bleeding	1.5[1-2]	1.5[1-2]	0.641
Post cardiothoracic surgery	1.5[1-2]	1.5[1-2]	0.421
Obstetric patients	1.5[1.1-2]	1.5[1-2]	0.241
Patients with Sepsis	1.5[1-1.9]	1.5[1-1.92]	0.355
Patients on ECMO	1.5[1-2]	1.5[1-2]	0.698
Patients with TBI or stroke	1.5[1.5-2]	1.5[1-2]	0.239
Type of ICU			0.094
Medical ICU	16(7%)	17(11%)	
Surgical ICU	46(19%)	18(11%)	
Mixed ICU	172(71%)	122(77%)	
Other	6(2%)	2(1%)	
Number of ICU beds			0.086
<10	56(23%)	39(25%)	
10-15	75(31%)	49(31%)	
16-20	38(16%)	26(16%)	
>20	71(29%)	45(28%)	

Table S4. Difference between respondents with and without an ICU-specific transfusion protocol *(continued)*

	No ICU-specific transfusion protocol (N= 242)	**ICU-specific transfusion protocol (N= 159)**	**P-value**
Type of institution			0.835
University hospital	111(46%)	67(42%)	
University affiliated hospital	56(23%)	48(30%)	
Non-university public hospital	57(24%)	25(16%)	
Private hospital	18(7%)	18(11%)	
Availability of transfusion guideline			
Hospital-wide transfusion protocol	141(58%)	39(25%)	p<0.001
Massive transfusion protocol	136(56%)	73(46%)	0.053

Supplement 4: Supplemental figures

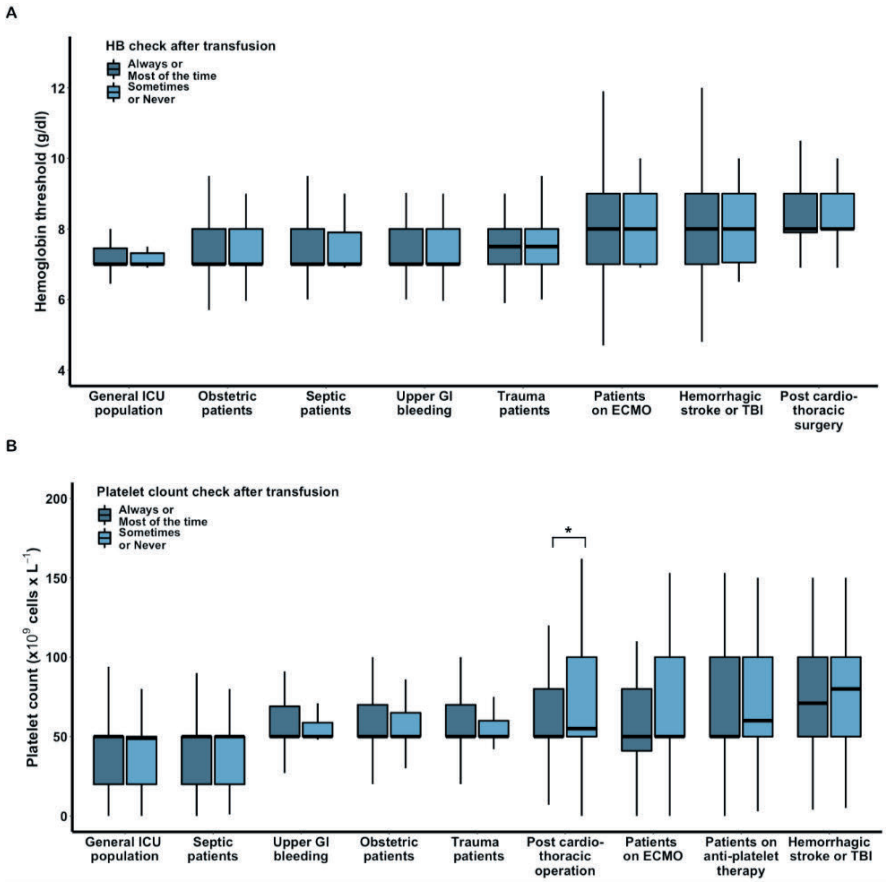

Figure S1. Effect of Hb check and platelet count check on Hb and platelet count threshold

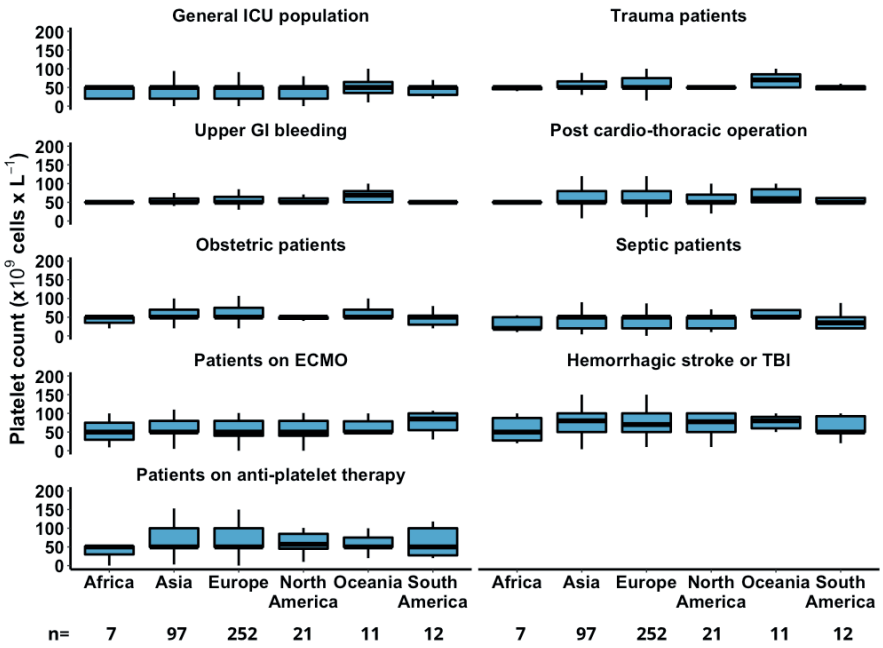

Figure S2 Platelet count threshold in different continents

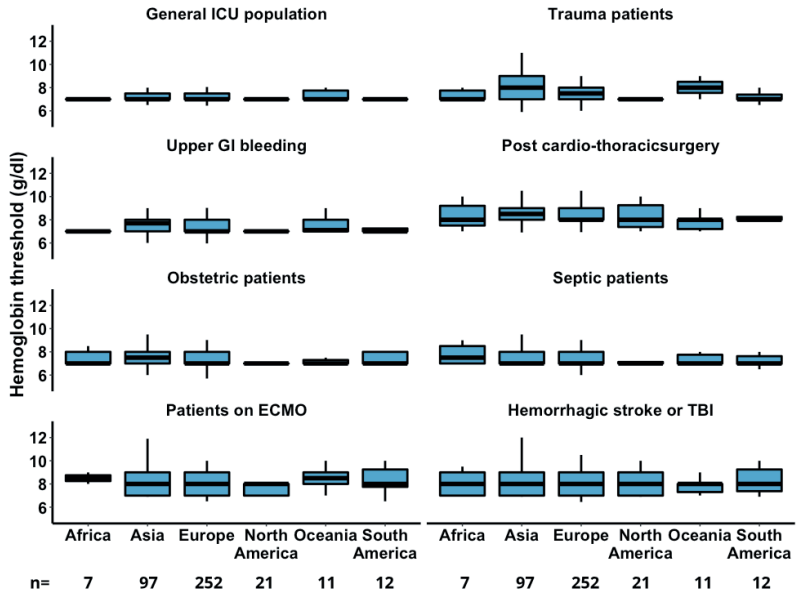

Figure S3 Hb threshold in different continents

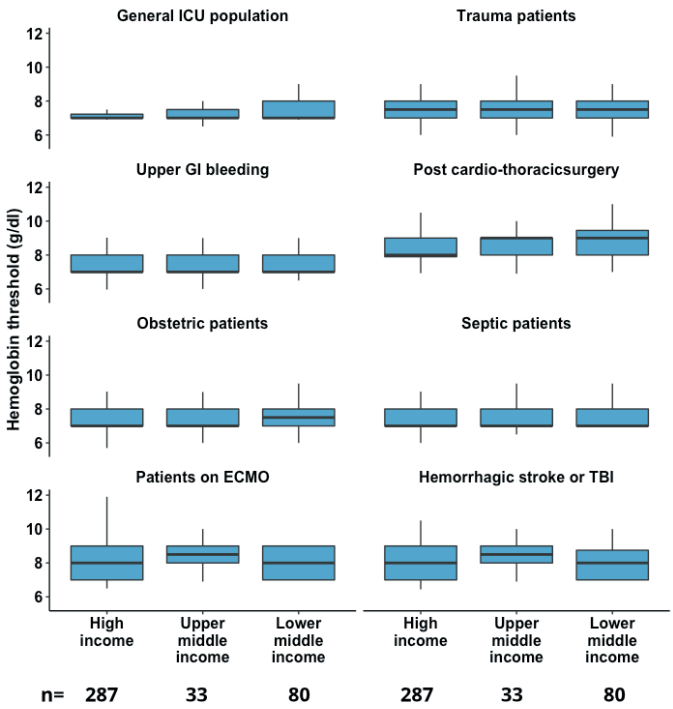

Figure S4 Hb threshold divided in high, upper middle and lower middle-income countries. Low income countries were not represented in this survey.

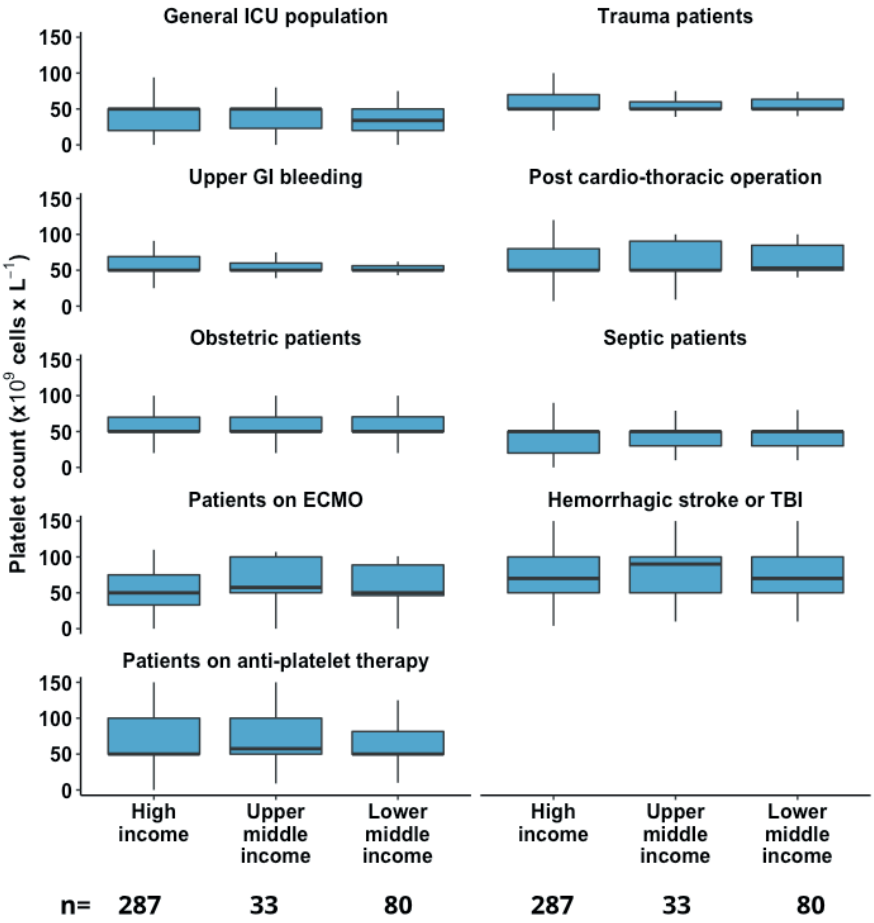

Figure S5 Platelet count threshold divided in high(n=287), upper middle(n=33) and lower middle-income (n=80 respondents) countries. Low income countries were not represented in this survey.

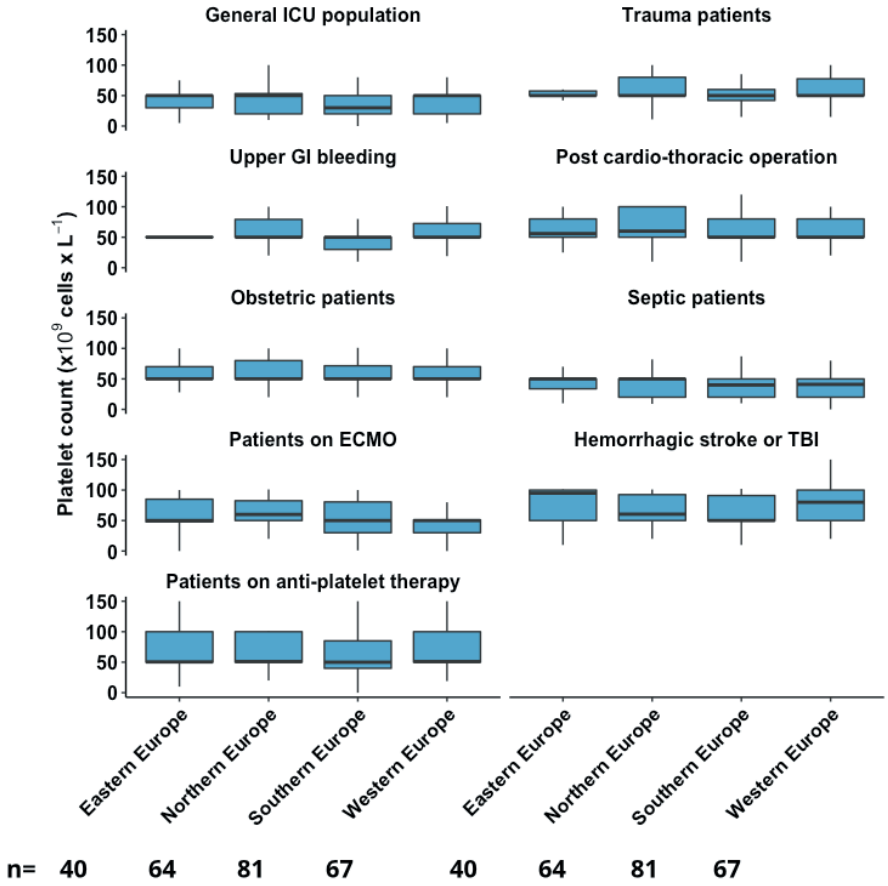

Figure S6 Hb threshold in different regions within Europe. Only regions with ³10 respondents are presented in this figure.

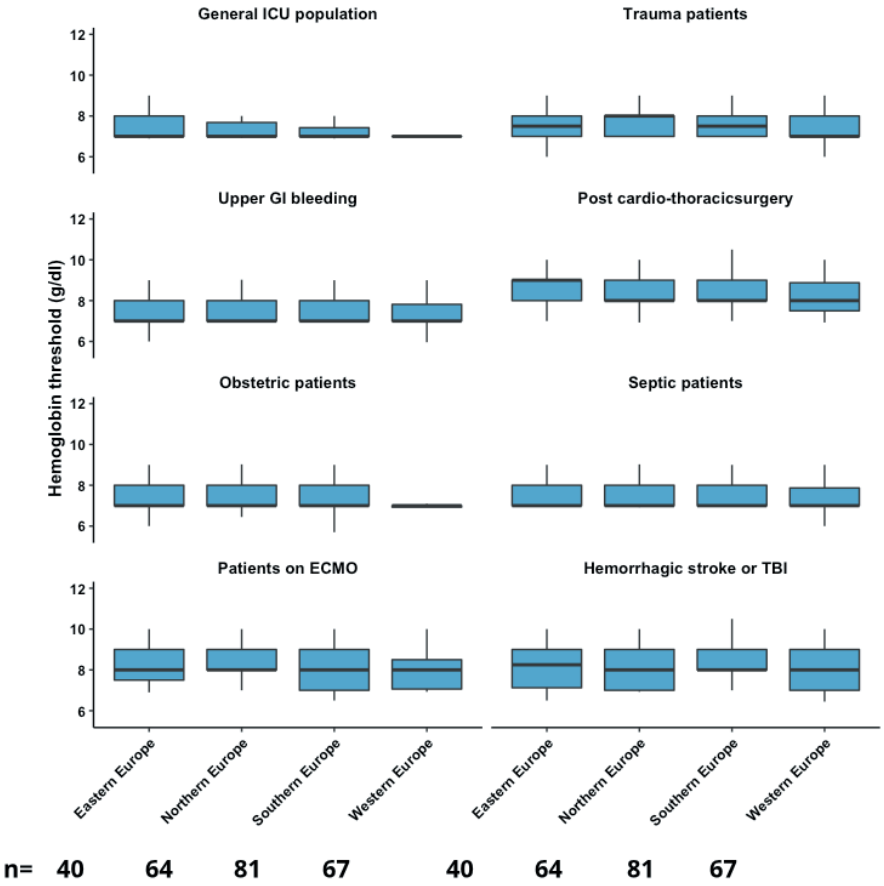

Figure S7 Hb threshold in different regions within Europe. Only regions with ³10 respondents are presented in this figure.

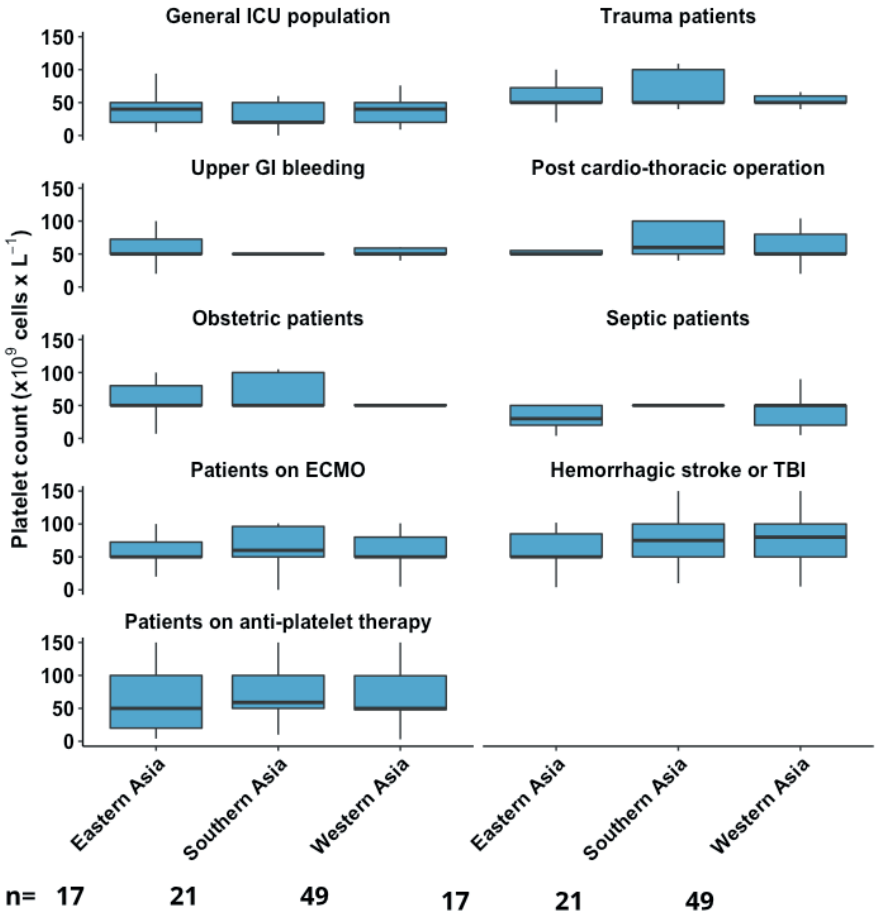

Figure S8 Platelet count thresholds in different regions within Asia. Only regions with ³10 respondents are presented in this figure.

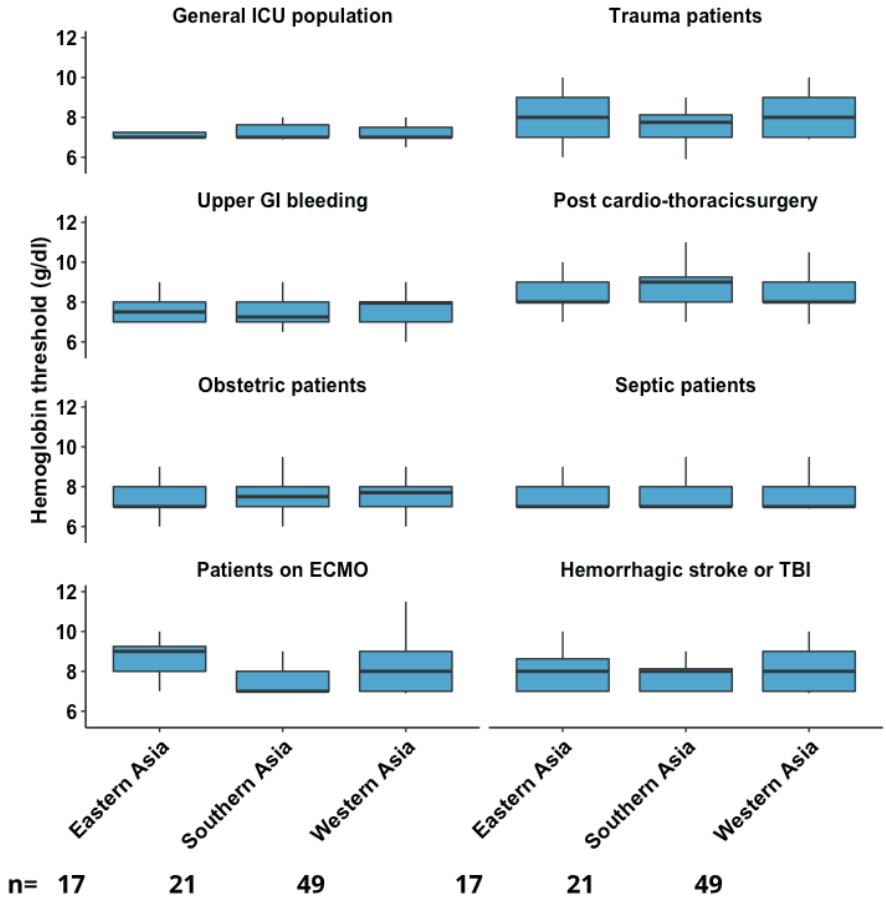

Figure S9 Platelet count threshold in different regions within Asia. Only regions with ³10 respondents are presented in this figure.

CHAPTER 4

International Point Prevalence Study of Intensive Care Unit Transfusion Practices – pilot study in the Netherlands

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Abstract

Background: Anaemia and coagulopathy are common issues in critically ill patients. Transfusion can be lifesaving, however, is associated with potential life threatening adverse events. As an international transfusion guideline for this specific patient population is lacking, we hypothesize that a high heterogeneity in transfusion practices exists. In this pilot-study we assessed transfusion practice in a university hospital in the Netherlands and tested the feasibility of this protocol for an international multi-centre study.

Methods: A prospective single centre cohort study was conducted. For seven days all consecutive non readmitted patients to the adult intensive care unit (ICU) were included and followed for 28 days. Patients were prospectively followed until ICU discharge or up to day 28. Patient outcome data was collected at day 28. Workload for this study protocol was scored in hours and missing data.

Results: In total 48 patients were included, needed in total three hours patient to include and collect all data, with 1.6% missing data showing the feasibility of the data acquisition. Six (12.5%) patients received red blood cells (RBCs), three patients (6.3%) received platelet concentrates, and two (4.2%) patients received plasma units. In total eight (16.7%) patients were transfused with one or more blood products. Median pre- and post-transfusion haemoglobin (Hb) levels were 7.6 (6.7-7.7) g/dL and 8.1 (7.6-8.7) g/dL, respectively.

Conclusion: In this pilot-study we proved the feasibility of our protocol and observed in this small population a restrictive transfusion practice for all blood products.

Introduction

Critically ill patients often receive a blood products to correct coagulopathy and/or improve oxygen delivery to peripheral tissues[1,2,3,4]. Administration of blood products can be lifesaving, but may also come with severe adverse events, varying from allergic reactions, acute haemolytic transfusion reactions, transfusion transmitted infections to transfusion related acute lung injury[5]. Since multiple large randomized controlled trials (RCTs) in the intensive care unit (ICU) proved the safety of a restrictive red blood cell (RBC) transfusion strategy[6–8], an overall reduction in the proportion of patients who received a RBC transfusion was observed [2,9]. However, the superiority of a liberal RBC transfusion strategy is still debatable for different subpopulations; particularly patients with acute coronary syndrome and elderly patients might benefit from a liberal transfusion strategy compared to a restrictive strategy[10,11]. For plasma and platelet transfusions, well powered randomized controlled trials are lacking[12], also the definitions for liberal and restrictive transfusion strategies are not yet well defined. This lack of definition and research gap may explain the heterogeneous transfusion practices described in multiple studies[3,4,13]. Current platelet transfusion practice is mainly based on studies conducted in haemato-oncological patients or in low quality studies in critically ill patients. Furthermore, the effectiveness of a large number of plasma transfusions is not proven, and might even be harmful[14,15]. For plasma transfusion multiple alternatives exist, including administration of vitamin K and prothrombin complex, depending on the cause of the coagulopathy. However, these alternatives cannot completely replace plasma transfusion in critically ill patients.

Multiple observational studies have investigated transfusion practice in critically ill patients using questionnaires or examining patient cohorts. Limitations of these studies include focussing on only one blood product[2–4] or being single country[4,13,16] or single centre studies[17]. Since the administration of different blood products might be correlated[18], there is a need for a large international multicentre observational study that examines the administration of different blood products and coagulations factors. Considering the challenging character of these kind of studies, a pilot study is necessary to optimize the study protocol before enrolling it worldwide. In this pilot study, we assessed current transfusion practice in a university centre in the Netherlands and also assessed the feasibility of this study protocol and the case record forms (CRF) in preparation for a worldwide study.

Methods

Study design

A prospective cohort pilot study was conducted to examine current transfusion practices in a 32 bed mixed medical-surgical ICU in a Dutch university hospital. The patient population consisted of mixed surgical and medical ICU patients, including cardiothoracic surgery and patients with traumatic brain injury and brain haemorrhages. The data were part of routine care and were extracted from the electronic patient files. During a period of seven consecutive days all consecutive non readmitted patients at the ICU older than 18 years were included. Patients were prospectively followed until discharge with a maximum of 28 days. At the 28th day, patient outcomes were scored, regardless whether they were discharged (see Figure 1).

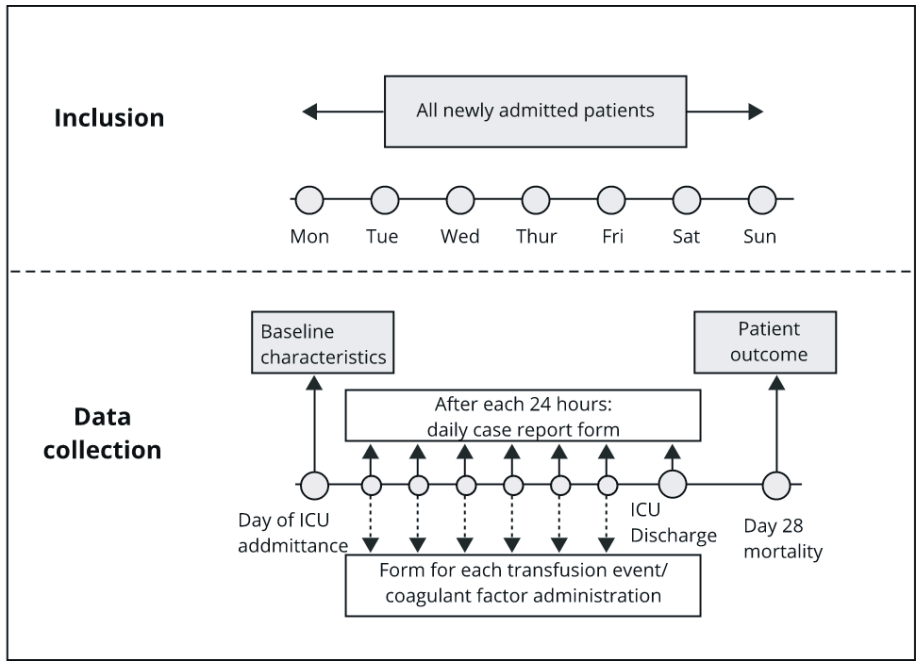

Figure 1. Study design

The Medical Research Ethics Committee of the Academical Medical Center in Amsterdam judged that this study is not subject to the requirements of the Medical research involving human subjects act. To use patient data, a written informed consent was obtained from the patient or a legal representative, using a deferred informed consent procedure.

When the patient or their legal representative did not give consent for study participation, the patient was excluded from the study and data was not used for analysis.

Data collection

Data was collected prospectively by a research student (see appendix 3 for CRF).

Baseline characteristics included information about the demographics, comorbidities, Acute Physiologic Assessment and Chronic Health Evaluation (APACHE) lV score, type and reason of admission. Clinical and laboratory data were collected daily to compare patients who received a transfusion to patients who did not receive a transfusion, including nadir haemoglobin (Hb), nadir platelet count, highest prothrombin time (PT) and highest activated partial thromboplastin time (aPTT). For each transfusion event a short transfusion CRF was completed. A transfusion event was defined as a transfusion of a blood derived product or the administration of a coagulation factor, ordered within one order in the electronic patient system. For each type of blood product or transfusion (RBCs, plasma, platelets, coagulation factors including prothrombin complex, fibrinogen and tranexamic acid and activation of massive transfusion protocol) a separate CRF was designed. The 28-day mortality and the location of the patient (home, ward, ICU) were also collected.

Outcomes

The primary outcome of this study was to assess the current transfusion practice at our ICU, described by the number of RBC, platelet and plasma transfusions and the administration of coagulation factors, correlated with Hb levels, platelet count, INR/PT and fibrinogen prior to and post transfusion. The secondary outcome was the feasibility of the study protocol, assessed by the workload, missing data and the number of transfusion and administration of coagulation factors in this patient cohort. Furthermore, the design of a worldwide follow-up study was based on this pilot data.

Data analysis

The statistical analysis was performed using the statistical program R version 3.5.2. Continuous normally distributed data are presented in means (standard deviation) and not normally distributed data as medians (first-third quartile). The Wilcoxon sum rank test was used to test the dependence of two grouping variables on continuous, non-normally distributed data. Categorical variables were expressed as number and percentage. Categorical data were compared using the chi-square test. P-values below 0.05 were considered to be statistically significant.

Results

Patient characteristics

During the study week, 49 patients were found eligible for study participation. One patient did not consent and was withdrawn from study participation. Therefore, data from 48 patients were obtained and analysed. The majority of the patients was male 33 (67%) and the age of the study population was 68.5 (53-72) years. During the first 24 hours, 27(56%) of the included patients received mechanical ventilation. Seventeen (35%) were admitted for treatment after cardiothoracic surgery, fifteen (31%) patients were admitted for non-surgical reasons. Other demographics, including reason of admission are shown in table 1.

Transfusion events

In total 30 transfusion events and eight coagulation factor administrations were reported. Eight patients (17%) received one or more blood products and/or coagulation factors (table 2). Forty patients (83%) did not receive any blood product or coagulation factor. The majority of the products were ordered by a resident (85%) and administered in the ICU (95%) (table 3). The remaining two products were transfused in the operating theatre; one platelet and one RBC concentrate.

Feasibility

The study related workload per included patient was on average three hours, including obtaining informed consent and data collection. The most time-consuming part of data collection was the baseline data, in particular calculating the APACHE IV score and EuroSCORE. Median time of collecting the baseline characteristics was 11 (9-14) minutes. The daily CRF was completed most often (167 times) with a median time of 4 (3-5) minutes per form.

Overall, the amount of missing data was limited (1.6%). In the demographics 0.8% of the data was missing, in all these cases the APACHE IV or EuroSCORE were missing. In the daily CRF, 1% of the data was missing: of this missing data the SOFA scores (40%), highest PT value (30%), and highest aPTT value (25%) were most frequently missing. The amount of missing data regarding transfusion events differed between the different blood products. Most frequently, data was missing in the coagulation factor form (11.9%), followed by plasma (3.1%), platelets (1.4%) and RBCs (1.0%) forms.

Table 1. Baseline characteristics.

Patient characteristics	
Age	68.5 (53.0-72.0) years
Gender men	32 (67%)
SOFA score	5 (3.0-7.0)
APACHE IV score	53 (40.0-71.5)
EuroSCORE	2.3 (1.0-3.4)
Type of admission	
Elective	15 (31%)
Urgent	2 (4%)
Emergency	31 (65%)
Referred from	
Operating theatre	22 (46%)
Other hospital	6 (12%)
General ward	11 (23%)
Emergency department	9 (19%)
Reason of admission	
Cardiovascular	18 (38%)
Gastro-intestinal	5 (10%)
Hematologic	1 (2%)
Metabolic	2 (4%)
Musculoskeletal/skin	3 (6%)
Neurological	8 (17%)
Respiratory	8 (17%)
Sepsis	2 (4%)
Other	1 (2%)
Type of patient	
Medical	15 (31%)
Cardiothoracic surgery	17 (35%)
Gastrointestinal surgery	6 (12%)
Neurosurgery	5 (10%)
Other surgery	5 (10%)
Presence of shock	
No	39 (81%)
Anaphylactic	1 (2%)
Cardiogenic	2 (4%)
Hypovolemic	1 (2%)
Neurogenic	1 (2%)
Septic	4 (8%)
Relevant comorbidities	
ARDS	2 (4%)
AKI	4 (8%)
ACS	0

Table 1. Baseline characteristics. *(continued)*

Patient characteristics	
Other	21 (44%)
Mechanical ventilation	27 (56%)
Supportive therapy	
CVVH	1 (2%)

Not normally distributed values are shown in median and interquartile range (IQR). Categorical variables are shown in number and percentage. *SOFA* sequential organ failure, *APACHE* Acute Physiologic Assessment and Chronic Health Evaluation, *ARDS* acute respiratory syndrome, *AKI* acute kidney injury, *ACS* acute coronary syndrome, *CVVH* continuous veno-venous hemofiltration.

Table 2. Transfusion forms.

Transfusion events	
Product ordered by	
Intensivist	2 (5%)
Specialist, non-intensivist	2 (5%)
Resident	34 (85%)
Student	2 (5%)
Location of transfusion	
ICU	36 (95%)
Operating theatre	2 (5%)
Red blood cell transfusion	
Number of red blood cell transfusion events	17
Reason of transfusion	
Low HB	12 (67%)
Active bleeding	7 (39%)
Hemodynamic instability	1 (6%)
Other physiological triggers	
None	16 (89%)
Tachycardia	1 (6%)
Lactate >2 mmol(L	1 (6%)
Hb level pre-transfusion	7.6 (6.7-7.7) g/dL
Hb level threshold	8.1 (6.9-8.1) g/dL
Hb level post-transfusion	8.1 (7.6-8.7) g/dL
Increment after transfusion	1.0 (0.5-1.1) g/dL
Number of transfused units	1.0 (1.0-2.0)
Platelet transfusion	
Number of platelet transfusions	11
Reason of transfusion	
Active bleeding	4 (36%)
Biopsy/ BAL	3 (27%)
Prophylactic without upcoming procedure	4 (36%)

Antiplatelet use trigger for transfusion	2 (18%)
Platelet count prior	10.0 (10.0-28.5) x10^9/L
Platelet count target	10.0 (10.0-50.0) x10^9/L
Platelet count post	13.0 (10.0-40.0) x10^9/L
Increment after transfusion	3.0 (0.0-15.5) x10^9/L
Number of transfused units	1.0 (1.0-1.5)
Plasma transfusion	
Number of plasma transfusion events	2
Reason of transfusion	
Active bleeding	2 (100%)
Pt prior	12.6 (12.1-13.2) s
Pt post	12.6 (12.2-12.9) s
Anticoagulant use trigger for transfusion	1 (50%)
Coagulation factors administration	
Number of coagulation factor events	8
Drug administrated	
Tranexamic acid	5 (63%)
Fibrinogen	2 (25%)
Prothrombin	1 (13%)
Reason of administration	
Active bleeding	5 (63%)
Biopsy	2 (25%)
Prophylactic without upcoming procedure	0 (0%)
Study	1 (13%)
Massive transfusion protocol	0

Not normally distributed values are shown in median and interquartile range (IQR). Categorical variables are shown in number and percentage. *ICU* intensive care unit, *HB* haemoglobin, *BAL* bronchoalveolar lavage, *Pt* prothrombin time.

RBC transfusion

Median Hb levels were 10.9 (9.3-12.4) g/dL at ICU admission and 8.8 (7.9-9.1) g/dL in the remaining patients who were still admitted (n=6) at day seven of ICU stay. During the study period, seventeen RBC transfusion events were registered. In total eight (12.5%) patients received one or more RBC transfusions. In eleven (65%) RBC transfusion events one RBC concentrate was transfused. In six (35%) events more than one unit was transfused. Non-single unit transfusion occurred only in bleeding patients. One patient received an irradiated RBC concentrate. RBC transfusion was mainly based on Hb triggers, in two cases other factors than Hb levels were considered (tachycardia and increased lactate levels).

Pre transfusion Hb levels in bleeding and non-bleeding patients were 7.7 (7.3-8.1) and 6.8 (6.5-7.7) g/dL, respectively, however, this difference was not statistically significant (p = 0.15). Haemoglobin increment after a RBC transfusion event was 1.0 (0.5-1.1) g/dL (Figure 3A), also this was similar in bleeding and non-bleeding patients (p=0.88).

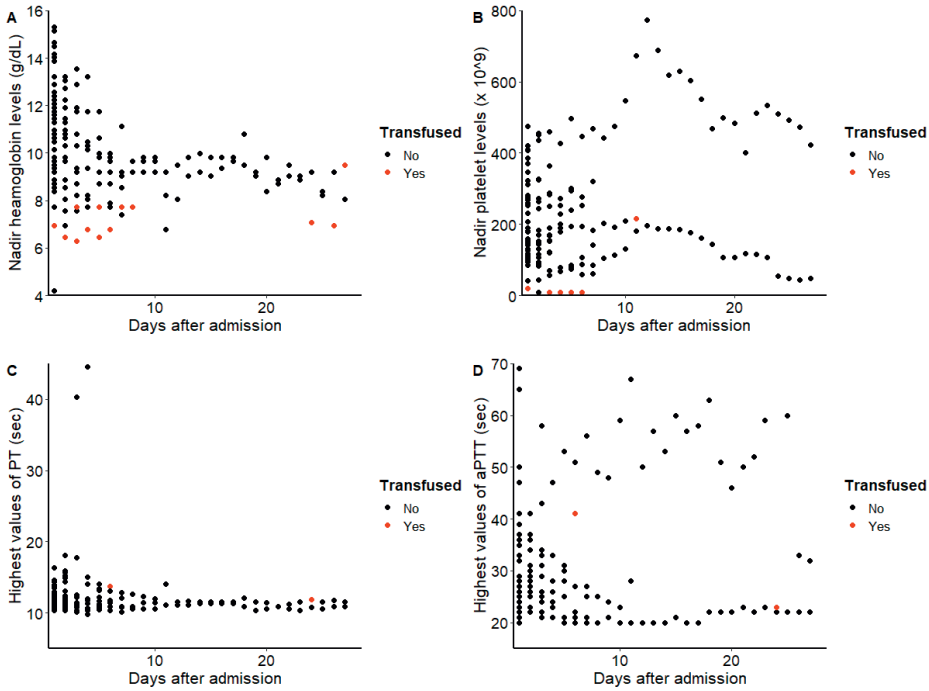

Figure 2. Levels of haemoglobin (A), platelets (B), PT (C) and aPTT (D) during the 28-day follow-up. Each dot represents a patient. Patients who received a RBC (A), a platelet (B) or a plasma(C-D) transfusion are presented with a red dot.

Platelet transfusion

During the first week of admittance, the mean nadir platelet count remained stable: the median nadir platelet count was 160 x 10^9 cells/L (122-276) and 163 x 10^9 cells/L (100-287) at day one (n=48) and day seven (n=6) of ICU stay respectively. During the ICU stay, in three (6.3%) patients eleven platelet transfusion events were reported resulting in the transfusion of fourteen platelet concentrates. Overall, in four (36%) cases, the main reason for transfusion was bleeding, in four (27%) cases the platelet concentrates were transfused prior to an invasive procedure, and in the remaining four (36%) solely the thrombocytopenia was the reason to transfuse. The highest platelet count prior

to a platelet transfusion was at 216x10^9/L, received by a patient who used double anti-platelet therapy, and these concentrates were administrated in the operating theatre.

Eight (72.7%) times one unit of platelets was transfused and three (27.3%) times two units were transfused. In all cases where more than one unit was transfused, patients were undergoing a procedure or were bleeding. In three (27.3%) cases, irradiated platelet concentrates were administered. One platelet concentrate (9%) was derived by apheresis and ten concentrates (91%) were pooled buffy coat products, obtained from five different donors. The majority (81.8%) of the platelet concentrates were administered in one single patient.

Plasma transfusion and coagulation factors

Prothrombin time (PT) remained stable during ICU stay. The median highest PT was 12.0 (10.9-12.5) sec at day one and 11.5 (10.8-13.6) sec at day seven of ICU stay. 1.7% of the reported PT values were greater than 20 sec (figure 2C).

A third of the patients had a normal aPTT during their whole ICU stay (aPTT <33 sec). The median aPTT was 26 (24-32) sec at day one and 23 (21-26.5) sec at day seven of ICU stay (Figure 2D). In nine (19%) of the patients an aPTT >40 sec was observed at some moment during their stay at the ICU.

Two patients received plasma transfusion during the study period. The mean PT was 12.6 sec pre- and 12.6 sec post-transfusion. In both cases, no ROTEM was performed prior transfusion. In three (6.3%) different patients, five times tranexamic acid administration was reported. In 80% of these cases, the main reason of administering was bleeding.

Three fibrinogen transfusions were recorded in one single patient, twice prior to an invasive procedure and once when actively bleeding. In one event, ROTEM was performed prior fibrinogen transfusion. In all three events, the fibrinogen target was >2.0 g/L. Prothrombin complex was transfused once during the study period.

Patient outcome

Patient outcome was measured at day 28 (table 3). Median observed ICU length of stay was two days (2-4.25). Six patients (12.2%) were admitted to the ICU for more than seven days. Two patients were still in ICU at 28 days. Mortality in the study cohort was thirteen percent. In patients without any RBC, platelet or plasma transfusion the mortality was 10% versus 25% in the group who did receive one or more RBC, platelet or plasma transfusions (p=0.24).

Table 3. Patient outcome.

Day 28	
Patients state: died	
Died at ICU (%)	6 (13%)
Died at another location than ICU	0
Days admitted on ICU	2.0 (2.0-4.3)
Location of alive patients	
ICU	2 (5%)
General ward	5 (12%)
Discharged	35 (83%)

Not normally distributed values are shown in median and interquartile range (IQR). Categorical variables are shown in number and percentage. *ICU* Intensive Care Unit.

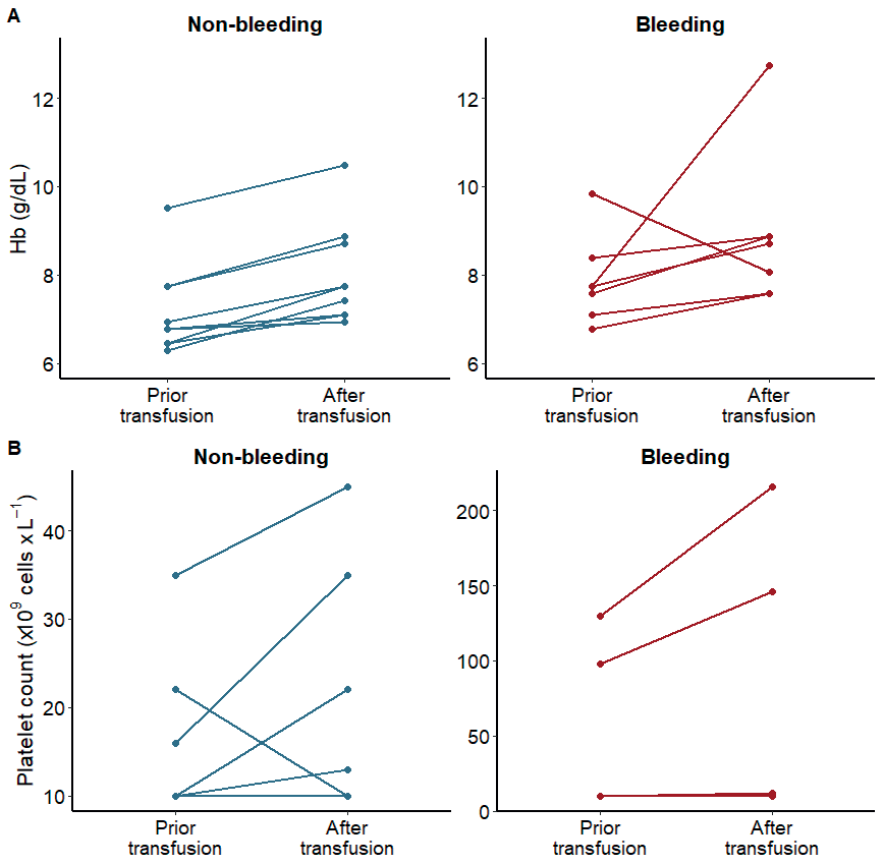

Figure 3. Pre and post red blood cell transfusion Hb levels (A) and pre and post platelet transfusion platelet count (B) after one or more RBC or platelet transfusions, respectively.

Discussion

In this pilot study, we observed that patients in our ICU were relatively restrictively transfused, and that pre- and post RBC transfusion Hb levels were similar in bleeding and non-bleeding patients. Furthermore, we showed the feasibility of our study protocol for an international point prevalence study of transfusion practice in ICUs. This study serves as a pilot study which is ready to be enrolled internationally as an observational cohort study.

In this small population the reported pre-RBC transfusion Hb levels of 7.6 (6.7-7.7) g/dL were lower than a recently published large observational cohort study in the ICU where a mean nadir Hb level on day of transfusion of 8.3 g/dL was reported[2]. Also the proportion of patients transfused with RBCs (12.5%) was lower than in this study where 26.3% received one or more RBC transfusions[2]. This lower proportion could be part of an ongoing downward trend in RBC transfusion, since the proportion of patients who received an RBC transfusion during ICU admittance has been declining the last decade. In 2002 37.0% of ICU patients was transfused[9], while in 2018 the same research group reported that only 26.3% received RBCs during their ICU stay[2].

Another explanation of the differences with current literature is the large proportion of post cardiothoracic surgery (35%) patients in this patient cohort. These patients were admitted with a relatively high haemoglobin level of 11.8 (10.5-12.1) g/dL and were admitted for a median length of ICU stay of two days, resulting in only one patient in this subpopulation receiving RBC concentrates.

Also, the proportion of patients receiving platelet concentrates in our study was only 6.3% of the patients during ICU admittance, which is slightly less than observed in the United Kingdom in 2012 where 9.0% of a national patient cohort received platelets during ICU stay [3]. Also, in that study more than 40% of the patients who received platelets had a platelet count ≥ 50 x 10^9 cells/L[3], while in our study, only 18% of the patients who received platelets had a platelet count ≥ 50 x 10^9 cells/L. In addition, all non-bleeding patients who received platelet concentrates prophylactically prior an invasive procedure or without any upcoming procedure had a platelet count of ≤ 50 x 10^9 cells/L and ≤ 10 x 10^9 cells/L, respectively. Only bleeding patients had a platelet count ≥ 50 x 10^9 cells/L prior to transfusion. This is in accordance to our local transfusion guideline. Unnecessary platelet transfusions should be avoided, especially since multiple RCTs in different patient populations have shown an increased mortality in patients transfused with a liberal platelet transfusion strategy[19,20].

While plasma transfusion is possibly beneficial in bleeding patients, plasma transfusion to correct prolonged coagulation time in the absence of bleeding is not recommended[14,15]. This is in accordance with our findings that only patients who were actively bleeding received plasma, but in contrast with a large study in the UK where only a third of the plasma transfusions was given to treat bleeding[21]. Also the proportion of patients of this cohort receiving plasma was two times higher (12.7%) than in our study[4]. The correction of the PT in our patient population was minimal after plasma transfusion.

In this study tranexamic acid was administered five times. Multiple studies showed a reduced need for transfusion during surgery when inhibiting fibrinolysis using tranexamic acid[22]. Also in trauma patients a decreased mortality was found when administering tranexamic acid early after trauma[23]. However, it is unclear how we can extrapolate these results to bleeding patients admitted to the ICU. To our knowledge, RCTs in this patient population comparing tranexamic acid to placebo have never been performed.

The major limitation of this study is the small number of included patients. With only 48 patients in this pilot study, the transfusion numbers are too dependent on a few specific patients; two patients were responsible for 30 transfusion events (79%). Furthermore, this was a single centre study, thus limiting generalizability of the findings.

A large international study is needed to draw valid conclusions on transfusion practices. Ideally, different subpopulations need to be included such as patients with and without bleeding and patients with acute coronary syndrome, brain injury and patients on extracorporeal membrane oxygenation. Current rationale is that these patient categories might need higher Hb levels, while evidence for this is limited. This protocol enables the researchers, when included a sufficient number of patients, to compare transfused and non-transfused patients and examine differences in why certain patients are transfused differently while having similar Hb levels, cell counts and coagulation tests. Numerous studies have studied RBC transfusions in large ICU cohorts, however, international studies examining plasma transfusion, platelet transfusion, and administration of coagulation factors are limited.

The feasibility of this protocol was shown by the acceptable amount of time per patient and number of registered blood product administration. However, based on this pilot study, the study protocol of the international observational cohort study was adjusted. Time consuming data collection that did not result in reliable and useful information was removed from the study protocol, including daily fluid balance and additional questions for the non-transfused patients. Also the workflow of the data collection was improved

using an electronic CRF. We expect this to reduce the amount of time to complete the different forms. Also obtaining informed consent might not be necessary in every centre depending on national and local regulations for observational research.

Conclusion

In this small patient cohort a restrictive transfusion strategy was observed. However, due to the small number of inclusions, clinically relevant conclusions cannot be drawn from this study. The feasibility of this protocol was shown in this pilot study and is suitable to compare transfused and non-transfused patients in different subpopulations for different blood products. The current study allowed us to optimize the protocol and as result, this adjusted study protocol will be used in an international multicentre observational point prevalence study. The authors can be contacted for participation in the upcoming international trial.

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CHAPTER 5

Metabolic changes in erythrocytes *in vivo*, during storage and after transfusion

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Abstract

Glucose metabolism plays an important role in several aspects in the survival and functionality of erythrocytes. Storage of red cell concentrates (RCCs) is characterized by several changes including metabolic changes. This area of research has developed rapidly the last decade thanks to the emergence of new technologies. Besides aging during storage, aging under physiological conditions *in vivo* has been extensively studied as well. These studies are mainly performed in the previous century while in the last decade more studies have been focusing on the biochemical changes during storage. Metabolic recovery from storage after transfusion is still a relatively unexplored field of research. Here we review the metabolic changes of erythrocytes and RCCs during physiological aging *in vivo*, during storage under blood bank conditions and during *in vivo* recovery from storage after transfusion.

It is known that certain similarities between physiological aging and aging during storage exist but there are also quite some differences. During aging *in vivo* the glycolysis, Luebering-Rapoport shunt and pentose phosphate pathway (PPP) activity gradually decrease, whereas during storage metabolic fluxes are dependent on the choice of the additive solution. Under standard storage conditions adenine triphosphate (ATP) levels decrease rapidly and after two weeks of storage 2,3-diphosphoglycerate (2,3-DPG) is almost completely depleted. However, when alkaline additive solutions are used, ATP and 2,3-DPG levels can be maintained. Even though ATP and 2,3-DPG levels decrease rapidly during storage, it is known that these compounds are regenerated after transfusion. Data about PPP activity and GSH regeneration after transfusion is still lacking. The goal of this review is to summarize all current knowledge about this topic and to determine what knowledge is still lacking to set goals for future studies.

Introduction

Red blood cell (RCC) transfusion is the most widely applied cellular therapy in medicine. To guarantee the supply of blood products it is inevitable to store red blood cells for a certain time. Storage of RCCs up to 49 days is allowed, depending on local regulations. The alterations which occur during storage are well studied and are known as the storage leasion[1,2]. These alterations include morphological and biochemical changes of which some are reversible, depending on the duration of storage[3].

The current opinion is that certain biochemical compounds are important for survival and functionality of erythrocytes like adenosine triphosphate (ATP), 2,3-diphosphoglycerate (2,3-DPG) and glutathione (GSH). The importance of these metabolites *in vivo* is seen in red blood cell disorders caused by deficiencies in enzymes that generate these compounds. For example, erythrocytes from patients suffering from glucose-6-phosphate-dehydrogenase (G6PD) deficiency contain lower GSH levels resulting in haemolysis when erythrocytes are exposed to oxidative stress[4]. Furthermore, a defect in one of the enzymes catalysing a step in the glycolysis, such as in pyruvate kinase and hexokinase deficiency, resulting in decreased ATP levels, leads to constant mild to severe haemolytic anemia[5]. Finally, 2,3-DPG levels are important for the correct oxygen carrying function of erythrocytes. Low 2,3-DPG levels result in increased oxygen affinity of haemoglobin leading to decreased oxygen offload to the peripheral tissue[6]. Thus low 2,3-DPG levels lead to impaired oxygen transport of erythrocytes to the tissues. This is exemplified in patients suffering from 2,3-DPG mutase deficiency, which results in lower 2,3-DPG levels and a compensatory polycythemia[7].

Over the years multiple research groups have studied the metabolic changes during *in vivo* and *in vitro* aging of erythrocytes. Significant differences exist in different metabolic pathways comparing *in vivo* aging with *in vitro* aging. These insights have helped blood banks to improve storage conditions over the years, and thereby improving the quality of stored blood products.

In the previous century metabolic changes in erythrocytes during *in vivo* an *in vitro* aging have been well studied. These studies were, however, mainly focused on a limited number of metabolites or enzymes which could lead to certain biases. The last two decades the scientific field studying this subject has advanced rapidly. Nowadays it is possible to measure a broad spectrum of metabolites and proteins at once using mass spectrometry approaches. These new approaches make it possible to do untargeted

metabolome or proteome analyses, identifying a metabolic fingerprint on a specific time point under specific conditions.

The big limitation of most studies is that these metabolic changes are only studied during normal aging and during *in vitro* storage of RCCs. Studies of the metabolic recovery of stored RCCs after transfusion are limited, while this information could determine how storage techniques should be adjusted to further improve RCC quality. Superiority of fresh RCCs above stored RCCs still remains questionable for at least two reasons. First, contradictory results have been published about clearance in comparing fresh with stored RCCs[8,9]. And secondly, no effect on mortality is seen in multiple randomized controlled trials comparing fresh blood with longer stored RCCs[10–13].

A review summarizing studies focusing on metabolic changes in erythrocytes and RCCs during aging *in vivo*, *in vitro* and after transfusion is still lacking. This review aims to summarize all current knowledge on the metabolic changes in these three different conditions.

Metabolism of aging erythrocytes

The life cycle of erythrocytes starts in the bone marrow. After the differentiation from hematopoietic stem cells to reticulocytes in the bone marrow, reticulocytes are released into the circulation[14]. Reticulocytes have already lost their nucleus, but still contain some residual RNA[15]. In two to three days, after being released into the circulation, the reticulocytes mature into erythrocytes[16]. This phase of maturation is characterized by the elimination of all internal membrane bound organelles including mitochondria[17]. The most relevant metabolic change of this phase for the red blood cells is that reticulocytes still have a citric acid cycle[18], while after maturation the erythrocytes are dependent on the energy production by the glycolysis to fulfil their function.

Glycolysis

During glycolysis glucose is converted into lactate via multiple enzymatic reactions, which leads to a net production of two molecules ATP per molecule glucose (see figure 1). In *in vivo* aged erythrocytes ATP levels decrease with 20-32%[19–22] while lactate is accumulating[21]. The current opinion is that glycolytic activity decreases when erythrocytes age. This hypothesis is supported by the fact that ATP levels decrease and the activity of different enzymes of the glycolysis is altered during aging[21,23–25]. The rate limiting enzyme in the glycolysis, phosphofructokinase (PFK), which converts fruc-

tose-6-phosphate into fructose-1,6-biphosphate is significantly influenced by aging of the erythrocyte. It has been observed that PFK activity is significantly lower in the oldest erythrocyte fraction compared to the youngest fraction[23]. Changes in enzyme activity are not limited to PFK, several other enzymes are also influenced by the aging process. Phosphohexokinase and aldolase activity progressively decrease during the aging of erythrocytes[21,23]. Pyruvate kinase activity, which is responsible for the penultimate step in the glycolysis, is also significantly decreased in the oldest erythrocyte fraction[24]. And finally, it is seen that some isoenzymes of lactate dehydrogenase (LDH), the enzyme responsible for converting pyruvic acid into lactate, are destroyed or inactivated over time[25]. Consequently, aged erythrocytes can utilize less glucose than younger cells[21]. Furthermore, it is hypothesized that in younger cells relatively more glucose is entering other pathways than the glycolysis, because relatively less glucose is converted into lactate[21].

Luebering-Rapoport shunt

A second important metabolic pathway parallel to the glycolysis is the Luebering-Rapoport shunt. In this pathway 1,3-bisphosphoglycerate is converted into 2,3-DPG (see figure 1). 2,3-DPG is important in the oxygen delivery capacity of the erythrocytes and the concentration of 2,3-DPG in erythrocytes is therefore relatively high compared to other cells in the human body[6]. When 2,3-DPG is produced, one step of the glycolysis is bypassed and thereby this process indirectly costs ATP. Similar to the glycolysis, also the Luebering-Rapoport shunt activity decreases as erythrocytes age. Multiple studies have shown an age dependent decrease of 2,3-DPG levels in the range of 13-42% [21,22,26]. However, the activity of 2,3-diphosphoglycerate mutase, the enzyme responsible for the conversion of 1,3-diphosphoglycerate into 2,3-DPG, remains constant over time[23]. This suggests that the decreased 2,3-DPG levels are caused by diminished glycolytic activity resulting in less substrate for this shunt.

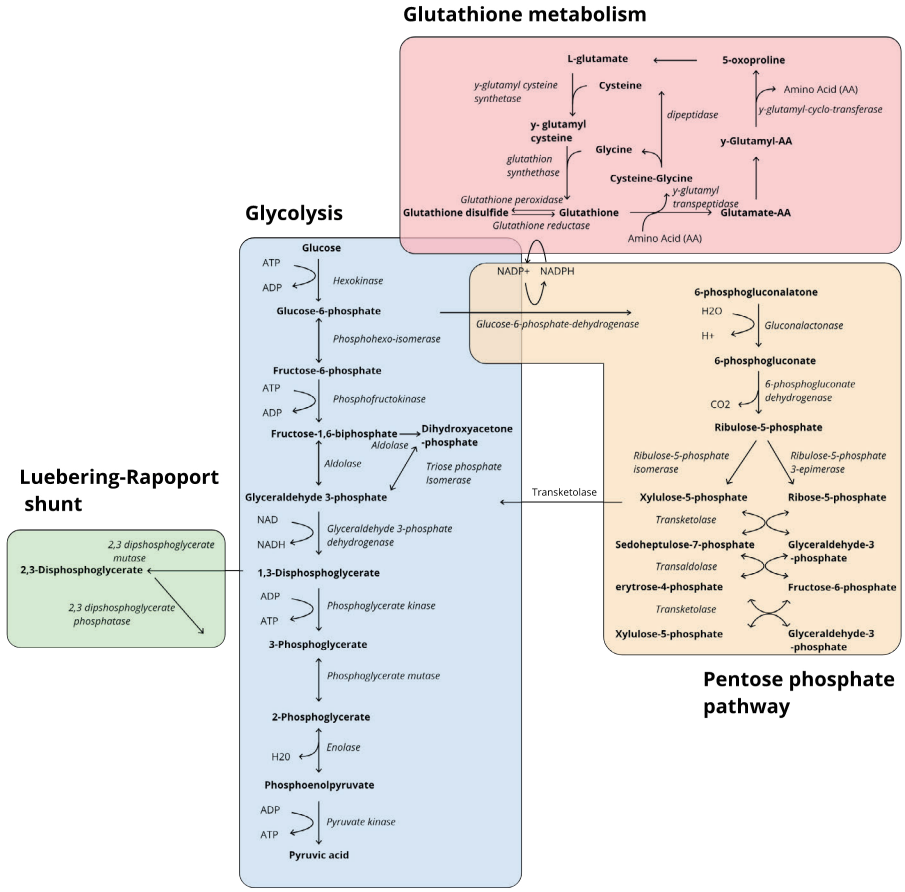

Figure 1. Overview metabolic pathways involving glucose and glutathione in erythrocytes

Pentose phosphate pathway and glutathione metabolism

The pentose phosphate pathway (PPP) consists of two parts, the oxidative phase and the non-oxidative phase (see figure 1). The non-oxidative part of the PPP consists of multiple reactions, which are responsible for the conversion of ribose-5P into glycolysis intermediates and vice versa. The main function of the oxidative PPP in erythrocytes is the production of the reduced electron carrier nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is converted from oxidized nicotinamide adenine dinucleotide phosphate (NADP+) during the oxidative phase while converting glucose-6P into ribulose-5P. NADPH is a cofactor for many reactions. In erythrocytes it is, among other things, essential for the reduction of GSH. GSH plays an important role in neutralizing reactive oxygen species (ROS) and is therefore essential during oxidative stress. Because

of the high concentration of oxygen in erythrocytes, reactive oxygen species are one of the most important stress factors in erythrocytes[27].

During increased oxidative stress young erythrocytes have a higher flux through the PPP than the oldest cell population. In contrast, when the cells are in steady state no differences in PPP flux between young and aged RBC were observed[28]. However, it is seen that in old erythrocytes the PPP related enzymes are less active, in particular G6PD[20,24,29–31]. Also, the activity of 6-phosphogluconate dehydrogenase, the enzyme responsible for the second step in the PPP, decreases but not as pronounced as G6PD[31].

Some contradictory results about the GSH levels have been published over the years. It has been described that GSH levels are higher in reticulocytes but remain constant after maturation[20,32], while other studies have described an age dependent decrease up to 70%[20,29,33,34]. Results about glutathione disulfide (GSSG) are more consistent, GSSG is oxidized GSH, and can be reduced to GSH using NADPH. Overall, a decrease in the GSH/GSSG ratio is seen in aged erythrocytes[29,32,34].

Metabolism during *in vitro* storage

To minimize the storage lesion an additive solution is added to a RCC which contains RBC and a small amount of plasma. Many different additive solutions with different concentrations of various substrates exist (see table 1). Each additive solution has its own impact on the metabolic changes during storage[35], however, the metabolism of stored RBC shows also some similarities when using the different additive solutions.

Table 1. Composition different additive solutions.

Ingredients (mmol/L)	Additive solution						
	SAGM	**AS-1**	**AS-3**	**AS-5**	**AS-7**	**PAGGGM**	**PAGGSM**
NaCl	150	154	70	150	-	-	72
NaHCO3	-	-	-	-	26	-	-
NaH2PO4	-	-	23	-	-	8	8
Na2HPO4	-	-	-	-	12	8	16
Na-gluconate	-	-	-	-	-	40	-
citric acid	-	-	2	-	-	-	-
Na-citrate	-	-	23	-	-	-	-
adenine	1.25	2	3	2.2	2	1.4	1.4
guanosine	-	-	-	-	-	1.4	1.4
glucose(dextrose)	50	111	55	45	80	47	47
mannitol	29	41	-	45.5	55	55	55
pH	6.2	5.5	5.8	5.5	8.5	8.2	6.0

Glycolysis and Luebering-Rapoport shunt

The glycolysis and Luebering-Rapoport shunt are important pathways for the generation of ATP and 2,3-DPG respectively. However, both ATP as well as 2,3-DPG are rapidly depleted during storage. This is more pronounced than during *in vivo* aging, especially for 2,3-DPG levels. Throughout storage 2,3-DPG levels rapidly decline up to 99% compared to baseline level[35–38] while *in vivo* 2,3-DPG levels decline up to 42% in the oldest cell fraction[21,22,26]. Due to the importance of these metabolites, new storage techniques aim to improve this, for instance by changing the composition of the additive solutions. In this review a distinction is made between non-alkaline additive solutions (e.g. SAGM, AS-1, AS-5) and alkaline additive solutions (e.g. AS-7 and PAGGGM). The latter are developed to maintain ATP and 2,3-DPG levels better during cold storage.

In non-alkaline additive solutions glycolysis is active during the first two weeks after which activity decreases resulting in lower ATP levels at the end of storage[35,38,39]. Glucose is rapidly converted into glucose-6-phosphate after uptake into the cells. After this, glucose-6-phosphate is converted via multiple steps into 2 molecules of glyceraldehyde-3-phosphate. In the first week of storage, these early glycolysis substrates increase but are almost completely depleted after two weeks[40]. In the second phase of the glycolysis, glyceraldehyde-3-phosphate is converted via five steps into pyruvate with a yield of 2 ATP molecules. In non-alkaline additive solutions, the glycolysis intermediates in these conversions: 1,3-diphosphoglycerate (1,3-DPG), 3-phosphoglycerate, 2-phosphoglycerate and phosphoenolpyruvate are all significantly decreased at the end of storage while pyruvate and lactate are accumulating[36,40]. Initially ATP levels remain constant during the first two-three weeks, but after this period ATP levels decline accompanied by increased levels of ATP breakdown products[35,41].

In non-alkaline storage solutions, 2,3-DPG levels decrease rapidly within two weeks with 99% compared to baseline level[36,37] while *in vivo* 2,3-DPG levels declined up to 42% in the oldest cell fraction[21,22,26]. The current opinion is that decreased glucose flux through the glycolysis results in less substrate for the Luebering-Rapoport shunt and thus less 2,3-DPG production. A second explanation could be increased breakdown of 2,3-DPG. There are indications that diphosphoglycerate mutase, the enzyme responsible for the conversion from 1,3-DPG into 2,3-DPG, is also able to catalyze the reverse reaction. According to an *in silico* model, 2,3-DPG could be reconverted by this enzyme to 1,3-DPG[42]. However, this is not yet confirmed *in vivo* or *in vitro*.

Thus ATP and 2,3-DPG are depleted during storage in non-alkaline additive solutions. The current hypothesis is that this is due to decreased pH caused by lactate accumu-

lation, which results in decreased PFK activity[43]. To counteract this phenomenon and thereby improve energy metabolism and oxygenation capacity in stored RCCs, alkaline additive solutions are developed[44–46]. The rationale behind this is that increasing the pH would lead to less inhibition of PFK activity resulting in more substrate for 2,3-DPG and ATP production. It is shown that RCCs stored in AS-7, an alkaline additive solution, utilize more glucose compared to AS-3, a non-alkaline additive solution[47]. Moreover, most glycolytic intermediate levels are higher in AS-7 compared to AS-3. Only pyruvate and lactate levels were similar in AS-3 and AS-7[47]. RCCs stored in PAGGGM consume more glucose and produce more lactate, resulting in higher ATP and 2,3-DPG levels compared to RCCs stored in SAGM[45,48]. In PAGGGM 2,3-DPG levels initially increase during the first two to three weeks. After this period the 2,3-DPG level gradually decreases to baseline level. This level is significantly higher than in non-alkaline additive solutions. ATP and 2,3-DPG molecules also inhibit PFK activity, however this negative feedback loop is inhibited under anaerobic conditions. In line with this, RCCs stored under anaerobic conditions in non-alkaline additive solutions showed higher ATP and 2,3-DPG levels despite a CO_2 dependent decline of pH [49–52].

The pentose phosphate pathway and glutathione metabolism

The oxidative and non-oxidative phase of the PPP are both influenced by cold storage. In general the oxidative part of the PPP remains active where the non-oxidative PPP part is inhibited. It is observed that the non-oxidative PPP activity gradually declines over time[40,42,53]. It is reported that in SAGM RCCs at the end of storage no glyceraldehyde-3-phosphate is produced through the PPP, thus suggesting a blockade in the non-oxidative PPP[40]. This part of the PPP consists of multiple reversible reactions. The direction in which the flux through the non-oxidative PPP occurs is dependent on storage time[42]. During the first ten days the non-oxidative PPP provides mainly glycolysis intermediates. From day ten until day seventeen the flux reverses and provides relatively more intermediates for adenine monophosphate production[42]. During the last phase of storage (day 18-42) the non-oxidative PPP provides ribose-5-phosphate as substrate for the glycolysis again[42].

Oxidative PPP activity is influenced by the additive solution used. In RCCs stored in AS-1 the PPP activity remains stable during storage[36]. In RCCs stored in AS-3, initially oxidative PPP intermediate levels increase during the first week of storage but from day 7 the activity declines gradually[53]. AS-7 stored RCCs showed higher PPP activation than AS-3[47] which can be explained by the fact that the optimal pH for G6PD activity is 8[54]. Therefore, an alkaline additive storage solution is beneficial for PPP activity. Contradictory results

on the PPP activity in SAGM stored RCCs have been published. Progressively decreasing activity but also increased oxidative PPP activity has been reported[40,42].

Higher flux through the PPP does not necessarily mean higher NADPH levels. The total NADPH and NADP+ pool gradually decreases during storage in AS-3[53]. These levels are similar in AS-7 stored RCCs even though PPP activation was higher in AS-7 than in AS-3[47]. Furthermore, nicotinamide accumulates during storage, which is a breakdown product of NADPH and NADP+[46]. The current hypothesis that this breakdown product has an inhibiting effect on G6PD activity and thus inhibits the oxidative PPP.

Even when PPP activity is increased, RCCs are unable to maintain their GSH levels during storage[40,53]. Total GSH and GSSG levels decrease over time independent of the additive solution[40,47,53]. GSH levels decrease rapidly during storage, and most studies reported decreased GSSG levels as well. However, one study showed increased GSSG levels[39]. Nevertheless, in literature there is consensus that the GSH/GSSG ratio decreases over time in RCCs[39,40,53]. This ratio is seen as the best parameter for antioxidant capacity. Until now, AS-7 stored RCCs seemed to maintain GSH levels best, resulting in a higher GSH/GSSG pool compared to AS-3 RCCs[47]. The breakdown of GSH seemed to be similar to that in other additive solutions because no difference in breakdown products were observed. Thus, in AS-7 GSH biosynthesis is upregulated or the reduction of GSSG to GSH is increased[47].

Decreased GSH levels are the result of a combination of increased turnover, increased reduction into GSSG or decreased GSH synthesis. One study showed that in AS-3 stored RCCs there is an increase in the GSH turnover product 5-oxproline and accumulation of glutathionyl-cysteine, implicating increased consumption of GSH[53]. However, there are also indications that GSH synthesis is impaired. Reduced NADPH levels result in less reduction of GSSG into GSH. Besides NADPH dependent conversion of GSSG into GSH, also the novo synthesis of GSH takes place[55,56]. In this ATP-dependent process, GSH is formed by three amino acids; cysteine, glutamate and glycine (see figure 1). This synthesis is decreased by 45% when stored in SAGM[57]. However, when extra amino acids were added, the *de novo* synthesis remained stable[57]. This suggests that decreased GSH is not only caused by lower ATP levels but also by decreased levels of intracellular substrates for de novo synthesis of GSH. However, the availability of the three precursors amino acids does not decrease during storage. On the contrary, one study has shown that the levels of all three precursors for GSH are increased during storage[41]. Another study reported also increased glycine and cysteine levels during storage while the third GSH precursor glutamate decreased compared to baseline levels[36].

A decreasing GSH/GSSG ratio despite higher PPP activity suggests that the oxidative stress exceeds the antioxidant capacity of RCCs. Several techniques have been tested to counteract this issue by broadly using two different kind of approaches; reducing the oxidative stress by reducing the ROS formation or increasing anti-oxidant capacity by adding more antioxidants to the additive solution [49,58].

It is hypothesized that by depleting oxygen from RCCs, less ROS will be formed which results in less oxidative stress. While accumulation of oxidative stress markers, such as prostaglandins and thromboxane were still observed during anaerobic storage[49], it is also shown that storage under hypoxic conditions resulted in a better preservation of glutathione homeostasis[52] and higher fluxes through the PPP[52]. In another study vitamin C (ascorbate) and N-acetylcysteine were added during storage, both known as anti-oxidants, which resulted in higher GSH levels and less accumulation of GSSH[58]. In addition, lower oxidative stress markers including malondialdehyde, were observed resulting in decreased haemolysis compared to controls[58]. Thus adding anti-oxidants could be a solution for reducing the damage caused by oxidative stress. However, this is at the cost of a reduced glucose uptake by RCCs because glucose and ascorbate both compete for the same transporter into the cell, leading to reduced flux through the glycolysis and PPP[58].

Donor erythrocyte metabolism after transfusion

In contrast to the large number of studies performed on metabolic changes during *in vivo* aging and during storage, little is known about the metabolic recovery from storage in RCCs after transfusion. It is known that certain aspects of the storage lesion are reversible[62–64]. This is underscored by different *in vitro* rejuvenation techniques[65]. Several studies have shown that by adding different substrates e.g. pyruvate, phosphate, adenine and inosine, several metabolite levels increase[65]. However, these rejuvenation techniques are labour intensive and therefore not well applicable in blood bank practice. There are a limited number of studies that address the recovery of donor erythrocytes after transfusion, which are presented below.

Glycolysis

Data on the recovery of glycolysis intermediates levels after transfusion are still lacking. However, limited data on the recovery of ATP levels, the main product of glycolysis are available[62,63]. A restoration of ATP levels can be used as a measure of glycolysis activity. It has been observed that ATP levels can restore from ATP depletion after transfusion

in healthy volunteers and surgical patients[62,63]. Depending on the additive solution, ATP levels restore almost completely from 35 days of storage to initial levels at the start of storage. This process takes four hours to seven days after transfusion depending on the additive solution[62]. ATP levels of donor erythrocytes recover fastest after storage in CPDA-1 and slowest after storage in AS-1 and AS-3[62]. Another study showed a restoration of ATP levels in SAGM stored RCCs within 12 hours. However, this was studied in 14 days stored blood, in which ATP levels were not completely depleted[63]. The mechanism of regaining ATP levels is yet unclear, although multiple explanations have been postulated. In the circulation there is plenty of substrate to fuel the glycolysis. Furthermore, the pH in the bloodstream is higher which results in increased PFK activity.

Luebering-Rapoport shunt

As mentioned above, 2,3-DPG is almost completely depleted after two weeks of storage in most commonly used additive solutions, such as SAGM. Several studies have been performed to investigate the regeneration of this metabolite after transfusion. In surgical patients 2,3-DPG levels were compared between patients transfused with stored allogenic RCCs and patients transfused with autologous salvaged blood. In the first group, significantly lower 2,3-DPG levels were observed. These 2,3-DPG levels did increase over time, but were not yet normalized at day three after transfusion[64]. In a second study patients undergoing gynaecological surgery were transfused with RCC stored for 10-14 days of storage. This study showed that 2,3-DPG levels in the donor RCCs regenerate to normal levels within 36-48 hours. Moreover, in a study performed on healthy volunteers 2,3-DPG levels restored almost completely within 72 hours to up to 95% of the initial 2,3-DPG level[62].

The clinical relevance of 2,3-DPG has been shown in humans and in several animal models[66–69]. Different experiments on rats showed that RCCs with higher 2,3-DPG concentration resulted in better oxygenation of peripheral tissues[66–68]. This does not imply that higher 2,3-DPG levels always lead to better oxygenation of tissues. An animal model showed that too high 2,3-DPG levels impair the oxygen uptake in the lungs which in the end results in decreased systemic oxygenation[70].

The effect of 2,3-DPG depletion in transfused RCCs is different in healthy volunteers and critically ill patients. A study performed on healthy volunteers showed no difference in oxygenation of the brain and thenar eminence muscle comparing a 7 days stored transfusion with a 42 days stored RCC[71]. This is not surprising, since in healthy volunteers no oxygenation deficit exists in contrast to critically ill patients. Furthermore, healthy volunteers have adequate compensation mechanisms to cope with a decreased

oxygenation transport capacity while in critically ill patients these mechanisms are often impaired. Indeed, in severe traumatic brain injured patients an effect of storage time on oxygenation capacity has been observed[69]. RCCs stored for less than 19 days improved the brain oxygenation while longer stored products had no beneficial effect on oxygenation.

Pentose phosphate pathway and glutathione metabolism

Data on PPP fluxes and glutathione metabolism of transfused RCCs are still lacking. GSH levels gradually decrease during storage, which can be reversed *in vitro* using rejuvenation solutions[65]. Therefore, we hypothesize that GSH levels in transfused cells normalize after transfusion. After normalization it is expected that these levels gradually decrease, similar as seen during physiological aging. Furthermore, we hypothesize that the oxidative stress in erythrocytes decreases after transfusion compared to storage. Consequently, less GSH needs to be utilized leading to a decreased flux into the PPP and normalization of GSH levels. Besides less utilization of GSH due to less oxidative stress, it is likely that regeneration of GSH increases as more precursors of GSH will become available.

Conclusions and opportunities for future investigations

Erythrocytes have a distinct metabolic pattern during aging. This physiological aging is characterized by decreased enzyme activity of almost all enzymes related to the glycolysis, Luebering-Rapoport shunt and PPP resulting in decreased ATP, 2,3DPG and GSH levels. Aging during storage shows a slightly different pattern. In non-alkaline additive solutions ATP levels also decline, but 2,3-DPG levels are almost completely depleted within two weeks. In alkaline additive solutions ATP and 2,3-DPG levels can be maintained for a longer period. PPP activity is also dependent on the additive solution used and can be either stable during storage or increased due to increased oxidative stress. Multiple studies have been focusing on preventing the storage lesion, but normalizing one pathway always seemed to be at expense of another. Besides all the different factors mentioned above we have to consider that not only environmental factors influence metabolic fluxes in cells, there is also a genetic component with a significant effect on the different pathways. It is seen in twin studies that a donor specific variability exists for at least GSH, GSSH and ATP levels of 79%, 60% and 53-64% respectively [59–61].

Remarkably, metabolic recovery of this storage lesion is only superficially investigated. One of the few aspects known is that ATP and 2,3-DPG levels are normalized within 4

hours-7 days and 24-72 hours, respectively. Data on PPP activity and glutathione metabolism after transfusion are still lacking. Investigating these compounds is a prerequisite to improve current storage conditions. Techniques, such as metabolomics, to determine metabolite levels after storage have greatly increased our knowledge on the behaviour of different metabolites during storage. Ideally, metabolite levels after transfusion in fresh and stored RCCs should be compared using different additive solutions, which has now become realistic through the emergence of metabolomics approaches that require much less cells than previous methods. Data obtained in this sort of analyses could lead to new insights to further improve storage techniques and it could confirm or refute the hypothesis that metabolic changes during storage are clinically relevant.

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CHAPTER 6

Storage of red blood cells in the alkaline storage solution PAGGGM improves metabolism after transfusion but has no effect on post transfusion recovery

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Abstract

Additive solutions are used to limit changes that red blood cells (RBCs) undergo during storage. Several studies have shown better preservation of glucose and redox metabolism using the alkaline additive solution PAGGGM (phosphate-adenine-glucose-guanosine-gluconate-mannitol). In this randomized open label intervention trial in 20 healthy volunteers, the effect of storage in PAGGGM on post transfusion recovery (PTR) and metabolic restoration after transfusion was assessed.

Subjects received either an autologous RBC concentrate stored for 35 days in SAGM or in PAGGGM. As a reference for the PTR, a 2-days stored autologous biotinylated RBC concentrate stored in SAGM was simultaneously transfused. RBC phenotype and PTR were assessed after transfusion. Biotinylated RBCs were isolated from the circulation for metabolomics analysis up to 24 hours after transfusion.

The PTR was significantly higher in 2 days stored RBCs than in 35 days stored RBCs after 2 and 7 days after transfusion: 96% [90-99] versus 72% [66-89] and 96% [90-99] versus 72% [66-89] respectively. PTR of SAGM and PAGGGM stored RBCs did not differ significantly. Glucose and redox metabolism were better preserved in PAGGGM stored RBCs. This difference was still present directly after transfusion but disappeared within one day after transfusion. No differences in RBC phenotype were found besides an increased complement C3 deposition on 35 days RBCs stored in PAGGGM.

Our data indicate that despite better metabolic preservation, storage in PAGGGM did not result in an increased PTR. Finally, RBC that were recovered from circulation after transfusion showed reversal of the metabolic storage lesion *in vivo*.

Introduction

Directly after red blood cell (RBC) transfusion, a significant number of RBCs is cleared from the circulation[1,2]. Several studies have reported that post-transfusion recovery (PTR), i.e. the percentage of RBCs that circulate at 24h after transfusion, is heterogeneous across the donor population, with units showing poor PTR (<75%) below Food and Drug Administration and European Council thresholds[3]. In some patients up to 38% of the transfused RBCs is cleared in the first 24 hours[1,2]. Similar observations on the heterogeneity of PTR as a function of genetic make-up of the donors have been recapitulated in mechanistic studies in rodents models showing an impact of iron and redox metabolism on PTR[4]. A low PTR might result in a larger need of blood products as well as a more frequent exposure to the potential harmful side effects of RBC transfusion[5,6].

RBC storage time is one of the factors associated with a decreased PTR[1]. During storage RBCs undergo several metabolic and morphological changes, known as the "storage lesion". The metabolic changes include decreased cellular levels of 2,3-diphosphoglycerate(2,3-DPG), adenosine triphosphate (ATP) and an impaired redox metabolism[7–11].

Decrease of the intracellular pH contributes significantly to the storage lesion. The rate limiting enzyme of glycolysis, phosphofructokinase (PFK), is inhibited at pH < 7.0[12]. The additive solutions that are used to preserve RBCs is another important factor in maintaining RBC integrity during storage. However, both citrate-phosphate-dextrose (CPD, anticoagulant), as well as the standard additive solution for storage of RBCs in Europe (saline-adenine-glucose-mannitol (SAGM)), have a pH of 5-6, resulting in a relatively low pH for the RBC concentrate. The low pH of the additive is readily buffered by RBC incubation to the solution during processing of the unit. However, the intracellular pH progressively acidifies during storage as a function of lactate formation from ongoing glycolysis.

It is unclear to what extent these metabolic changes are reversible. ATP and 2,3-DPG levels can restore within 72 hours after transfusion[13–15]. However, storage also impacts the main antioxidant pathways[16] (e.g., the pentose phosphate pathway – PPP and glutathione - GSH metabolism), purine[16] and lipid oxidation[17]. It is unknown whether these metabolic lesions also improve after transfusion.

Reduction of storage time or improvement of additive solutions may result in increased RBC quality and PTR. Several studies have shown better preservation of glycolysis activity and redox metabolism using alkaline, chloride free additive solutions[18,19]. Storage

in alkaline additive solutions results in chloride shifts from intracellular to extracellular, with an opposite flux of hydroxyl ions into the RBC. The hydroxyl ions increase the intracellular pH and thereby activate PFK. An experimental additive solution PAGGGM (phosphate-adenine-glucose-guanosine-gluconate-mannitol) showed better *in vitro* preservation of 2,3-DPG levels and higher ATP levels throughout storage but is not tested *in vivo* so far[18].

We hypothesized that: 1) storing RBCs in PAGGGM results in improved RBC metabolism during storage which in turn would improve post-transfusion survival; 2) storing RBCs in PAGGGM changes expression of eat-me and don't eat me signals on stored RBCs.To investigate these hypotheses we performed a randomized controlled trial, comparing RBCs stored in SAGM with RBCs in PAGGGM. To this end, differentially biotinylated RBCs were used, which allowed the post transfusion quantification, isolation and phenotypic analysis of donor RBCs. In this manner, the metabolic status of the donor RBCs before and on different time points after transfusion was assessed by metabolomics. In addition, the expression of several "eat me" and "don't eat me" markers were quantified on the transfused cells.

Material and methods

Study design

An open label randomized controlled trial was conducted to study the effect of storage time and additive solution on the PTR of RBCs. Subjects were randomly allocated to receive autologous biotinylated RBC products stored in saline-adenine-glucose-mannitol (SAGM) or the experimental additive solution phosphate-adenine-glucose-guanosine-gluconate-mannitol (PAGGGM). Volunteers received 2 and 35- days stored RBC concentrates.

Twenty healthy male subjects were recruited. Prior to inclusion, each subject was screened by a research physician and was approved as an autologous blood donor by the national blood bank. Screening included a health questionnaire, physical examination, electrocardiogram and blood examination. Healthy males between the age of 18 and 35 were included. Potential subjects who used medication on doctor prescription, with alcohol and/or drugs abuse, donated/lost >500 ml of blood three months before start of study or had abnormalities during the medical screening were excluded from study participation. Participants were compensated for time lost and received travel allowance.

The study was approved by the Institutional Review Board of the Academic Medical Center, Amsterdam the Netherlands and registered in the Dutch trial register (NTR6492). Written informed consent was obtained from each subject prior to screening.

Blood donation

Twenty subjects donated 500 mL and 200 mL of whole blood in citrate-phosphate-dextrose at the national blood bank 35 and 2 days prior to the transfusion, respectively. After overnight storage at 20 – 24°C, the units whole blood were processed according to the Dutch Blood Bank standards: separated into plasma, buffycoat and leukoreduced RBCs. RBC storage was randomly allocated to saline-adenine-glucose-mannitol (SAGM, 150 mmol/L NaCl, 1.25 mmol/L adenine, 50 mmol/L glucose, 29 mmol/L mannitol, pH 5-6, Fresenius Kabi) or the experimental additive solution (PAGGGM, 1.44 mmol/L adenine, 1.44 mmol/L guanosine, 47.5 mmol/L glucose, 40 mmol/L Na-gluconate, 8 mmol/ $NaH_2PO_4 \cdot 2H_2O$, 8 mmol $Na_2HPO_4 \cdot 2H_2O$, 55 mmol/L mannitol, pH 8-9). Blood was stored according to standard blood bank practice until biotinylation.

Biotinylation of red blood cells

One day prior to transfusion, 25 ml of each donation was labelled with 15 µg /ml (low density) or with 48ug/ml (high density) biotin. Directly after labelling, the biotinylation was checked using flow cytometry, to ensure that the different populations were distinguishable. To exclude any effect of biotin label concentration, half of each group received fresh RBCs labelled with a low biotin concentration and stored RBCs labelled with a high concentration. The other half received fresh RBCs labelled with a high concentration biotin and stored RBCs labelled with a low concentration of biotin. All units were biotinylated in a closed system according to methods described previously[20].

In short, biotin (EZ-link Sulfo-NHS-LC-Biotin, 100mg; Thermo scientific) was diluted in SAGM (Fresenius Kabi) in two different concentrations: 48 µg /ml and 15 µg /ml, and sterilized using a 0.22 µ filter (Fresenius HemoCare, Fresenius Kabi) in a closed system using a sterile connection device (Sterile Turbing Welder-Terumo BCT, TSCD II). A portion of 25 mL red cell concentrate from each donation was transferred to a sample bag in a closed system and washed once with 125 mL SAGM (2000 RPM with $5.00X10^7$ ACE) to avoid binding of the biotin to plasma proteins. Supernatant was removed with a plasma clamp, after which the cell pellet was resuspended in biotin diluted in SAGM and incubated for 60 minutes at 22 °C. The biotinylated RBCs (bioRBCs) were washed twice with 125 mL SAGM (2000 RPM with $5.00X10^7$ ACE). After removing the supernatant, the cell pellet of the 2 days stored RBCs was resuspended in SAGM to its original haematocrit.

35-days stored bioRBCs were resuspended in their allocated additive solution (SAGM or PAGGGM).

Study day

Subjects were admitted for one day at the research unit of the Department of Intensive Care. Prior to the study procedures, a research physician medically checked the subjects and blood was cross matched as additional safety procedure. Fresh and stored bioRBCs were mixed prior to infusion in a closed system. 50 mL mixed bioRBC sample was infused within ten minutes. The infusion system was flushed with 100 mL 0.9% saline (Baxter, Viaflo).

Ten minutes, 30 minutes, 1, 2, 4, 6 and 8 hours after infusion blood samples were drawn from an indwelling venous cannula and stored in the dark at 4 °C until further analysis. Temperature, blood pressure, heart rate and respiration rate were monitored up to eight hours after transfusion. Whole blood samples were drawn using EDTA tubes (BD Plymouth, United Kingdom).

In the hospital's general laboratory of clinical biochemistry, total bilirubin, lactate dehydrogenase (LDH), aspartate aminotransferase (ASAT) and haptoglobin were measured with the Roche cobas c702 chemical analyser (Roche Diagnostics, Indianapolis, USA).

Follow up:

All study subjects were followed for 90 days after transfusion: blood was sampled 1, 2, 7, 30 and 90 days after transfusion. On return visits, blood samples were collected using venipunctures. All samples were stored in the dark at 4 °C until further analysis.

Antibodies

The following antibodies were used in our study: CD44 (conjugate FITC, Diaclone, product code: 852601010, 1:5), CD47 (2D3) (conjugate FITC, eBioscience, San Diego, USA, product code: 11-0478-42, 1:5), CD47 (B6H12) (Santa Cruz Biotechnology,sc-12730, 1:250), CD55 (conjugate FITC, Sanquin, Amsterdam, The Netherlands, product code: M2192, 1:5), CD59 (conjugate FITC, Diaclone, Besançon, France, product code: 954361010, 1:5), CD147 (conjugate PE, eBioscience, San Diego, USA, product code: 12-1472-42, 1:10), ICAM-4 (Abnova, Taipei, Taiwan, product code: H00003386-B01P, 1:20), Band 3 (EMA staining) (conjugate FITC/PE, Biotum, Fremont, USA, product code: 92013, 25 µl (undiluted)), Annexin V (conjugate FITC, VPS diagnostics, Hoeven, The Netherlands, product code: A705, 1:200), Lactadherin (conjugate FITC, Bio Connect, Huissen, The Netherlands, product code: BLAC-FITC, 1:100), Anti-lactadherin (conjugate PE, R&D systems, Minneapolis,

USA, product code: IC27671P, 1:10), GAS-6 (R&D systems, Minneapolis, USA, product code: MAB885, 1:5), Protein-S (Abcam, Camebridge UK, product code: ab61364, 1:5), Anti complement iC3b (Quidel, San Diego, USA, product code: A209, 1:5), Wheat germ agglutinin (conjugate alexa 488, Invitrogen, Carlsbad, USA, product code: W11261, 1:500), Anti-thrombospondin (A6.1) (Thermo Scientific, Landsmeer, The Netherlands, product code: MA5-13398, 1:50), CR1 (rabbit polyclonal; kind gift from Prof. Dr. M. R. Daha, Leiden University Medical Center, the Netherlands, 1:500), Bcam (R&D systems, productcode AF148, 1:100), C3-19 (a kind gift from Diana Wouters, Department of Immunopathology, Sanquin, Amsterdam, The Netherlands, 1:100), 4N1K (4N1K, amino acid sequence; KRFYVVMWKK, 1:100), 4NGG (amino acid sequence; KRFYGGMWKK, 1:100), Streptavidin (conjugate alexa 488/647, Invitrogen, Carlsbad, USA, product code: S-32354, 1:200), Mouse isotype control IgG1 (Invitrogen, Carlsbad, USA, product code: MG100, 1:5), Mouse isotype control IgG1 (conjugate alexa 488, Invitrogen, Carlsbad, USA, product code: MG120, 1:5), Mouse isotype control IgG2A (Invitrogen, Carlsbad, USA, product code: MG2a00, 1:5), Mouse isotype control IgG2A (conjugate alexa 488, Invitrogen, Carlsbad, USA, product code: MG2a20, 1:5), Mouse isotype control IgG2A (conjugate PE, Sanquin, 1:50), Rabbit isotype control IgG (Invitrogen, Carlsbad, USA, product code: 026102 , 1:200), Goat anti-human IgG (conjugate alexa 647, Invitrogen, Carlsbad, USA, product code: A-21445, 1:200), Goat anti-mouse IgG (conjugate alexa 488, Invitrogen, Carlsbad, USA, product code: A-11001, 1:200), Goat anti-rabbit (conjugate FITC, Invitrogen, Carlsbad, USA, product code: A11008, 1:200), Rabbit anti-goat (conjugate FITC, Invitrogen, Carlsbad, USA, product code: A11078, 1:200)

Flow cytometry

EDTA anticoagulated blood samples were analysed one day after sampling. The amount of bioRBCs was quantified by flow cytometry after staining with Streptavidin Alexa Fluor 647 conjugate (Thermo Fisher Scientific, catalogue number: S32357). The PTR of bioRBC was calculated with respect to the percentage bioRBCs present in the circulation 10 minutes after transfusion. The PTR was assessed until 90 days after transfusion. To assess the clearance of RBCs in the first 10 minutes, the distribution of fresh and stored bioRBCs in the mixed RBC concentrate prior to infusion was compared with the distribution of fresh and stored RBCs recovered from the circulation after transfusion. A difference in distribution indicates that one of the populations is faster cleared from the circulation.

To investigate the role of several "eat me" and "don't eat me" markers double and triple staining was used to quantify the clearance markers separately in the different populations. Membrane markers were expressed as percentage of fluorescent positive cells or as mean fluorescent intensity (MFI) per population.

All flow cytometry experiments were performed with a flow cytometer (BD FACS, BD Fortessa + HTS, BD Biosciences). The data were analysed with computer software (FACS-Diva v.8.0.1, BD Biosciences).

Glucose-6-phosphate dehydrogenase activity

The G6PD activity was assessed per individual RBC using a flowcytometric assay as previously described[9,21]. In short, 10 µl RBC suspension was suspended in 90 µl PBS and 100 µl 0.125 mmol/l sodium nitrate (Sigma St. Lous, USA) and incubated for 20 minutes at room temperature for 20 minutes at a roller bench to convert all oxyhemoglobin into methemoglobin. RBCs were washed three times with PBS (200 RCF, 1 minute) and resuspended in 1 ml PBS.

In the next step methemoglobin was reduced to oxyhemoglobin in a NADPH and thus G6PD dependent matter. To do so, the RBC suspension was incubated for 90 minutes at 37˚C with 100 µl PBS, 18 µl 0.28 mmol/L glucose (Sigma), and 6 µl 0.3mmol/L methylene blue sulphate (Merck, Darmstadt Germany). After incubation 2.5 µl 400 mmol/L potassium cyanide (Sigma) was added and incubated at room temperature for 5 minutes, followed by incubating 5 µl sample in 100 µl 1 mmol/L H_2O_2 (Sigma) in PBS for 3 minutes. After incubation, the cells were stained with streptavidin alexa fluor 647 (Invitrogen, S32357, 1:200) for 30 minutes at 4 degrees Celsius. Finally, the samples were washed twice (PBS, 1500 RCF 3 minutes) and analysed on the flowcytometer (BD FACS, BD Fortessa + HTS, BD Biosciences).

Cell sorting procedure

BioRBCs were isolated in two separate steps from the EDTA whole blood samples. This procedure is based on the differences in biotin density. In the first step, bioRBCs were isolated using magnetic beads, followed by the second step using flowcytometric cell sorting (supplemental Figure S1).

Blood samples were washed twice to remove plasma and the buffy coat (SAGM, 1000 RCF for 15 minutes, 4˚C and 2500 RCF, 5 minutes, 4˚C). RBCs were incubated for 30 minutes at 0˚C with streptavidin-647 (1:100, Thermo fisher scientific) and washed (2500 RCF, 5 minutes, 4˚C), followed by incubation for 15 minutes at 4˚C with 80 µl anti-Alexa fluor 647 micro beads (Milteny biotec). Incubated cells were flushed through an LS column (Milteny biotec) while put in a magnetic field. This column was flushed three times with a flushing buffer containing PBS (Fresenius Kabi), 10% tri sodiumcitrate (Merck) and 0.5% human serum albumin (Brocacef) to remove the unlabeled cells. The retained BioRBCs in the LS column were eluted in the flushing buffer after the column was removed from

the magnetic field. This cell suspension, containing bioRBCs with two different densities biotin, was sorted using flowcytometry. After flow cytometry, the cell suspension was centrifuged (2500 RCF, 5 minutes, 4˚C), removing the supernatant. The cell pellet was stored at -80˚C until further analysis.

Liquid chromatography mass spectrometry

Metabolomics analyses were performed as extensively described in previous studies[22–24]. A volume of 50µl of frozen RBC aliquots was extracted in 450µl of methanol:acetonitrile:water (5:3:2, *v/v/v*). After vortexing at 4°C for 30 min, extracts were separated from the protein pellet by centrifugation for 10 min at 10,000g at 4°C and stored at −80°C until analysis. Ultra-High-Pressure Liquid Chromatography-Mass Spectrometry analyses were performed using a Vanquish UHPLC coupled online to a Q Exactive mass spectrometer (Thermo Fisher, Bremen, Germany). Samples were analyzed using a 5-minute gradient as described. Solvents were supplemented with 0.1% formic acid for positive mode runs and 1 mM ammonium acetate for negative mode runs. MS acquisition, data analysis and elaboration was performed as described[25–27].

Statistical analysis

Data was visually checked for distribution. Normally distributed and non- normally distributed data was reported as mean (standard deviation) or as median [first quartile-third quartile], respectively. Normally distributed data were analysed using the students t test or ANOVA analysis and non-parametric data was first ranked prior to analysis or analysed with the Wilcoxon-sum rank or Kruskal-Wallis test. Significance between two groups (SAGM versus PAGGGM and 2 days versus 35 days stored) was evaluated using a two-way ANOVA with grouping variables additive solution or storage time and time after transfusion. To assess the effect of additive solution, only the 35 days stored RBCs were included for analysis. $P < 0.05$ was considered to be statistically significant.

Metabolomics analysis was considered to be exploratory, therefore no Bonferroni correction was applied. As predefined in the trial registry (NTR6492), only glycolysis, pentose phosphate pathway and redox metabolism related metabolites were analysed. 35 days stored SAGM RBCs were compared with 35 days stored PAGGGM RBCs. Two days stored SAGM RBCs were compared with 35 days stored SAGM RBCs. Data was analysed using computer software (R studio version, R version 3.5.2).

Results

Clinical effects

Twenty subjects were transfused with 25 ml 35 days stored autologous RBCs, either stored in SAGM or PAGGGM, divided in groups of 10 volunteers each. As a reference, 25 ml 2 days stored autologous RBCs, all in SAGM, were simultaneously transfused. In 5 subjects only the 35 days stored RBCs were administered due to logistic issues concerning the 2-day stored RBCs. Four of these subjects were allocated to the SAGM group and one subject who received only RBC concentrate was allocated to the PAGGGM group. No adverse events were reported. After transfusion of the RBCs, no effects on bilirubin, haptoglobin, lactate dehydrogenase (LDH) and aspartate aminotransferase (ASAT) were observed in either group (Figure S2). In addition, transfusion of SAGM-RBCs and PAGGGM-RBCs did not affect body temperature, blood pressure, respiratory rate and heart rate during the study day (data not shown).

Storage in PAGGGM does not result in higher PTR

The PTR of 35 days stored PAGGGM-RBCs was compared with the PTR of 35 days stored SAGM-RBCs. The PTR was significantly lower for the PAGGGM-RBCs over 90 days (Figure 1, $p=0.002$). However, in the post-hoc analysis of each separate timepoint, no significant differences were observed (supplement table S1). The 24 hours PTR of 35 days stored PAGGGM-RBCs was 68% [59-87] and 81% [70-93] in SAGM stored RBCs ($p=0.16$).

35 days of storage results in a decreased PTR

As anticipated, the duration of storage had a significant effect on the PTR of 35 days stored RBCs in SAGM ($p<0.05$, Figure 1B.). Post hoc analysis showed that the PTR was significantly higher in 2 days stored RBCs at 2 days (96% [90-99] versus 72% [66-89], $p<0.05$) and seven days (96% [90-99] versus 72% [66-89], $p<0.05$) after transfusion compared to the 35 days stored RBCs. The 24 hours PTR of 2 days stored SAGM-RBCs was 97% [97-105] and 81% [70-93] in 35 days SAGM-RBCs ($p=0.056$).

A similar effect was observed in the PAGGGM group. Here we compared 2 days stored RBCs in SAGM with 35 days stored RBCs in PAGGGM. The 35 days stored RBCs had a significant lower PTR over time ($p<0.01$). In this post-hoc analysis, the PTR was significantly lower at 8 hours, 2 days, 30 days and 90 days after transfusion (supplemental table S2).

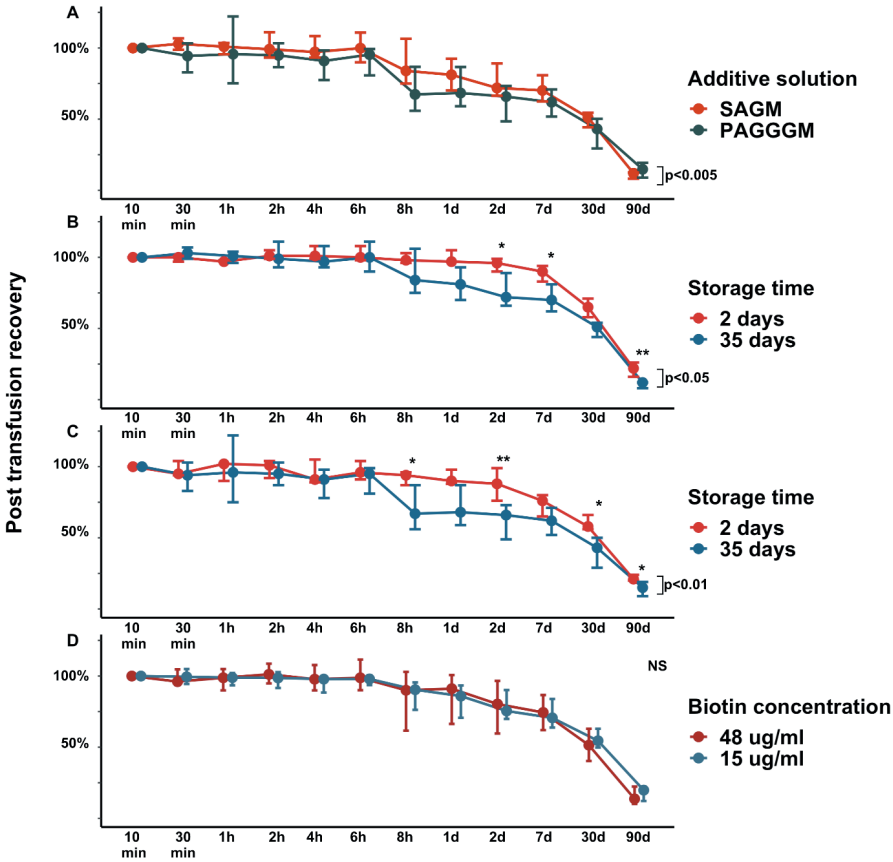

Figure 1. Post transfusion recovery (PTR) is significantly different between 35 days stored SAGM and PAGGGM RBCs over time, but not on a specific time point (A). 35 days of storage in SAGM (B) and PAGGGM (C) results in decreased PTR compared with 2 days of storage in SAGM. Density of biotin did not affect the PTR of RBCs (D). The line plot indicates the median with 25th and 75th percentile of PTR over time, up to 90 days after transfusion.

In addition to the PTR, which was calculated using the 10 minutes after transfusion reference value, we also aimed to assess the clearance of RBCs in the first 10 minutes. We compared the ratio of fresh and stored RBCs in the RBC concentrate prior to transfusion with the ratio of these two populations in the whole blood sample taken 10 minutes after transfusion (supplemental figure S3 and S4). Six subjects, allocated to the SAGM group, received both 2-days stored RBCs and 35 days stored RBCs. In half of them (n=3) relatively more 2 days stored RBCs were cleared in the first 10 minutes, while in the other half more 35-days stored RBCs were cleared. nine subjects allocated to storage in PAGGGM received both RBC concentrates. In three subjects 2-days stored RBCs were cleared at

a higher rate compared to six subjects in whom the 35 days stored RBCs were cleared at a higher rate. However, for the group as a whole there was no statistically significant difference between the ratio of fresh and stored RBCs before and after transfusion.

To exclude any effect of the differential biotinylation on our results, half of each group received 35 days stored RBCs with the high-density biotin and the 2 days stored RBCs low density of biotin. The other half of the participants received RBCs that were labelled in the opposite manner. Our data revealed no effect of the density of biotin on the PTR (Figure 1D).

Expression of "eat me" and "don't eat me" signals after transfusion

In addition to the analysis of the PTR, attempts were made to identify the occurrence of changes of the membrane of the transfused RBCs in time. Transfusion of stored RBCs offers a window in which a relatively large proportion of the RBC is cleared within a short time, thus allowing to detect changes on the RBC that precede their clearance, which may be linked to the clearance. In particular, the expression of several "eat me" and "don't eat me" signals, which have been linked to increased phagocytosis of the RBCs in vitro, were tested. Among these were phosphatidylserine exposure, IgG binding, complement deposition and CD47 expression28. No changes in the expression of potential "eat me" or "don't eat me" signals were found, except for complement deposition on RBCs stored in PAGGGM. PAGGGM-RBCs showed significantly higher C3 deposition 10 minutes, 30 minutes, one hour and two hours after transfusion (supplement Figure S5A). However, also higher iC3b deposition was found on these time points (supplement Figure S5B).

Increased glycolytic activity in PAGGGM RBCs

Besides the PTR and the expression of several membrane markers related to RBC clearance, the metabolic profile of the donor RBC was also determined prior and after the transfusion. To this end, donor RBCs were isolated from whole blood samples after transfusion by a protocol in which the biotinylated RBCs were first enriched for by magnetic bead isolation and then subjected to cell sorting by FACS. Using this protocol, pure populations of high and low biotinylated RBCs were extracted from blood samples of the healthy volunteers (Supplementary figure 1). After their sorting and processing, metabolomics was performed on the different cell fractions, and the metabolic profile of the RBCs was determined in time. As described before, glycolysis was more active during storage in PAGGGM (Figure 2).

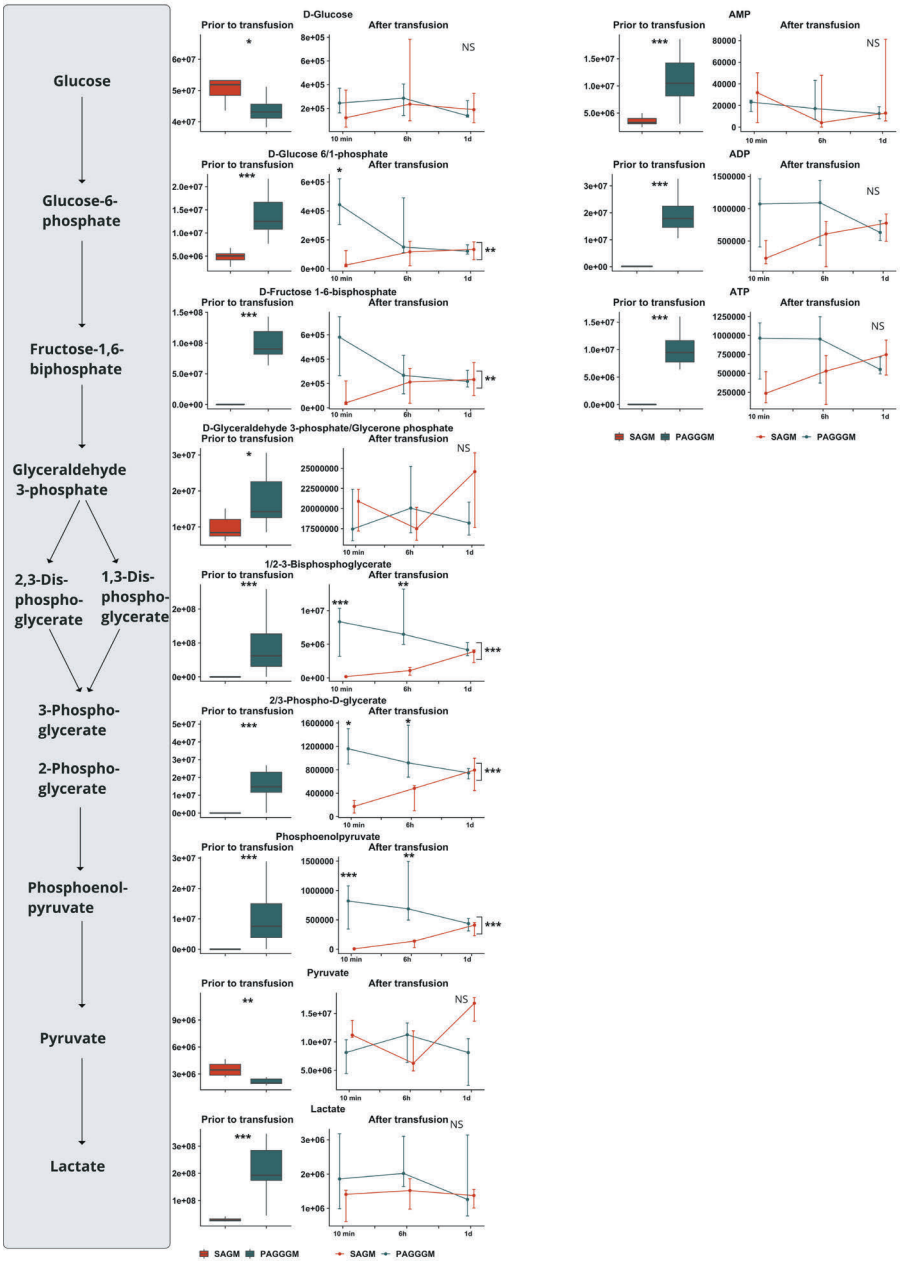

Figure 2. Metabolomics data of glycolysis metabolites comparing 35 days stored RBCs in SAGM with 35 days stored RBCs in PAGGGM. The boxplots show metabolite levels in RBCs prior to transfusion. Line plots indicate the median with 25th and 75th percentile of metabolite levels over time in the recovered RBCs, up to one day after transfusion.* p<0.05, ** p<0.01, *** p<0.0001, NS not statistically significant

As a result, ATP levels were significantly higher and less glucose was present in PAGGGM stored RBCs, resulting in increased levels of 7 metabolites of the glycolysis including hexose phosphate (including unresolved isomers glucose 1 or 6-phosphate and fructose 6-phosphate with the chromatographic conditions adopted in this study), fructose-1,6-biphosphate, glyceraldehyde-3-phosphate, diphosphoglycerate (including isomers 1,3 and 2,3-DPG), phosphoglycerate, phosphoenolpyruvate and lactate. Several differences remained shortly after transfusion. Glucose-phosphate was higher 10 minutes after transfusion in the PAGGGM stored RBCs but was similar after 6 hours. DPG, phosphoglycerate and phosphoenolpyruvate were significantly higher up to six hours after transfusion. In SAGM RBCs, these levels increased after transfusion, while in PAGGGM RBCs a decrease was observed. Consequently, one day after transfusion, no differences were found between SAGM and PAGGGM stored RBCs that were still circulating at 24h. Also, no differences were found in AMP, ADP and ATP levels after transfusion.

Redox metabolism in PAGGGM RBCs

In addition to the metabolomics analysis, G6PD activity was also assessed using a flow cytometric assay. PAGGGM stored RBCs showed an increased PPP flux prior to transfusion indicated by higher levels of 6-phosphogluconate levels in the metabolomics analysis (Figure 3) and increased G6PD activity as determined by our flow cytometric assay (Figure 6). Immediately after transfusion, the differences between 6-phosphogluconate levels and G6PD activity were corrected. On the other hand, ribose phosphate – the final product of the PPP – was significantly higher in transfused PAGGGM RBCs at 10 min, 6h and 1d after transfusion (Figure 3). Sedoheptulose phosphate levels were higher 10 minutes after transfusion in PAGGGM stored RBCs, while prior to transfusion higher levels were found in SAGM stored RBCs. Glutathione disulfide (GSSG) levels were significantly higher in PAGGGM RBCs prior to transfusion. After transfusion these differences rapidly disappeared. Reduced glutathione (GSH) and the breakdown product of GSH, 5-oxoprolin was not significantly different on any of the time point prior or after transfusion.

Purine metabolism in PAGGGM RBCs

Hypoxanthine is a deaminated purine, which is a product of ATP, AMP and adenosine metabolism. In PAGGGM RBCs hypoxanthine levels were significantly higher compared to 35 days stored SAGM RBCs. This difference remained present up to 6 hours after transfusion in the restored RBCs (Figure 4). Hypoxanthine can be converted via xanthine to urate with hydrogen peroxide as by-product. Xanthine was significantly higher in PAGGGM prior to transfusion but not in the RBC that were recovered after transfusion. In contrast, urate levels were similar in SAGM and PAGGGM RBCs before and after transfusion.

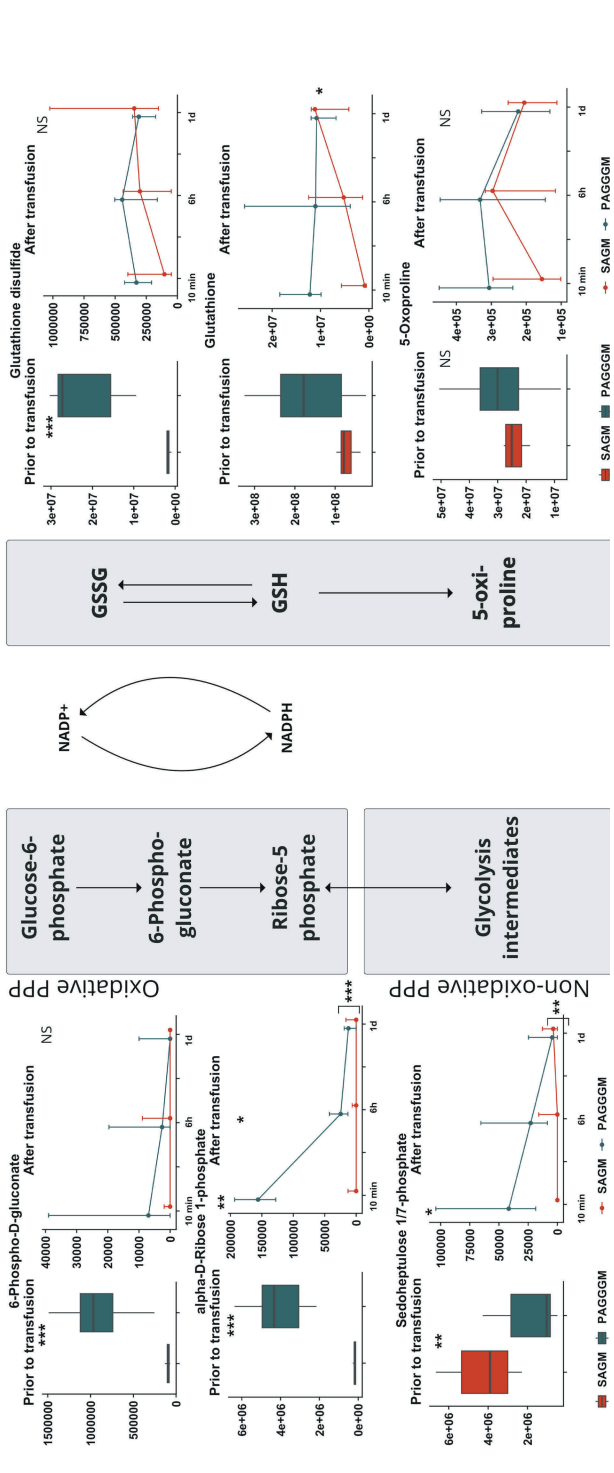

Figure 3. Metabolomics data of PPP and glutathione related metabolites comparing 35 days stored RBCs in SAGM and 35 days stored RBCs in PAGGGM. The boxplots show metabolite levels in RBCs prior to transfusion. Line plots indicate the median with 25th and 75th percentile of metabolite levels over time in the recovered RBCs, up to one day after transfusion.
* p<0.05, ** p<0.01, *** p<0.0001, NS not statistically significant

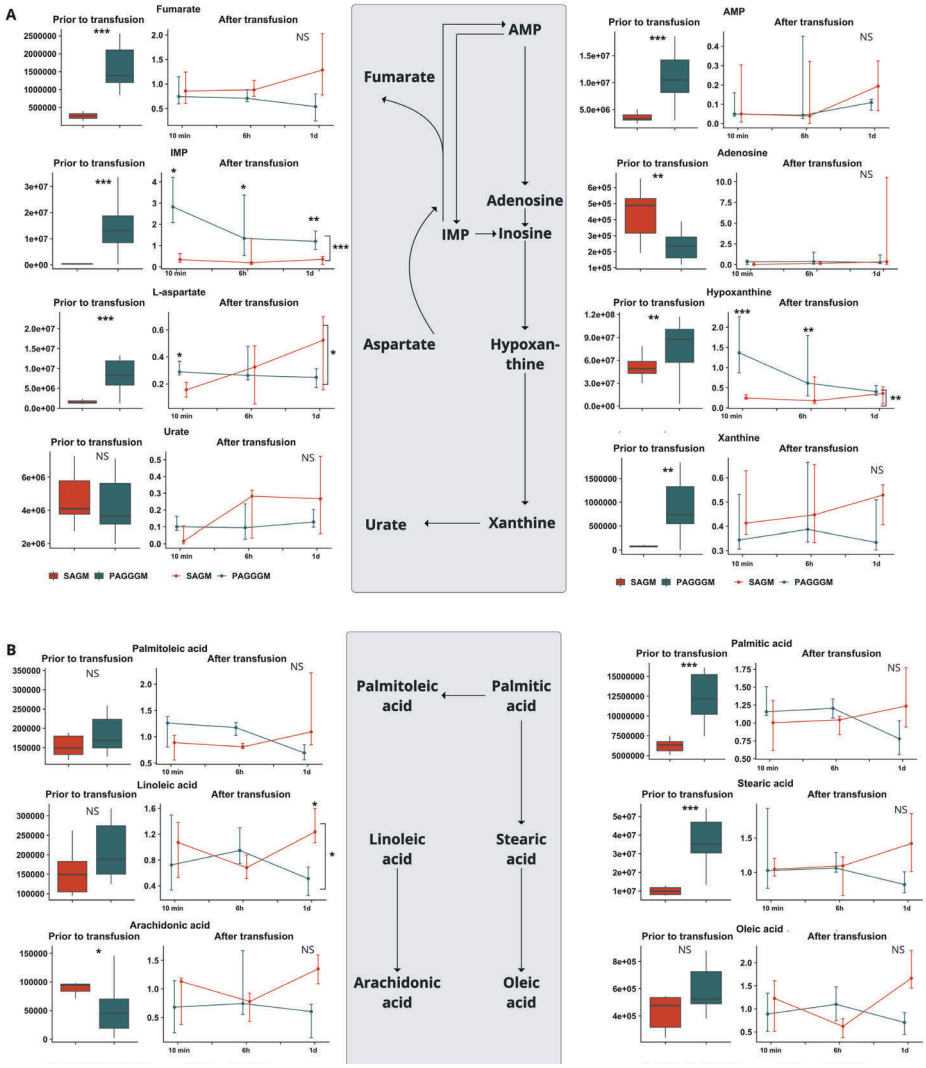

Figure 4. Purine (A) and free fatty acid (B) metabolism comparing 35 days stored RBCs in SAGM and 35 days stored RBCs in PAGGGM. The boxplots show metabolite levels in RBCs prior to transfusion. Line plots indicate the median with 25th and 75th percentile of metabolite levels over time in the recovered RBCs, up to one day after transfusion. * p<0.05, ** p<0.01, *** p<0.0001, NS not statistically significant

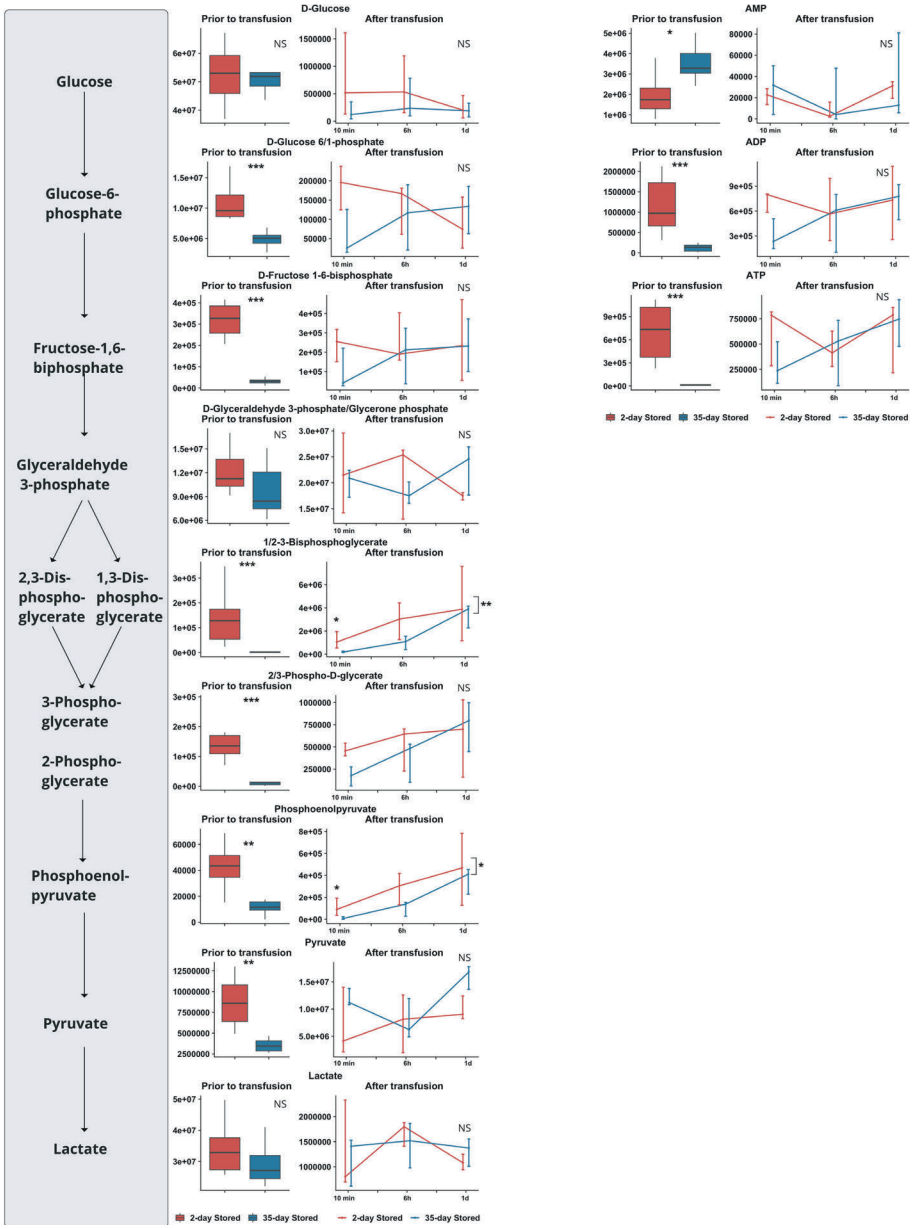

Figure 5. Metabolomics data of glycolysis related metabolites comparing RBCs stored for 2 days and 35 days RBCs in SAGM. The boxplots show metabolite levels in RBCs prior to transfusion. Line plots indicate the median with 25th and 75th percentile of metabolite levels over time in the recovered RBCs, up to one day after transfusion. * p<0.05, ** p<0.01, *** p<0.0001, NS not statistically significant

Free fatty acids in PAGGGM RBCs

Palmitic and stearic acid were significantly higher in PAGGGM RBCs prior to transfusion, while in the restored cells no difference was found. Palmitoleic acid and oleic acid, were similar in the PAGGGM and SAGM RBCs. Lineolic acid was similar in PAGGGM and SAGM RBCs prior to transfusion, while 1 day after transfusion lower levels were found in the PAGGGM RBCs. Arachidonic acid showed increased levels in PAGGGM RBCs prior to transfusion. However, there were no differences after transfusion.

Glycolysis metabolism 35 days after SAGM storage

After 35 days of storage glycolysis activity is decreased (Figure 5). This was indicated by complete depletion of ATP levels, decreased DPG levels and increased AMP levels compared to 2 days stored RBCs. In addition, the levels of several glycolysis metabolites (hexose phosphate, fructose-1,6-biphosphate, 2/3-phosphoglycerate, phosphoenolpyruvate and pyruvate) were decreased in the 35 days SAGM stored RBCs compared to 2 days SAGM stored RBCs.

Directly after transfusion, glycolysis activity recovered, illustrated by immediate recovery of ATP levels. Furthermore, AMP and ADP levels were similar after transfusion. DPG and phosphoenolpyruvate remained higher in 2 days stored RBCs until 10 minutes after transfusion, despite inter-donor heterogeneity.

Redox metabolism

No evident effect of storage time was found in G6PD activity, which was similar in the 2- and 35 days stored RBCs (Figure 6). Only 6-phosphogluconate levels were significantly higher in 2 days stored RBCs prior to transfusion. However, this normalized directly after transfusion. Ribose-phosphate was higher in 2 days stored RBCs 6 hours after transfusion, while prior to transfusion no effect was observed. In addition, no differences were found in sedoheptulose levels (Figure 7).

We did observe an increased breakdown of GSH and GSSG in the 35 days stored RBCs. Oxoproline levels were significantly higher in 35 days stored RBCs with similar levels of GSH but lower levels of GSSG, indicating an increased breakdown of GSH. After transfusion, no differences in these levels were found in the cells that recovered.

Figure 6

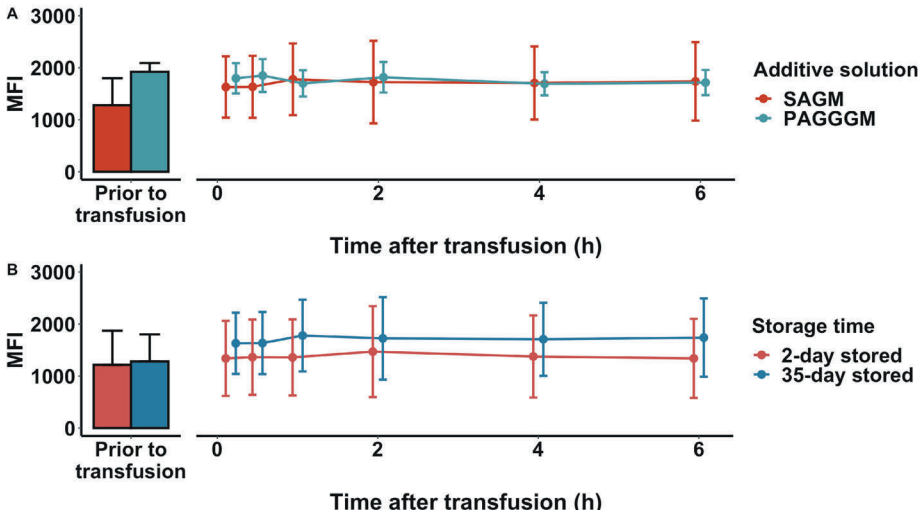

Figure 6. G6PD activity in 35 days stored RBCs. (A) comparing storage in SAGM with PAGGGM (B) and comparing 2 and 35 days stored RBCs. The bar chart represents the mean + standard deviation of G6PD activity of RBCs in the transfusion bag prior to transfusion. The line chart depicts the mean ± standard deviation of G6PD activity in the recovered RBCs after transfusion.

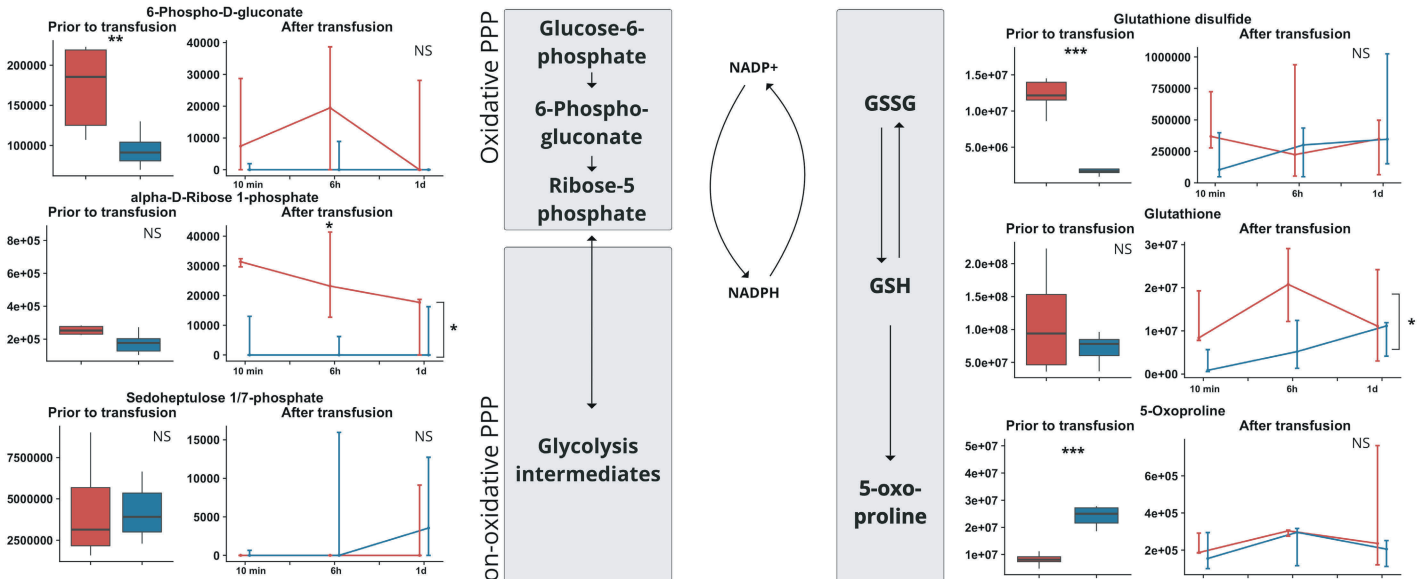

Figure 7. Metabolomics data of PPP and glutathione related metabolites comparing RBCs stored for 2 days or 35 days RBCs in SAGM. The boxplots show metabolite levels in RBCs prior to transfusion. Line plots indicate the median with 25th and 75th percentile of metabolite levels over time in the recovered RBCs, up to one day after transfusion. * p<0.05, ** p<0.01, *** p<0.0001, NS not statistically significant

Purine metabolism and free fatty acids metabolism in 35 days stored SAGM RBCs

During storage in SAGM, increased purine metabolism was found. Hypoxanthine and xanthine levels were significantly higher in 35 days stored SAGM RBCs compared to 2-days stored SAGM RBCs. In addition, the precursors AMP, adenosine and fumarate were also significantly higher after 35 days of storage. However, directly after transfusions, none of the differences between fresh and stored RBCs remained.

Arachidonic acid was significantly higher prior to transfusion in the 35-days stored RBCs. After transfusion, no difference in arachidonic acid levels was found in the restored cells. The other investigated fatty acids (palmitoleic acid, palmitic acid, stearic acid, oleic acid and linoleic acid), were not affected by storage time in this study.

Discussion

This is the first randomized trial investigating the PTR and metabolic recovery of transfused RBCs in different additive solutions using biotinylated autologous RBCs. The main findings of this study are: 1) longer storage time of RBCs is associated with a decreased PTR at 2-7 days after transfusion, 2) despite a better preservation of the glycolysis, PPP and redox metabolism, storage of RBCs in PAGGGM did not result in an increased PTR and 3) despite the metabolic storage lesion, transfused RBCs that can survive the first day after transfusion are characterized by normalization of glycolysis the pentose phosphate pathway, purine metabolism and fatty acid metabolism.

A negative effect of storage time on PTR has been reported previously[1]. However, in our study we found a different timing of this effect. We found a significantly lower PTR two-seven days after transfusion, but no significant difference in the first 24 hours. This is in contrast with the study of Luten *et al.* where, longer stored RBCs had a decreased 24-hour PTR. Moreover, in the study by Luten *et al.* the cells that survived the first 24 hours had a similar lifespan as the shorter stored RBCs[1]. First of all, it is worth noting that Luten et al. had relied on classic ^{51}Chromium radiolabeling to determine PTR, while here we employed biotinylation strategies. The discrepancies between the ^{51}Cr labeling method and biotinylation may be attributable to the different findings. Other significant differences include the type of recipient, while we examined healthy volunteers, in the study of Luten *et al.* PTR was assessed in haematological patients. It has been shown that underlying disease such as sepsis is associated with markers of increased clearance including decreased deformability[28,29]. Another explanation

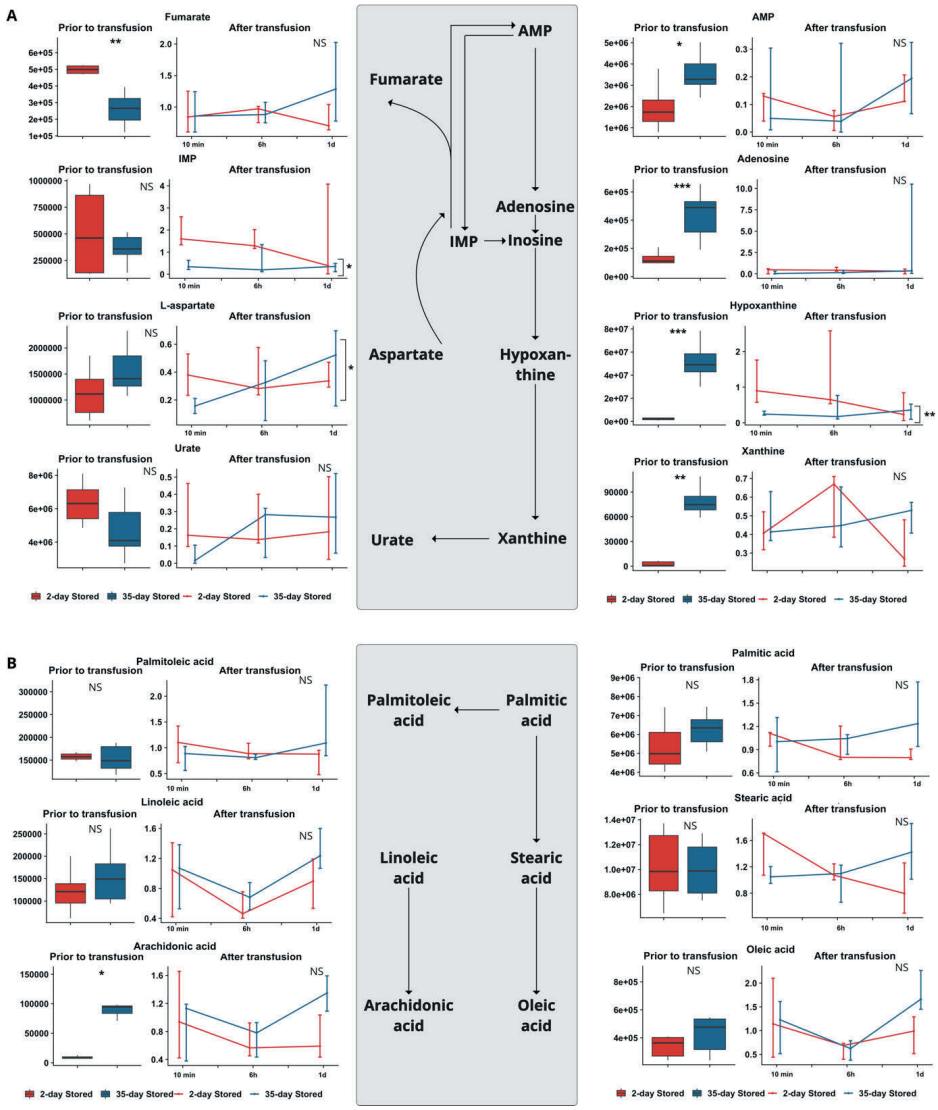

Figure 8. Purine (A) and free fatty acid (B) metabolism comparing RBCs stored for 2 days or 35 days RBCs in SAGM. The boxplots show metabolite levels in RBCs prior to transfusion. Line plots indicate the median with 25th and 75th percentile of metabolite levels over time in the recovered RBCs, up to one day after transfusion. * p<0.05, ** p<0.01, *** p<0.0001, NS not statistically significant

may be the origin of the blood product, as we examined an autologous RBC transfusion. An allogeneic component could result in an increased clearance compared to an autologous transfusion. In a comparable study in healthy volunteers, which assessed

the PTR up to six hours after transfusion, also no difference in PTR between 2 and 35 days stored RBCs was found[30].

Here we show that, in concordance with previous published work, storage in PAGGGM resulted in better preservation of glycolysis, redox metabolism and purine metabolism[10,18]. However, that this did not result in an increased PTR was unexpected, especially in light of recent double-blind randomized studies showing that improvements in metabolic phenotypes correlate to improved PTR[31]. Analogously, boosting RBC metabolism through storage in additive solution 7 (AS-7), which is a similar alkaline additive solution, showed an increased PTR[19,32]. The main difference between these additive solutions is that AS-7 contains bicarbonate instead of guanosine and gluconate. How this may affect RBC clearance remains unclear.

After transfusion, the RBCs metabolic profile improved. This recovery can be explained by a technical bias of the study design, cells that are amenable to recover from storage, biotinylation and transfusion are those that survived intra- and extra-vascular haemolysis via splenic sequestration and phagocytosis. Recent studies have indeed shown that RBCs with the highest degree of morphological alterations (small microcytic erythrocytes) are the most likely to be removed rapidly from circulation upon transfusion in humans and mouse models[33]. Acknowledged the potential survival bias of this study, we can also speculate on the effect of exposure to the physiological extracellular pH in the blood circulation after transfusion. After 35 days of storage the pH of RBC concentrates decreased to approximately 6.5[18] while the pH in the circulation is 7.45 under physiological circumstances. By infusing the RBCs in the circulation, the PFK and G6PD activity may normalize and thereby restore the glucose flux through glycolysis and PPP, provided the enzymatic activities are not irreversibly compromised by oxidant stress-induced fragmentation of metabolism regulatory elements (e.g., N-terminus of band 3) or post-translational modifications, such as beta-elimination of thiols to dehydroalanine or deamidation of asparagines – as reported for all glycolytic enzymes in end of storage units[34–39].

To investigate factors involved in RBC-clearance we assessed several eat me and don't eat me signals. An increased C3 deposition was found in 35 days stored PAGGGM RBCs. It is unclear whether this concerns active C3, since also the deposition of the non-active component iC3b was increased. Furthermore, none of the other assessed markers differed significantly. This may be explained by the chosen timeframe in which we measured these markers. The phenotype of the donor RBC was assessed up to six hours after transfusion, while in this timeframe the RBC clearance was limited. Another possibility

is that RBCs with a different phenotype were immediately captured in the spleen and were thus not represented in the blood samples after transfusion.

In this study we showed that the metabolic profile is better in fresh RBCs and second, after two days the PTR of fresh RBCs is higher than longer stored RBCs. Better preservation of 2,3-DPG levels could potentially increase the capacity of RBCs to transport oxygen. This has several potential clinical benefits: improved preservation of 2,3-DPG levels could potentially increase the capacity of RBCs to transport oxygen. Furthermore, compared to 35 day old RBCs, fresh RBCs were shown to have higher levels of PPP metabolites up to 1 day post transfusion (despite similar G6PD activity), suggestive of an increased overall antioxidant potential. In addition, increased PTR could reduce the number of RBC transfusions, reducing harmfull side-effects and costs. However, in critically ill patients, it is shown that transfusion of fresh RBCs does not improve outcome compared to longer stored RBCs[40–42]. Nonetheless, for patients with a long term dependency on RBC transfusion due to chronic blood disorders such as sickle cell anemia and thalasemia, it would be beneficial to increase the interval between transfusions. We hypothesize that in these patients, transfusion of fresh RBC could increase the interval between RBC transfusion resulting in a reduced number of transfusions in the longterm, and thereby limiting long term side effects of transfusion including iron overload.

This study has various strengths including the use of an autologous transfusion in healthy volunteers. Using this approach, we ensured that each volunteer served as their own control and we thereby excluded the immunological effects of antigen mismatched blood. The most important limitation of this study is that the PTR was not assessed in the first 10 minutes after transfusion. It is therefore unknown whether transfused RBCs are cleared in the first 10 minutes. Subjects received a 2 days stored and a 35 days stored RBC concentrate simultaneously. We calculated the distribution of the two populations in the RBC concentrate and the distribution of the transfused products in the recovered RBCs after transfusion. If a difference in the ratio before and after transfusion was found, this would suggest that one population was cleared faster than the other. As 35-days stored RBCs suffer more metabolic damage from storage than 2-days stored RBCs, we expected increased clearance of the 35-days stored RBCs. On an individual level, differences were found between the ratio in the transfusion product and the ratio in the recovered RBCs after transfusion. But on group level, no statistically significant difference was found to indicate a relatively faster clearance of 2 days or 35 days stored RBCs in the first 10 minutes. However, it is possible that our study was not sufficiently powered to detect these differences. A more precise method to estimate clearance in the first 10 min would be to precisely assess circulating volume, followed by calculation of the

expected fraction of bioRBCs and comparison with the measured fraction of bioRBCs. Another limitation is that we infused 25 ml per RBC concentrate. It is unclear how fast the spleen is saturated by RBCs and therefore how these results can be extrapolated to a standard RBC concentrate that contains 300 ml. Our study is also biased towards testing metabolism of the cells that could be recovered upon transfusion, making the cells to be more likely to be metabolically divergent (i.e., the ones carrying the most damage upon storage) less likely to be recovered from the bloodstream of autologous recipients. In this view, it is worth noting that prior studies on the metabolic impact of autologous transfusion of end of storage units indeed reported significant alteration of plasma and RBC metabolism in the recipient[43], suggesting that any difference in the present study may at least in part, go undetected due to the limited volume transfused (25 ml vs half a unit in prior studies). Yet, the present study shows a proof of feasibility with respect to the capacity to recover in vivo biotinylated RBCs for omics analyses upon transfusion. And finally, due to the small sample size and the exploratory character of this study no Bonferroni correction was applied in the metabolomics analysis. This may have resulted in a type 1 error. However, we assessed multiple metabolites in several pre-specified pathways, and as the majority of these metabolite were affected, we believe that the chance of a type 1 error is limited.

In conclusion, despite a better metabolic profile of PAGGGM RBCs, PAGGGM storage did not lead to a higher PTR. Furthermore, the metabolic storage lesion was corrected within a day after transfusion in cells that did not undergo intra- or extra-vascular haemolysis and still circulated 24h after transfusion. Finally, 35 days of storage resulted in a lower PTR compared to 2 days of storage.

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Supplemental material

Table S1.

	Additive solution		
Time after transfusion	**SAGM**	**PAGGGM**	**p value**
10 minutes	100[100-100]	100[100-100]	1.000
30 minutes	103[99-107]	94[83-103]	0.190
1 hour	101[96-104]	96[75-122]	0.853
2 hours	99[93-111]	95[87-103]	0.529
4 hours	97[93-108]	91[78-98]	0.190
6 hours	100[90-111]	95[81-99]	0.481
8 hours	84[75-106]	67[56-87]	0.052
1 day	81[70-93]	68[59-87]	0.165
2 days	72[66-89]	66[49-73]	0.190
7 days	70[62-81]	62[52-71]	0.190
30 days	51[44-54]	43[29-50]	0.236
90 days	12[8-12]	15[9-19]	0.400

Table S2. (n=6 subjects receiving both 2-days stored and 35-days stored RBCs)

Time after transfusion	**SAGM-RBCs**		
	2 days stored (%)	**35 days stored (%)**	**p value**
10 minutes	100[100-100]	100[100-100]	1.000
30 minutes	100[97-101]	103[99-107]	0.5622
1 hour	97[97-98]	101[96-104]	0.4923
2 hours	101[99-105]	99[93-111]	0.8749
4 hours	101[99-108]	97[93-108]	0.3132
6 hours	100[98-108]	100[90-111]	0.7925
8 hours	98[96-103]	84[75-106]	0.4278
1 day	97[97-105]	81[70-93]	0.0559
2 days	96[90-99]	72[66-89]	0.0420
7 days	90[83-94]	70[62-81]	0.0496
30 days	65[58-71]	51[44-54]	0.0879
90 days	22[16-26]	12[8-12]	0.0076

Table S3. (n=9 subjects receiving both 2-days and 35 days stored RBCs)

Time after transfusion	**PAGGGM-RBCs**		
	2 days stored (%)	**35 days stored (%)**	**p value**
10 minutes	100[100-100]	100[100-100]	1.000
30 minutes	95[94-104]	94[83-103]	0.6038
1 hour	102[90-102]	96[75-122]	0.9048
2 hours	101[92-104]	95[87-103]	0.6607

Table S3. (n=9 subjects receiving both 2-days and 35 days stored RBCs) *(continued)*

Time after transfusion	PAGGGM-RBCs		
	2 days stored (%)	35 days stored (%)	p value
4 hours	91[89-105]	91[78-98]	0.5490
6 hours	96[91-104]	95[81-99]	0.9048
8 hours	94[87-96]	67[56-87]	0.0435
1 day	90[88-98]	68[59-87]	0.1128
2 days	88[76-99]	66[49-73]	0.0076
7 days	76[65-80]	62[52-71]	0.1135
30 days	58[56-66]	43[29-50]	0.0140
90 days	21[20-24]	15[9-19]	0.0172

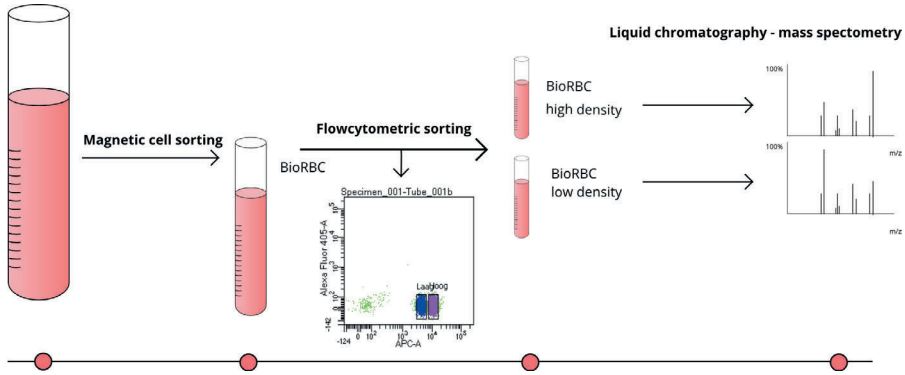
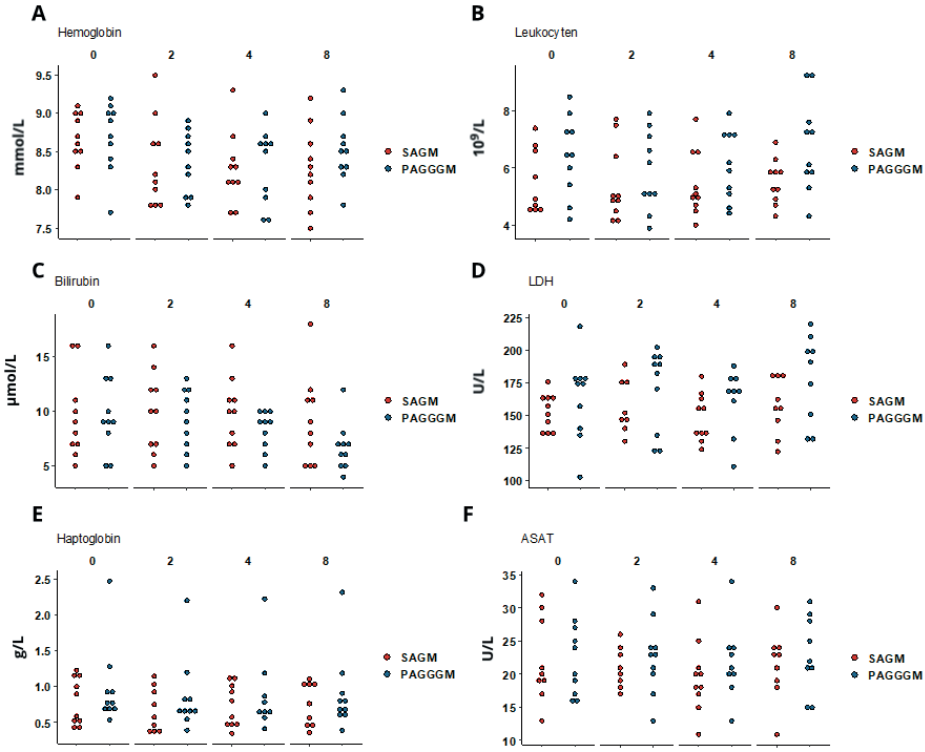


Figure S1


A
Hemoglobin
mmol/L
B
Leukocyten
10^9/L
C
Bilirubin
µmol/L
D
LDH
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SAGM
PAGGGM

Figure S2.

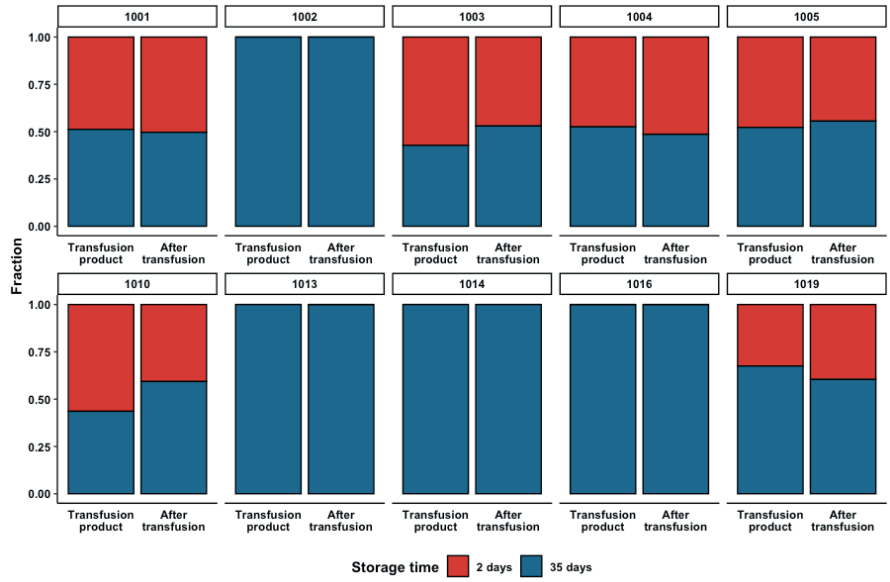


Figure S3. Distribution of 2 days and 35 days stored RBCs in the transfusion product and in the measured bioRBCs 10 minutes after transfusion in subjects allocated to storage in SAGM.

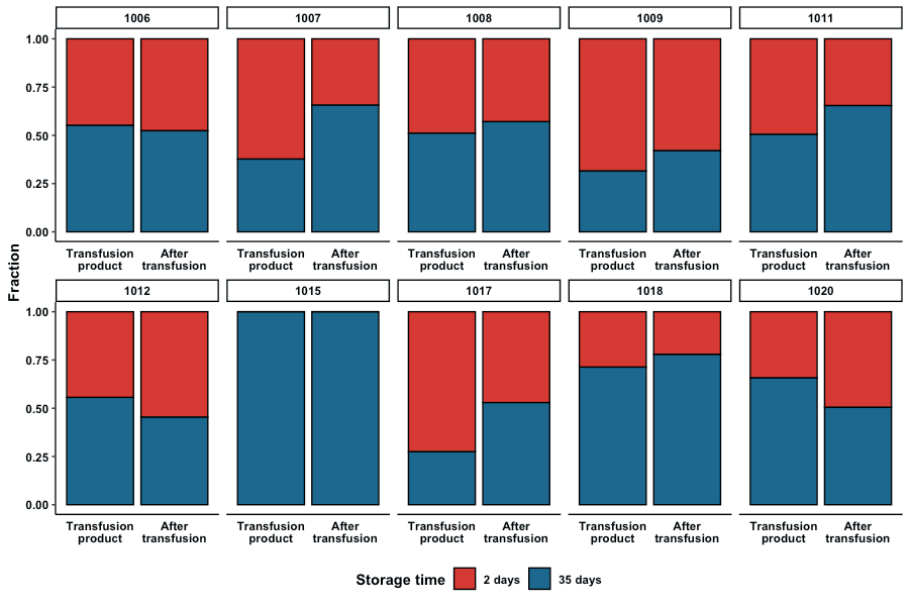


Figure S4. Distribution of 2 days and 35 days stored RBCs in the transfusion product and in the measured bioRBCs 10 minutes after transfusion in subjects allocated to storage in PAGGGM.

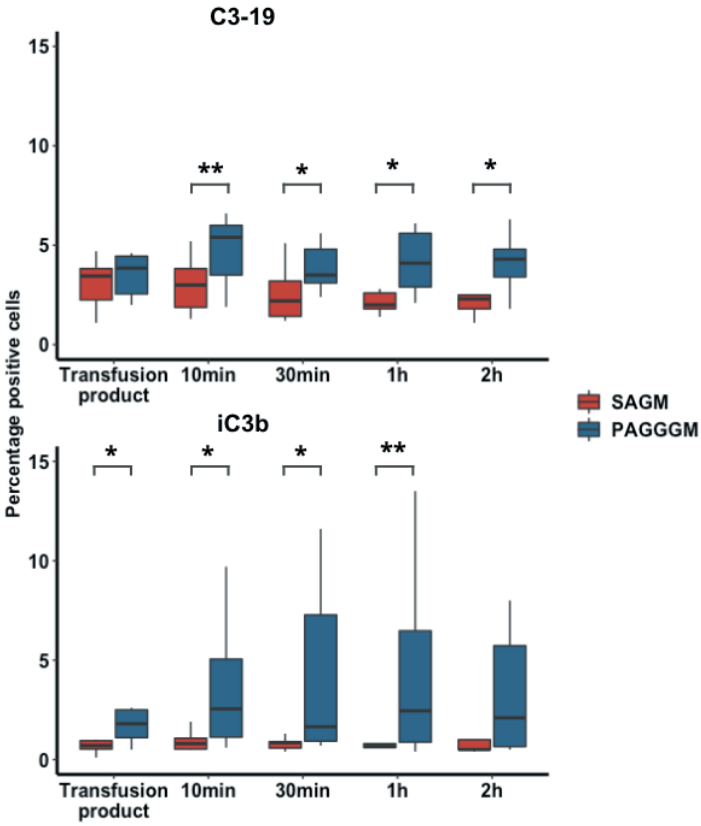
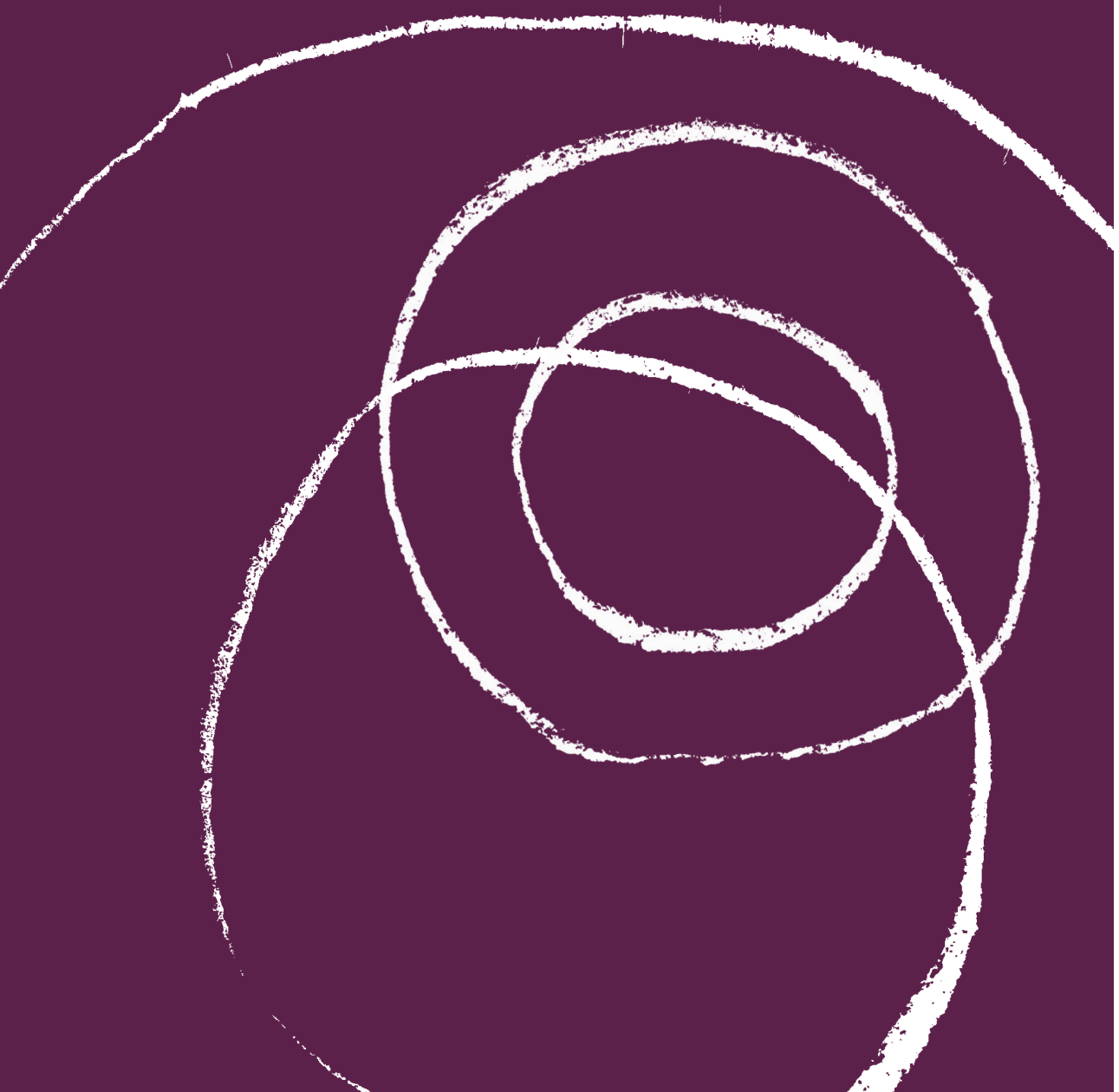


Figure S5. Significantly more RBCs displayed C3-19 binding after transfusion($p<0.05$), but not in the transfusion product prior to transfusion ($p=0.110$, panel A). Prior to transfusion and until one hour after transfusion significantly more iC3b was bound to SAGM-RBCs compared to PAGGGM-RBCs (panel B). But after two hours this difference is not statistically significant ($p=0.086$).



CHAPTER 7

Biotinylation of platelets for transfusion purposes: a novel method to label platelets in a closed system

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Summary

Background: Labeling of platelets is required to measure the recovery and survival of transfused platelets *in vivo*. Currently a radioactive method is used to label platelets. However, application of those radio-labeling methods are limited by both safety issues and the inability to isolate transfused platelets from the circulation. Biotinlabeled platelets (bioPLT) are an attractive non-radioactive option. However, no validated protocol to biotinylate platelets is currently available for human studies.**Study design and methods:** Six platelet concentrates (PCs) derived from pooled buffy coats were sub-aliquoted and biotinylated at day 1 and day 7 of storage. To distinguish the effect of the processing steps from the effects of biotin incubation, two control groups were used: 1) 'sham' samples were processed on day 1 and day 7 but without the biotinylation reagent and 2) control samples were assessed on day 1 and day 7 but without any processing other than the PC isolation. For the biotinylation procedure, 50 ml of PCs was washed twice and incubated with 5 mg/L biotin for 30 minutes in a closed system. Stability of the biotin label after irradiation and storage of the biotin-solution was quantified. As measure of platelet activation, phosphatidylserine exposure and CD62p expression were assessed.

Results: The biotin labeling density was reproducible. After biotinylation, 98.4% ± 0.9% of platelets were labeled. Platelet counts, pH and 'swirling' were within the range accepted by the Dutch blood bank for standard platelet products. Biotinylated platelets were not more activated compared to sham samples, but were more activated than the controls.

Conclusion: We developed a standardized and reproducible protocol according to Good Practice Guidelines (GPG) standards, for biotin-labeling of platelets for clinical purposes. This method can be applied as non-radioactive alternative assess survival and recovery of transfused platelets *in vivo*.

Introduction

Labeling of platelets is required to distinguish transfused platelets from the recipient's own circulating platelets. This enables the measurement of recovery and survival of transfused platelets *in vivo*.

Radiolabeling of platelets with the radioactive isotopes ^{111}Indium-oxin and/or ^{51}Chromium is currently the golden standard to test for survival and recovery of platelets.[1] This method is used to evaluate the effects of donor-, recipient- and platelet storage factors on platelet survival after transfusion in the recipient.[2] Also, radiolabeling is required by the FDA to analyze the effect of altered platelet storage protocols, such as new additive solutions and pathogen-reduction technologies.[3] However, radiolabeling exposes the recipient to potential harmful ionization. Therefore, this method cannot be used in vulnerable patients, in particular pediatric patients and neonates. Moreover, the use of radiolabeled platelets is strictly regulated, which limits it's applicability for research purposes. In Europe, radiolabeling is restricted to studies in healthy volunteers only. Therefore, a non-radioactive alternative to label platelets is desired. Biotin, a water-soluble vitamin (B8) can be used as a non-radioactive label for various cells.[4-9] The N-hydroxysuccinimide ester of the biotin reagent binds in a non-specific manner to cellsurface proteins of the platelet (Figure 1A, biotinylation of platelets). Unlike radiolabeling, biotin-labeling has the major advantage that it is possible to selectively isolate biotin-labeled platelets from the recipients' circulation. Also, a biotin label can even be used to trace multiple platelet populations concurrently by using different densities of the biotin label per platelet.

Recently, our group reported a protocol for the biotinylation of red cell concentrates, according to Good Practice Guidelines (GPG).[10] This product is currently available for clinical use and the first trials have started recruiting patients. (Registered at trialregister.nl: NTR 6596, NTR6492). However, labeling of platelets is difficult compared to erythrocytes, due to their propensity to become activated.

Biotin-labeling of platelets has been used in various animal models[7,11,12] and twenty years ago, biotin-labeled platelets (bioPLTs) were safely infused in humans for the first time.[13] In this pilot study, platelet recovery was measured after infusion of biotinylated platelets in ten healthy male subjects. However, this study was hampered by activation of the platelets during biotinylation. We developed a method to minimize platelet activation during biotinylation. Because platelets concentrates are particularly prone to bacterial

contamination compared to other blood products, a method for biotinylating platelets in a closed system was developed.

In this manuscript, we describe the results of a method to produce a biotinylated platelet product in a closed system, in accordance to GPG, with minimal platelet activation, which can be used in clinical research in humans.

Material and Methods

Platelet concentrates

Platelet concentrates (PCs) were manufactured and stored by Sanquin Blood Bank, according to the Dutch Blood Bank standards. Whole blood (WB) collections (500 ml) were obtained from volunteer, non-remunerated donors. WB was centrifuged and separated after overnight hold into red cell concentrates, plasma and buffy coats. To obtain a PC, pooled buffy coats from five donors were re-suspended in 100% plasma or 65% PAS-E (Terumo BCT, Inc Lakewood CO USA) and 35% plasma and leukoreduced by filtration. Single donor apheresis PCs were obtained according to the manufacturer's instructions (Trima, Terumo BCT). PCs were stored under gentle agitation, at 20-24°C. Informed consent to use their blood for research purposes was obtained from all donors. The validation protocol was approved by the Sanquin department of Quality Assurance.

Preparation of the biotin solution

Sulfo-NHS-biotin was dissolved in phosphate-buffered saline (PBS,140.3 mmol/L NaCl, 10.9 mmol/L $Na_2HPO_4.2H_2O$, 1.8 mmol/L $NaH_2PO_4.2H_2O$, pH 7.4, Fresenius Kabi) to a concentration of 50 mg/L (EZ-link Sulfo-NHS--Biotin, 100mg; Thermo scientific). The sulfo-NHS-biotin-solution was sterilized by passing it through a 0.22 µM filter (Fresenius HemoCare, Fresenius Kabi) using a sterile connection device (Sterile Turbing Welder-Terumo BCT, TSCD II) and a 600-mL container (Compoflex, Fresenius Kabi). After filtration of the sulfo-NHS- biotin-solution, the biotinylation took place in a closed system, to prevent microbiological contamination. To obtain the final concentration of 5 mg/L, the sulfo-NHS-biotin solution was diluted 1:9 in Platelet Additive Solution (PAS-E: 0.030% $MgCl.12H_2O$, 0.037% KCl, 0.105% $NaH_2PO_4.2H_2O$, 0.318% $C_6H_5Na_3O_7.2H_2O$, 0.405% NaCl, 0.442 %, $C_2H_3NaO_2.3H_2O$, 0.769% $Na_2HPO_4.12H_2O$, pH: 7.1-7.5) (Terumo BCT), and divided in portions of 100 mL, and used within 30 minutes after diluting

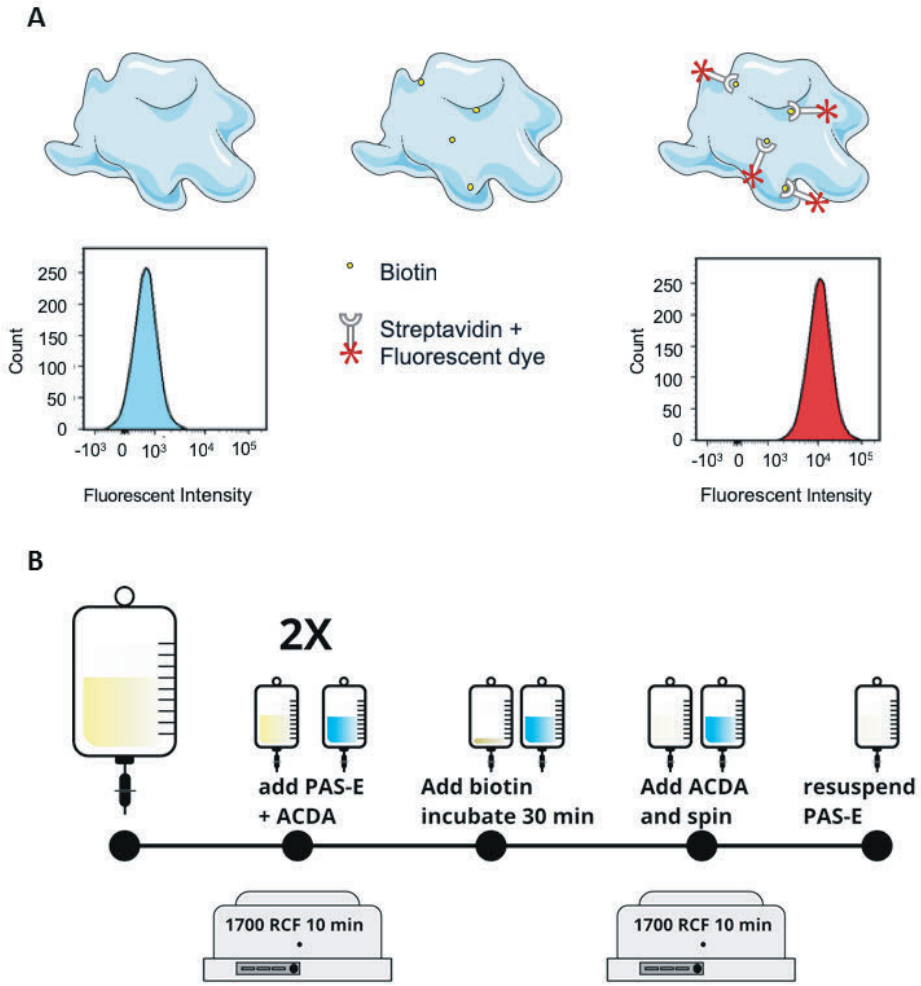

Figure 1. Biotinylation of platelets and biotinylation procedure. **A.** Platelets (top left), are incubated with sulfo-NHS-biotin (top middle), bioPLTs are counterstained with fluorescently labeled streptavidin (top right). Unlabeled platelets show a characteristic FACs histogram, the fluorescently labeled biotin-streptavidin causes the peak of the histogram to shift horizontally. **B.** A proportion of the PC was washed twice in PAS-E. Prior to each washing step, the PC was acidified to 10% ACD-A. Platelets were incubated with 5 mg/L biotin, diluted in PBS-PAS-E (1:9), for 30 minutes. BioPLTs were washed and resuspended in PAS-E.

Standardized platelet biotinylation procedure

The biotinylation procedure is depicted in Figure 1B. Biotinylation of platelets was performed in a closed system. The full protocol for biotinylation of platelets is provided in Appendix 1. A 50 ml portion of PC was transferred to a small transfer bag. Transfusion of a biotinylated PC aliquot of 50 ml would theoretically achieve a bioPLT enrichment of 2.5-7% in healthy subjects. Plasma proteins that could interfere with biotinylation were reduced by washing the platelet concentrate twice for PCs stored in plasma or once for PCs stored in 65% PAS-E. Prior to centrifugation, samples were acidified by ACD-A (Anticoagulant citrate dextrose Solution A, Terumo BCT) to prevent irreversible clumping during centrifuging (1700 x g for 10 minutes). For the first washing step, 100 ml of 8.5:1.5 PAS-E / ACD-A solution was added to the 50 mL of platelet concentrate. 150 ml of a 9:1 PAS-E/ACD-A solution was added or the second washing step. The washed platelets were incubated with the 100 mL biotin solution, for 30 minutes, under gentile agitation, at 22º Celsius. Twelve ml of ACD-A was added prior to centrifugation (1700 x g for 10 minutes), after which the biotinylated platelets were resuspended in PAS-E, to their original volume of 50 ml. To confirm biotinylation, samples of both biotinylated and unbiotinylated platelets were counterstained with Streptavidin 488 (1:200), Alexa Fluor 488 conjugate (Thermo Fisher Scientific, catalogue number: S32354), incubated for 30 minutes at room temperature, washed (1700 x g for 10 minutes) and measured by flow cytometry on a LSRII + HTS (BD Biosciences). Data were analyzed with FlowJo v(CFC).

Validation experiment

For the validation procedure, aliquots of six in plasma stored PCs were biotinylated at day 1 and day 7 of storage. At these time points a 'sham'-sample was also obtained from the same unit, in which all processing steps were identical to the biotinylated samples, except the incubation with sulfo-NHS-biotin, which was replaced by incubation with a 1:9 PBS-PAS-E solution. Platelets that were biotinylated after one day of storage were subsequently stored for two more days and tested for biotinylation and quality parameters (hence, at day three of storage after donation). To show applicability to various types of PCs, also three platelet concentrates that were obtained via apheresis and three PCs stored in 65% PAS-E were biotinylated at day 1 of storage.

Storage of bioPLT

Two methods of storage of bioPLT were tested: first 50 mL bioPLT and sham platelets were stored for three days after processing and then tested for quality parameters (Supplement Figure 1B). Since this led to inacceptable high platelet activation a second method was tested. We hypothesized that storage of a small sample in this storage bag caused the high platelet activation. Therefore, 50 ml of bioPLT was returned to the

retained original PC, resulting in the original volume of approximately 330 ml consisting of labeled and unlabeled platelets (Supplement Figure 1C). Therefore triple staining was necessary to distinguish platelet activation of the unlabeled and labeled platelets, at day of biotinylation and day 4 to day 7 of storage.

Additional conditions

To evaluate the effect of storage of the sulfo-NHS-biotin solution on biotinylation, a part of the sulfo-NHS-biotin-PBS- solution was stored within 30 minutes after dilution at -30 (± 5) °C. After42 days the frozen sulfo-NHS- biotin-PBS- solution was thawed at 37°C in 10 minutes to approximately 20°C . After thawing, the sulfo-NHS-biotin-PBS- solution was diluted 10 times with PAS-E (at 20°C) to a final concentration of 5 mg/L, aliquoted to portions of 100 ml, and used within 30 minutes.

To analyze the effect of irradiation on the biotin label, biotin-labeled platelets were irradiated after labeling with a dose of 25 Gray (according to standard blood bank regulations). Samples were obtained prior and after irradiation to assess the effect of this treatment on the biotin label.

Platelet quality parameters

Ranges for quality parameters were pre-defined according to local blood bank guidelines and are expressed in Table 1. Blood gas analysis was performed to determine the pH of the platelet concentrates. (Rapidlab 1265, Siemens Medical Solution Diagnostics). Platelet counts were measured on an Advia 2120 (Siemens Medical Solutions Diagnostics).

The morphology of the platelets was assessed both non-invasive (swirl) and invasive (microscopically). To perform the platelet swirling test, the motion of the platelets was assessed visually by gently moving the bag in front of a light source. The swirl was recorded as positive (3), moderate (2), impaired (1) or absent (0). The test was performed by an independent, experienced, laboratory staff member, who was blinded for the intervention. Platelet morphology was also assessed by light microscopy, for which 50 µl of platelet concentrate was mixed with 250 µl 0.5% glutaraldehyde (Merck, Darmstadt Germany) in PBS. The fixed platelets were visualized with a 1000 times magnification (Axio, Zeis, Breda, the Netherlands).

Baseline platelet activation was assessed by CD62P expression.[14] The samples were incubated for 20 minutes at RT with PE-mouse anti-human CD62P (BD Pharmingen Biosciences) and FITC mouse antihuman CD61 (BD Pharmingen Biosciences. For the

triple staining platelets were also incubated with Streptavidin 647 (1:200), Alexa Fluor 647 conjugate (Thermo Fisher Scientific, catalogue number: S32357). Immunoglobulin G1 Mouse PE conjugated (Immunotech SAS, Beckman Coulter, Marseille France) was used as isotype control for the CD62P activation. To assess platelets' ability to become activated, platelets were incubated simultaneously with CD61 and CD62P. Agonists used were 625 nM of thrombin receptor-activating peptide, (TRAP-6-amide/trifluoracetate salt, Bachem AG, Switzerland) or 125 µl/ L of adenosine di-phosphate (ADP, Chronolog, Havertown, USA). The reaction was stopped after 20 minutes by adding formaldehyde 1% (Merck) in PBS.[15] Annexin V binding was assessed as a marker for phosphatidylserine (PS), as described.[16] Flow cytometry was performed using a LSRII + HTS (BD Biosciences). Data were analyzed with FlowJo v(CFC).

The nucleotide content of the platelets was assessed in neutralized perchloric acid extracts, which were stored at -80°C, until batch analysis with high-performance liquid chromatography using a cation exchange, column as described previously.[4,17]

Platelet concentrates were cultured by BacT/ALERT(Bio Merieux), both before and after the biotinylation procedure, to rule out bacterial contamination.

Statistical analysis

Statistical analyses were performed in R, version 3.5.0. Variables were assessed for normality and corresponding statistical tests were performed (paired t-test for parametric data, Mann-Whitney U test for non-parametric data). Differences were considered to be statistically significant if the p-value was < 0.05.

Results

Biotinylation of platelet concentrates

Six pooled buffy-coat derived PCs in plasma, three pooled buffy-coat derived PCs in 65% PAS-E/35% plasma and three apheresis PCs in plasma were biotinylated as described in the protocol (Appendix 1). After the biotinylation-procedure, 98.4% ± 0.9% and 99.0% % ± 0.9% of platelets were labeled with biotin at day 1 and day 7 of storage respectively (Figure 2). There was no difference in biotinylation of PCs obtained from pooled buffy coats and stored in plasma as compared to apheresis PCs and PCs stored in PAS-E. The unbiotinylated fraction and the biotinylated fraction of the PC could be visualized as two distinctive populations on flow cytometry. We confirmed that biotin labeling of platelets is still successful after 42 days of storage of the dissolved biotin solution at

-30° (Figure 3). Irradiation of the biotin-labeled PC with a standard dose of 25 Gray did not affect the degree of biotinylation. (Figure 3). It was not possible to incubate the platelets with biotin and add ACD-A simultaneously. (Figure 3). This would have reduced one processing step. The reduction of biotinylation after additions of ACD-A is probably due to a lower pH.

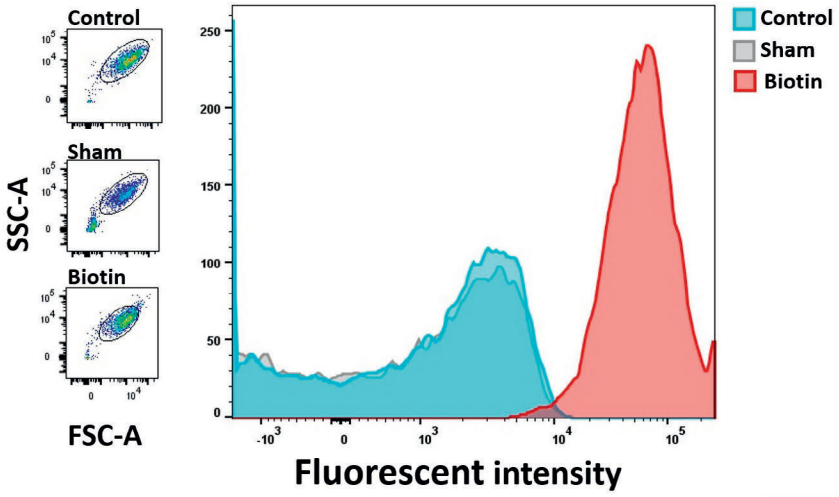

Figure 2. Flow-cytometric analysis of unlabeled (blue), sham (grey) and bioPLTs (red), after incubation with streptavidin-488. The bioPLT show a significant higher fluorescent intensity as compared to the sham and control platelets. 98.4% ± 0.9% of bioPLTs were biotinylated, and can be visualized as a distinct population. Scatters of all three populations are similar (left). Images are from a selected PC, but are representative for the other experiments (n=6).

Effects of the biotinylation procedure on platelet quality parameters

Platelet quality parameters were assessed to assure bioPLTs met the requirements of the Dutch blood bank. Ranges for quality parameters were pre-defined and are expressed in Table 1. Platelet counts, pH and 'swirling' score were within the range accepted by the Dutch blood bank for all products. Morphology scores were higher for control platelets, as compared to sham and bioPLTs. There was no significant difference between sham and bioPLT morphology scores, indicating that the difference to control was caused by the processing steps with repeated centrifugation steps and not by incubation with biotin. The procedure led to a marginal decrease in platelet count. Annexin V binding was not affected by the procedure (Figure 4). CD62P expression was increased to the same extent in both the sham and bioPLT (Figure 5). Hence, the processing steps, but not biotin itself led to activation of platelets.

Table 1. Platelet quality parameters

Reference	**Biotinylation**	**platelet count**	**pH**	**Swirl**	**Morphology**	**ATP***
values	**on day**	**(800-1600 x 109)**	**(6.3-7.5)**	**(>1)**	**>200**	
Control (N=6)	day 1	1126 (1016-1202)	7.2 (7.2- 7.2)	3 (3-3)	270 (260-288)	42.6(5.3)
	day 7	1083 (1004-1152)	7.1 (7.1-7.2)	3 (3-3)	245 (233-250)	40.6(5.3)
Sham (N=6)	day 1	971 (890-1034)	7.1 (7.0-7.1)	3 (3-3)	245 (233-269)	34.2(3.6)
	day 7	997 (934-1113)	7.0 (7.0-7.1)	3 (3-3)	210 (205-215)	27.3(3.4)
Biotin (N=6)	day 1	1034 (965-1166)	7.0 (7.0-7.0)	3 (3-2.3)	258 (240-275)	33.2(4.4)
	day 7	949 (928-992)	7.01 (7.1-7.1)	3 (3-3)	213 (199-226)	29.6(4.9)

*No predefined range exists for ATP levels according to our local blood bank guideline

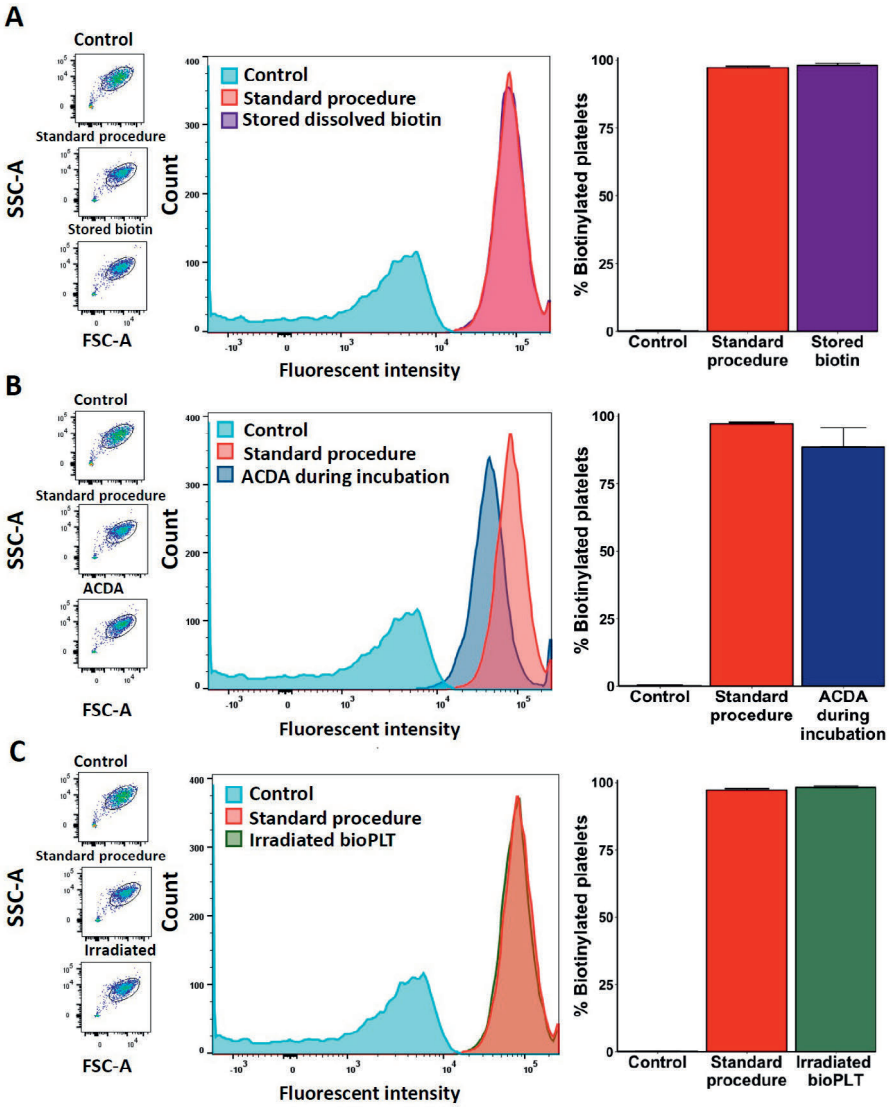

Figure 3. Biotinylation in various conditions. **A.** Biotinylation performed with biotin stored in PBS for 42 days at -30. There was no difference between freshly dissolved biotin and a stored biotin solution (mean of 97.9 and 97.1% respectively, n=3). **B.** Adding ACD-A simultaneoulsy with the biotin, led to a decrease of the amount of bioPLTs (mean of 88.5% versus 97.10%, n=3). **C.** Irradiation with 25 Grey did not influence the amount of bioPLTs (mean of 98.1%) .

The percentage of CD62P activation met blood bank standard. All samples showed maximal response to thrombin receptor-activating peptide (Figure 5). Similar results were observed in apheresis derived PCs and in PAS-E stored PCs (supplement, table S1). All blood products were culture negative before and after biotinylation

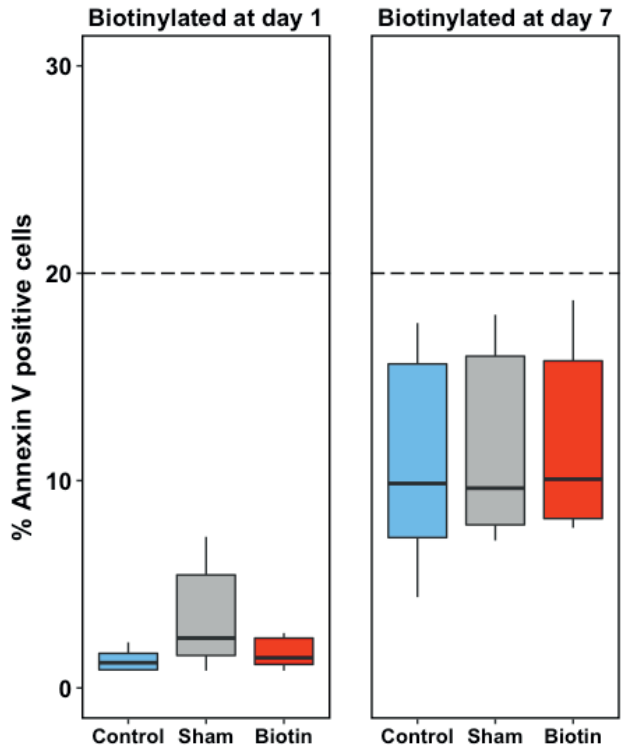

Figure 4. Annexin V expression of a fresh PC (day 1, left panel, n=6) and a stored PC (day 7, right panel, n=6). On day 1, the bioPLT did not show a statistically significant difference in Annexin V positive cells, as compared to control: 1.2% (0.9%-1.7%), p=0.56 and sham: 3.4% (1.6%-5.5%), p=0.16. At day 7, the bioPLT also showed no significant difference in annexin V positive cells: bioPLT: 10.1% (8.2%-15.8%) compared with the control: 9.9%(7.3%-15.6%), p=0.09 and the sham 9.6%(7.9%-16.0%), p=0.16.

The effect of storage on platelet quality parameters

Platelet quality parameters were measured directly after biotinylation of a 50 mL aliquot of fresh (day 1) and stored (day 7) buffy-coat derived PCs. Both fresh and stored platelets fulfilled platelet quality standards after biotinylation (Table 1). Platelets that were biotinylated at day one of storage were subsequently stored for three more days and tested for stability of the biotin-label and platelet quality parameters. Storage of

bioPLTs in the small aliquot volume of 50 ml led to substantial decrease of platelets quality (data not shown). This might be due to the sub-optimal storage and not to biotinylation itself. Therefore, for three PCs we transferred the biotinylated aliquot back to the retained fraction of the original PC and stored this PC for 7 days. BioPLT could be distinguished from the unbiotinylated platelets (Figure 6A). Both biotinylated and unbiotinylated showed minimal platelet activation, as expressed by CD62P expression (Figure 6B). We confirmed bioPLTs can be stored for 7 days using this method, all platelet quality markers met Dutch blood bank quality standard.

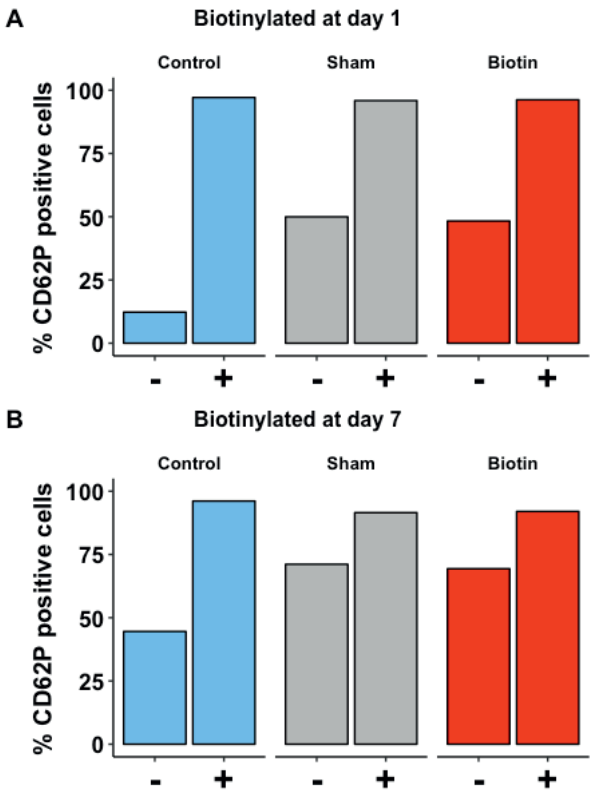

Figure 5. CD62P expression, of a fresh PC (day 1, panel A) and a stored PC (day 7, panel B). For each condition, left bars (-) represent the unstimulated state, right bars (+) represent CD62P expression in response to TRAP. After incubation with TRAP, all samples showed an increase in CD62P positive cells. At day 1, the number of CD62P positive cells increased in both the bioPLT 48.4%(41.7%-56.2%) and the sham 50.0%(41.7%-56.2%), as compared to control platelets 12,3%(9.5%-12.7%), p= 0.03. On day 7, more cells were CD62P positive in the bioPLT 69.60%(64.5%-70.3%) and sham 71.2%(66.4-75.7%) as compared to the control platelets: 44.6%(39.7%-50.3), p=0.03. No statistically significant difference was observed in comparing CD62P expression after incubation with TRAP in bioPLT with control (day 1: p=0.84, day 7 0.69) or sham platelets (day 1: p=0.11, day 7: 0.13).

Discussion

Here, we describe a reproducible protocol for biotin-labeling of PCs under GPG conditions in a closed system. Showing a low within and unit to unit variation. The findings will be of interest to blood banks and clinical researchers. BioPLTs can be used to evaluate the *in vivo* effect of new additive solutions, donor variability and the effect of transfusion in various patient categories.

The major advantage of biotin is that it enables *in vivo* tracing of transfused platelets without exposing the recipient to radiation. Moreover, bioPLTs enable tracing of multiple populations concurrently. Also, bioPLTs can be isolated from venous blood samples, thereby permitting direct population analysis for surface markers and metabolomics composition of the platelet subgroups. HLA discrepancy is another non-radioactive method to discriminate transfused platelets from the patients' circulating platelets.[18] However, this method inherently requires a HLA discrepancy, which excludes the possibility of tracing HLA-matched platelet transfusions or autologous transfusions in the recipient. Also, isolation and subanalyses of platelets are not possible with this method.

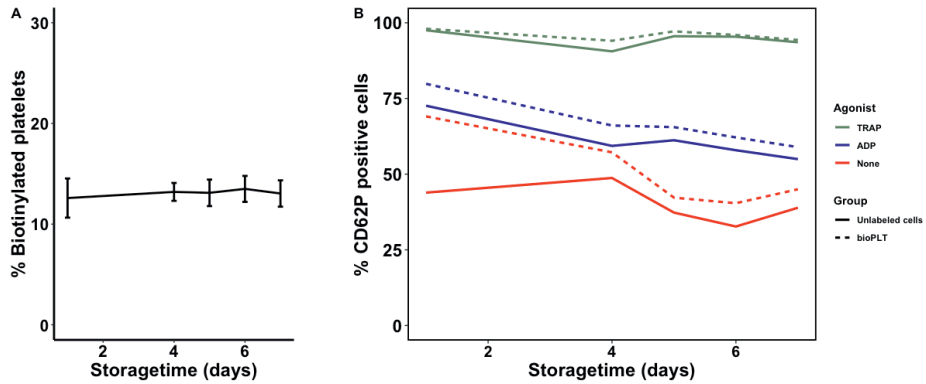

Figure 6. Stability of biotin label and CD62P expression of stored bioPLT. BioPLTs were returned to the retained fraction of the original PC, to enhance storage conditions. (n=3). **A** The percentage of biotinylated platelets remained stable througout 7 days of storage. (mean of 12.6% at day 1 and 13.0% at day 7). **B.** CD62P expression was assessed in unstimulated platelets (red), after stimulation with ADP (blue) and TRAP (green). CD62P expression was determined in both labelled (dotted lines) and unlabelled cells (continuous line). Measurements were performed at day 1,4,5,6 and 7. N=3

Biotinylation of platelets has previously been described under invalidated, experimental conditions.[1,13] Our protocol fulfils GPG requirements. Since US Food and Drug Administration (FDA) guidelines differ from European GPG requirements, the protocol needs to be validated according to the FDA standard before it can be implemented in the US. Our work can serve as a reference method.

We adapted the previous protocol on various crucial points (Table 2).[13] After sterile filtration of the sulfo-NHS-biotin-solution, the procedure took place in a closed-system, minimizing the risk of bacterial contamination. We added an extra washing step, to minimize nonspecific biotinylation of plasma proteins in the PC. Our group showed that PAS-E can be used to optimize platelet storage.[19] We tested whether PAS-E could be used to store the sulfo-NHS-biotin solution (for 42 days at (< -30°C). However, the quality of biotinylation decreased after storage of dissolved biotin in PAS-E. The biotin label remained stable when dissolved and stored at < -30ºC in PBS. Therefore, sulfo-NHS-biotin was dissolved and stored in PBS at a concentration of 50 mg/L. Shortly before biotinylation, the sulfo-NHS-biotin solution was diluted in PAS-E in a 1:9 ratio, to obtain a concentration of 5 mg/L. Since the reactive group of dissolved sulfo-NHS-biotin is instable, the sulfo-NHS-biotin-solution should either be used within 30 minutes, or after frozen storage at < -30º C, to be used within 42 days of storage. After thawing, the solution should be used within 30 minutes, to avoid hydrolysis. Since >97% of all platelets were biotinylated using our protocol, we found 30 minutes of incubation to be sufficient to adequately biotinylate platelets, instead of the previously described 45 minutes.[1]

TABLE 2. Adaptations on the previously described protocol

Protocol described by van der Meer[13]	Our protocol	Advantage
A platelet sample is centrifuged, the supernatant replaced by the biotin solution.	Centrifuged and supernatant removed twice before incubation.	Limits rest-biotinylation of proteins in PC.
Incubation in saline in which biotin is added at a final concentration of 25 mg/L.	The biotin solution was diluted 1:9 in PAS-E.	Less activation of the platelets.
Incubation for 45 minutes	Incubation for 30 minutes.	Time reducing.
Resuspended in saline/ACD.	Resuspended in PAS-E.	Superior storage conditions, mimics PC more realistically.
250 ml	50 ml	Minimal amount of traceable platelets.

BioPLTs and sham samples showed equal decreases in platelet quality parameters as compared to control platelets. Hence, platelets were affected by the processing steps,

but not by biotin itself. The processing steps are similar to the steps in obtaining platelet volume reduced products, which have been in use for several years and showed, after correction for platelet loss during preparation, similar cell count increment as standard platelet concentrates.[20-22] Centrifugation of platelets has previously been suggested to activate platelets[23], which is not completely prevented by the addition of ACD-A. Radiolabeling of platelets also requires centrifugation steps.[24] Since the processing steps, and not the biotin led to platelet activation, similar results can be expected for radiolabeling. We found that exposure to saline or PBS is detrimental to platelet quality; incubating platelets in a PBS-sulfo-NHS-biotin or saline solution led to unacceptable high activation of platelets (data not shown). Therefore the PBS-biotin was diluted in PAS-E. To our knowledge, no data are available on the effect of radiolabeling on platelet quality parameters. However, in previous labeling studies, platelets were incubated in saline, both for radiolabeling and biotin-labeling,[13,24] which might be not optimal with respect to platelet quality. We recommend a comparative study to assess platelet activation in both bioPLTs and radiolabeled platelets.

An important limitation of our study is that biotin labeling has not yet been compared to radioactive labeling in humans. However, in dogs, survival of bioPLTs was comparable to platelets labeled with both ^{111}Indium-oxine or ^{51}Chromium.[7] BioPLTs survived normally after transfusion, and could be used for determining platelet life spans *in vivo.* The platelet lifespan curves of bioPLTs were in agreement with radiolabeled platelets.

Red blood cell labeling studies showed modification of antigens and the risk of antibody-formation against the biotin.[25,26] These antibodies did not affect red blood cell recovery and survival in the recipient. However, this limits the possibility of repeated transfusions with bioPLTs for clinical research. To minimize the risk of antibody formation we labeled at the lowest possible traceable biotin density. Future research should include the assessment of the formation of antibodies against bioPLTs. BioPLTs will be first administered in an autologous transfusion model in healthy volunteers (registered at trialregister.nl, NTR6493).

In conclusion, we developed a standardized, simple, reproducible, protocol according to GMP standards for biotin-labeling of platelets, as non-radioactive alternative to trace and isolate transfused platelets *in vivo*.

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Appendices

Appendix 1. *Protocol to biotinylate platelets*

1.0 Purpose

The aim of this protocol is to describe a technique for biotin-labeling of fresh or stored platelets with Sulfo-NHS-LC-Biotin, in a closed system. This protocol describes how to biotinylate 50 ml of platelet concentrate.

2.0 Equipment, supplies, and reagents

2.1. Sterile docking station, sealing device.
2.2. Calibrated, variable speed, swinging bucket centrifuge.
2.3. Device to remove supernatant of centrifuged platelets
2.4. Calibrated analytical electronic balance, accurate to ±0.00002 g.
2.5. Calibrated generic electronic scale, accurate to 0.5 g.
2.6. Kochers, clamps.
2.7. 0.22 µ sterilizing filter unit.
2.8. PBS: Phosphate buffered saline.
2.9. ACD-A: anticoagulant citrate dextrose solution, Formula A.
2.10. PAS-E : Platelet Additive Solution.
2.11. Product bags for platelets, 150 ml.

3.0 Preparation of biotin solution

Note: Dissolved biotin needs to be used directly, or immediately after thawing of frozen product, as dissolved biotin is vulnerable to hydrolysis. Bound biotin is stable.

3.1. Biotin is dissolved in PBS at a concentration of 50 mg/L, and transferred in a 150 ml bag.
3.2. Filtrate biotin-solution by passing a 0.22 µ sterilizing filter.
3.3. Store within 15 minutes at -30º, or use within 30 minutes.

4.1 Preparation of required solutions

4.5. Prepare 150 ml of 15% ACD-A in 85% PAS-E in a sterile, labeled bag.

4.6. Prepare 100 ml of 10% ACD-A in 90% PAS-E in a sterile, labeled bag.
4.7. Prepare 11 ml of ACD-A in a sterile, labeled bag.
4.8. Maximal 30 minutes prior to the biotinylation procedure, thaw the stored 50 mg/L biotin-PBS solution, and mix 1 part biotin-PBS-solution to 9 parts PAS-E, to obtain a 5 mg/L biotin-PBS-PAS-E-solution. Fill out 100 ml in a sterile, labeled, bag.

5.1. Obtain the proportion of 50 ml platelet concentrate.

5.2. Transfer 52 grams (50 ml) of platelet concentrate to a sterile, labeled bag, using a sterile docking device.

6.0 Washing steps

Note: Before every centrifugation step, add a total of 10% ACD-A, to prevent thrombus formation due to centrifugation.

6.1. Dock the bag containing 50 ml of platelet concentrate to the 150 ml 15%ACD-A/85% PAS-E-solution bag. Add this 15%-ACD-A solution to the platelet concentrate.
6.2. Centrifuge the bags at 1700 x g for 10 minutes.
6.3. Remove the supernatant by using the empty bag, discard this bag.
6.4. Dock the bag containing the platelet pallet to the 100 ml 10% ACD-A/90% PAS-E bag. Resuspend the platelet pallet by gentle manipulation.

7.0 Incubation with biotin

7.1. Dock the 5 mg/L biotin-PBS-PAS-E-solution to the platelet pellet. Resuspend the platelet pellet by gentle manipulation.
7.2. Incubate for 30 minutes, at room temperature, under gently agitation.

8.0 Washing step and resuspending to original volume

8.1. Add the bag containing 12 ml of ACD-A to the biotinylated platelets.
8.2. Centrifuge at 1700 x g for 10 minutes.
8.3. Remove the supernatant by using the empty bag, discard this bag.
8.4. Dock PAS-E to the platelet pallet, bring to original volume (50 ml).
8.5. Resuspend the platelets pallet by gently manipulation.
8.9. Store the platelets under gentile agitation, at room temperature until use.

9.0 Test for biotinylation

9.1. Obtain sample, incubate for 30 min with streptavidin 488.

9.2. Flowcytometrically analyze the percentage of biotinylated platelets.

Appendix 2. Supplemental figures

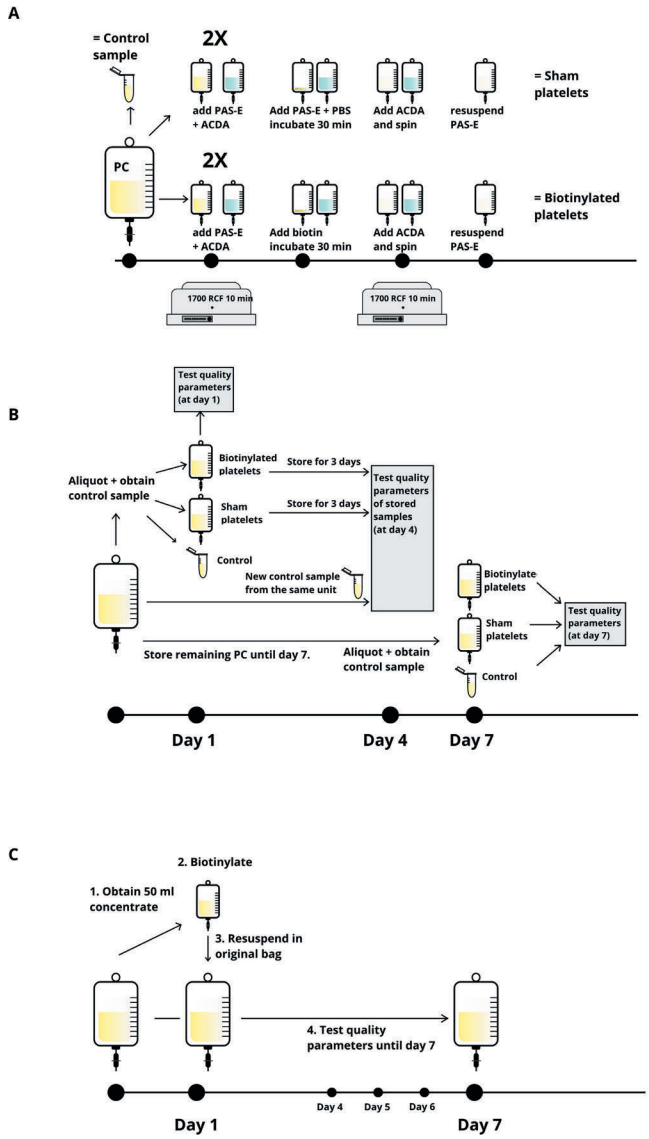

Figure S1. A) Procedural steps to obtain biotinylated platelet, sham platelets and the control sample. B) Validation experiment in which platelets were labeled at day 1 or day 7 of storage. Platelets labeled on day 1 of storage were also stored for 3 days separately to assess the effect of storage on the quality of the platelets. However this method of storage led to unacceptable high platelet activation. Therefor another method was designed: C) After labeling, the bioPLT were returned in its original bag. Here, the various parameters tested in biotinylated platelets fulfilled the quality criteria.

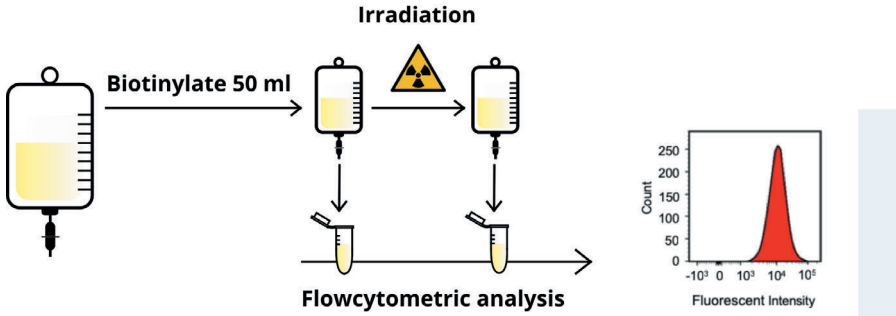

Figure S2. Samples from the biotinylated unit were obtained prior and after irradiation. From each unit both samples were tested for fluorescent intensity on the flocwcytometer to assess the effect of irradiation on the biotin label (n=3).

Appendix 3. Supplemental tables

Table S1. Platelet quality markers in apheresis PCs and PCs stored in 65% PAS-E

Apheresis PCs	**Platelet count**	**pH**	**Annexin V positive**	**Morphology**	**CD62P expression (%)**		
(n=3)			**cells (%)**	**score**	**Baseline**	**ADP stimulation**	**TRAP stimulation**
Control	1277 (134)	7.21 (0.054)	4 (1.5)	252 (12)	20.4 (5.8)	72.3 (2.7)	82.5 (27.8)
Sham	1129 (116)	7.062 (0.048)	3.7 (0.8)	252 (13)	57.1 (3)	63.4 (5.6)	75.9 (38.9)
Biotin	1095 (120)	7.089 (0.011)	4.2 (1.7)	227 (13)	54.5 (1.6)	59.2 (3)	98.5 (0.1)

PAS-E PCs	**Platelet count**	**pH**	**Annexin V positive**	**Morphology**	**CD62P expression (%)**		
(n=3)			**cells (%)**	**score**	**Baseline**	**ADP stimulation**	**TRAP stimulation**
Control	867 (46)	7.162 (0.013)	4.1 (2.5)	267 (13)	19.1 (5)	48.4 (12.7)	97.9 (0.3)
Sham	917 (106)	7.058 (0.021)	4.8 (1.7)	265 (10)	42.8 (3.1)	48.9 (7)	97.6 (0.2)
Biotin	851 (80)	7.06 (0.019)	5.1 (1.6)	267 (13)	44.1 (6.8)	51.3 (3)	97.3 (0.3)

CHAPTER 8

General summary

General summary

In summary, this thesis focussed on several aspects of transfusion medicine. In **chapter 2-4** current transfusion practice was assessed in critically ill patients. In **chapter 5** metabolic changes in red blood cells (RBCs) were reviewed, including changes during physiologically aging *in* vivo, aging during storage and in donor RBCs after transfusion. Followed by **chapter 6** in which the metabolic state and post transfusion recovery (PTR) was assessed in RBCs that were stored in a new alkaline additive solution. Finally, in **chapter 7** we described a method to label platelets with biotin as an alternative for radioactive labelling.

Due to the lack of international guidelines specific for critically ill patients, we hypothesized that we would find large heterogeneity in transfusion practice. To assess current transfusion practices and identifying knowledge gaps, we conducted two online surveys, which are described in **chapter 2** and **chapter 3.** In these surveys transfusion practice was assessed in non-bleeding and bleeding critically ill patients, respectively. The surveys included questions on RBC, platelet and plasma transfusion practices, transfusion triggers, the use of coagulation factors and the use of transfusion guidelines.

The most important findings were that RBC transfusion is restrictive in most critically ill subpopulations. For both bleeding and non-bleeding critically ill patients the median reported haemoglobin (Hb) threshold was 7 g/dL. RBC transfusion thresholds were most variable for patients on extracorporeal membrane oxygenation (ECMO): 7[7-9] g/dL in bleeding patients and 8[7-9] g/dL in non-bleeding patients. Some populations, including non-bleeding patients with acute myocardial infarction and bleeding patients after cardiothoracic surgery, were transfused at significantly higher Hb levels compared to other patient categories: 9.0 [8-9.7] g/dL and 8 [7-9] g/dL respectively.

The clinical practices of platelet and plasma transfusions was more heterogeneous and less restrictive than RBC transfusion. About 60% of the respondents would consider plasma transfusion to correct vitamin K induced prolonged international normalized ratio (INR) prior to an invasive procedure.

The presence of local transfusion guidelines influenced the transfusion practice of the respondents. We found that RBC transfusion was more restrictive in non-bleeding patients when a local transfusion guideline was available. Furthermore, the presence of a massive transfusion protocol was associated with a more frequent use of fixed transfu-

sion ratio's in massively bleeding patients. In non-bleeding patients, also an association between transfusion practices and base specialty was found.

Physicians with a base specialty in internal medicine transfused relatively more restrictive than physicians with a base specialty in anaesthesiology. Furthermore, the use of other transfusion triggers than Hb levels was common for RBC transfusion according to our survey. More than 50% of the respondents always or most of the time use other triggers than Hb levels.

While surveys provide insights how physicians believe patients are currently transfused, they don't reveal how patients are actually transfused in daily practice. For this, a large prospective observational study is necessary. To optimize this trial, a feasibility prospective observational study on transfusion practice was performed. This study was described in **chapter 4.** We conducted a prospective observational single centre study, in which for seven consecutive days all newly admitted patients were followed until ICU discharge. Information on baseline characteristics, daily clinical and laboratory values, data on each transfusion event (including a questionnaire for the physician why a transfusion was ordered) and 28-day mortality were collected. Workload was scored to assess study feasibility. We included 48 patients in this single centre study. During the study period 30 transfusion events were reported in eight patients including 17 RBC transfusions, 11 platelet transfusions and two plasma transfusions. The average time of data collection was three hours per patient. This study showed that the large prospective study is feasible, and currently this study is enrolling worldwide.

In **chapter 5** the metabolic changes *in vivo* of RBCs, *in vitro* during storage, and the metabolic changes of RBCs after transfusion were reviewed. Several similarities exist between aging *in vivo* and *in vitro* during storage. Glycolysis and pentose phosphate pathway (PPP) metabolism slow down during physiological aging, but not as prominent during storage. Mainly due to the decrease in pH, ATP and 2,3-DPG levels decrease significantly during storage. Also, PPP activity decreases during storage. The degree of changes depends on the composition of the additive solution that is used to preserve the RBC. Alkaline, chloride-free, additive solutions are designed to counteract the inhibition of rate limiting enzyme activity in these pathways. Consequently, 2,3-DPG and ATP levels are better preserved in these additive solutions. In addition, the redox metabolism remains more active when RBCs are stored under alkaline conditions. Literature on metabolic restoration after transfusion was limited, but it was observed that ATP and 2,3-DPG levels restore within 72 hours after transfusion. Other pathways, such as the PPP have not been studied after transfusion.

In **chapter 6** we conducted a randomized controlled trial to assess whether storage of RBCs in the experimental alkaline, chloride-free, additive solution PAGGGM (phosphate-adenine-glucose-guanosine-gluconate-mannitol)) resulted in a higher post transfusion recovery (PTR) and better preservation of metabolism after transfusion compared to storage in the current standard SAGM. We found that glycolysis and redox metabolism were better preserved in PAGGGM stored RBCs compared to SAGM stored RBCs. However, this did not result in a higher PTR. Furthermore, 2 days stored RBCs stored in SAGM, had a higher 2- to 7-day PTR, compared to 35 days stored RBCs. Finally, the metabolic storage lesion was reversible in RBCs that remain in circulation after transfusion. The differences in glycolysis, redox and purine metabolism remained present in RBCs, up to one day after transfusion.

In **chapter 7** we validated a novel method to label platelets with biotin. The current gold standard is labelling with radioactive isotopes including Indium-111 and Chromium-51. Since the use of radioactive labelling is in most EU countries, including the Netherlands, not allowed in vulnerable patient groups, a non-radioactive alternative is desirable. We showed that biotin is a feasible alternative. Platelet labelling with biotin itself did not affect the quality of platelets. The procedural steps resulted in a small increased platelet activation. However, this biotinylated platelet product still fulfilled all quality criteria from the Dutch blood bank (Including: annexin V expression and CD62P activity). Biotinylated platelets may be a safe alternative for radioactive labelled platelets in vulnerable patients including critically ill patients. Besides giving important insights in the PTR of donor PLTs in different patient cohorts, the biotinylation of PLTs allows assessment of the quality of new blood products. Finally, labelling with biotin has as advantage that biotin labelled platelets can be isolated from the recipient's circulation after transfusion.

CHAPTER 9

General discussion

The aims of this thesis

Research on transfusion practice has gained considerable traction in recent years. Improving transfusion practice involves adequate guidelines for physicians and awareness of the benefits and potential adverse effects of transfusion. It also includes research to improve storage conditions, providing the basis for safer and more efficient blood products. Transfusion practice is becoming more and more restrictive in order to limit the potential harmful adverse effects of transfusion in patients. Simultaneously, blood products are constantly being improved. This thesis focused on several aspects of the current transfusion research field, including the following key aspects:

1. To study transfusion practice and transfusion triggers on the ICU
2. To design a study protocol in which transfusion practice is evaluated, including the use of transfusion triggers other than laboratory values.
3. To assess whether storage of RBCs in an alkaline additive solution resulted in a higher post transfusion recovery and better metabolic restoration after transfusion.
4. To develop a standardized method to label platelets with biotin, a product that can be used to asses post transfusion platelet recovery, even in vulnerable patient populations.

Transfusion practice on the Intensive care unit (ICU)

In **chapter 2-4** clinical practice in critically ill patients is assessed in two surveys among physicians working on the ICU and one small prospective cohort study with 48 critically ill patients. One of our main findings is that RBC transfusion is restrictive in most ICU subpopulations. However, the clinical practice of platelet and plasma transfusion is more heterogeneous and less restrictive.

In this thesis we showed a continuation of the trend towards a more restrictive transfusion strategy for red blood cell transfusion practices with the exception of different subpopulations. Patients with acute myocardial infarction were transfused at significantly higher Hb levels compared to other patient populations. This is in line with current guidelines that recommend a liberal transfusion strategy for patients with acute coronary syndrome[1–3]. Consequently, these patients are excluded in large RCTs comparing different transfusion strategies. Recently, the REALITY trial showed that in anaemic patients with acute coronary syndrome a restrictive transfusion strategy (Hb threshold 8g/dL) was non-inferior compared with a liberal transfusion strategy (Hb threshold of

10 g/dL) for the primary outcome: major adverse cardiovascular event and/or all-cause mortality[4]. In addition, patients allocated to a restrictive transfusion strategy developed less infections and less acute lung injury. A second RCT in anaemic patients with myocardial ischemia is currently recruiting patients, (MINT, NCT02981407), comparing the same transfusion strategies. If both the MINT and the REALITY can show the safety of a Hb trigger of 8 g/dL, this would be a major change in current transfusion practice. After implementation of a more restrictive transfusion strategy for these patients, the next step could be to assess the safety of an even lower transfusion trigger of 7 g/dL.

Currently a transfusion trigger of 7 g/dl is considered restrictive, but no studies have assessed lower thresholds yet. Therefore, it remains unclear what the optimal Hb level trigger is. The threshold of 7 g/dL is partly determined from data in studies on Jehovah witnesses who refuse blood transfusion for religious reasons. In these patients, a higher mortality and morbidity rate was observed when Hb levels dropped below 7 g/dL. Due to the retrospective character of these data, these conclusions might be confounded. One of the most likely confounding factors is that patients who are more severely ill more often developed a severe anaemia. We could also state that despite a low Hb level, still a large proportion of the patients survived without major complications. Furthermore, in resting healthy volunteers, it is shown that inducing an acute isovolumic anaemia of 5 g/dL by acutely removing blood did not result in impaired critical oxygen delivery, which was assessed by oxygen consumption (VO_2), lactate levels and ST changes on electrocardiogram[5]. This suggest that there is room to investigate, preferably in a stepwise manner, if a Hb transfusion trigger lower than 7 g/dL is safe in patients. To do so, the first step is to identify patients who are most likely to benefit from a more restrictive strategy. We would argue that non-bleeding female patients could be the first subpopulation transfused at lower Hb levels. Under normal conditions women have significantly lower Hb levels (12-16 g/dL) than men (14-18 g/dL). Consequently, women are used to lower Hb levels than men, and therefore they might be more resilient to lower Hb levels.

We found that Hb level remains the most important RBC transfusion trigger. While other RBC transfusion triggers than Hb levels such as hypotension, tachycardia and increased lactate levels are commonly used according to our surveys from **chapter 2 and 3**, this was not the case in our single centre study in **chapter 4**. In only two out of seventeen RBC transfusion events in this study, tachycardia and increased lactate levels played a role in the decision to transfuse the patient. Due to the small sample size in this single centre study, it is not possible to generalize these findings. Therefore, the use of other transfusion triggers will be studied in a larger population of patients in multiple centres. This large international study is currently recruiting patients, in succession to the pilot

study in **chapter 4.** In this international study, the use of other transfusion triggers than Hb levels will be assessed. Although so far no study showed other transfusion triggers than Hb levels to be superior to determine the need for RBC transfusion, the additive value of other triggers besides the use of Hb levels can be a step towards personalized medicine. In recent transfusion guidelines, recommendations are based on a combination of underlying illness and Hb levels[3,6]. Possibly, a combination of physiological changes, comorbidities, underlying disease and Hb levels will guide in the future the decision to transfuse or not. The international study will also reveal potential implementation issues of current evidence regarding transfusion in the critically ill patients. This could be a starting point for focussed education and implementation projects.

Platelet and plasma transfusion practice in non-bleeding patients is far more heterogeneous. This is observed in **chapter 2 and 3.** For these products it is recognized that INR/PT and platelet count are not the only determinants in the decision to transfuse a patient. Two RCTs in other vulnerable patient populations have shown that liberal platelet transfusion strategies resulted in worse clinical outcomes: neonates who were randomized to a platelet threshold of 50 x 10^9 cells/L died or suffered more often from major bleeding significantly than neonates who were allocated to a threshold of 10 x 10^9 cells/L[7]. Furthermore, patients with a haemorrhagic stroke who used antiplatelet therapy, showed a higher mortality and dependency rate when they were allocated to platelet transfusion[8]. Even though these studies included different patient cohorts, they hint at potential harmful side effects of platelet transfusion. These trials were investigating prophylactic platelet transfusion. The next step is whether platelet transfusion is needed in severe thrombopenic patients undergoing an invasive procedure. Although small underpowered studies have been performed, the PACER trial will be the first large well powered non-inferiority RCT in critical ill patients, the PACER trial, which compares different platelet thresholds in severe thrombopenic patients undergoing an ultrasound guided central venous catheter (CVC) placement, is currently recruiting[9]. If the PACER study provides conclusive results, showing the safety of lower platelet count transfusion thresholds during CVC placement, it would pave the way to study lower platelet count transfusion thresholds during more invasive interventions. This could be the start of a shift towards a more restrictive platelet transfusion practice in thrombopenic critically ill patients. Furthermore the PACER trial only allows enrolment when the physician is experienced and uses ultrasound during the CVC placement procedure. Hence, instead of preparing the thrombopenic patient with platelet transfusion for a less experienced physician the trial applies a personalized strategy in severe thrombopenic critically ill patients hopefully preventing allogenic platelet transfusion.

Plasma transfusion has been studied more extensively in the ICU in non-bleeding patients, compared to platelet transfusion. Several studies have compared different plasma strategies in critically ill patients. Although these studies were prematurely stopped due to low inclusion rates, none of these studies were able to find any benefit of plasma transfusion[10,11].

Despite the lack of evidence for a positive effect of plasma transfusion in these studies, but in line with the limited attention on the efficacy of plasma transfusion we found a large degree of heterogeneity in plasma transfusion practice. This is in line with several observational studies that aimed to study the incidence of inappropriate plasma transfusion. Even these studies did not have an agreement on the definition of plasma transfusion. For example, the use of plasma prior to an upcoming procedure in patients suffering from coagulopathy was a particular point on which the authors did not agree[12,13]. Based on our survey, an ICU specific transfusion guideline is published that advises against plasma in non-bleeding critically ill patients[3]. Possibly, this will influence current practice on plasma transfusion and reduce the number of plasma transfusions.

Future directives

As mentioned above, the INPUT study (NTR9049), a large international point prevalence study, is currently enrolling patients. In **chapter 4** the protocol for this study was tested. In this international study we will assess if the findings in our surveys are similar in actual clinical practice. The aim of this project includes identifying commonly used clinical transfusion triggers besides or in addition to Hb levels, platelet levels and INR/PT. These triggers could be the next step in designing algorithms that allow tailor made transfusion practice.

An important remark is that surveys and observational studies cannot identify the most optimal transfusion practice. The goal of this sort of studies is to investigate the current opinion on transfusion practices. Furthermore, it shows the gaps in current knowledge and may find correlations between transfusion practice and outcome. To prove causality, additional RCTs are necessary.

After determining when transfusion is inappropriate, the next step will be to implement these new insights in daily practice. Organisational interventions such as educational interventions and audits have shown to reduce rates of inappropriate transfusion[14].

Effect of an alkaline additive solution on post transfusion recovery and metabolic restoration of RBCs

In **chapter 6** we found that RBCs stored in an alkaline chloride-free additive solution did not have a higher post transfusion recovery (PTR) despite a better metabolic state in these RBCs. Especially the redox state and glycolysis were significantly better preserved in PAGGGM stored RBCs. This study shows that the metabolic state of RBCs is not the main driver of RBC clearance. While adenosine triphosphate (ATP) levels still might be important for the function of a RBC, we were not able to confirm the finding of a previous study which showed a correlation between ATP levels and PTR[15].

An important limitation in assessing RBC PTR after transfusion is the lack of knowledge on the survival of the transfused RBC in the first ten minutes after transfusion. In our study, as done in most studies[15,16], we calculated the PTR using the percentage of labelled RBCs in the first blood sample, drawn 10 minutes after transfusion, as reference. Thus, using this method, RBC that are immediately cleared from the circulation, within the first ten minutes, are not considered. Ideally, the number of circulating RBCs and the number of administered biotinylated RBCs would be known at the time the transfusion was finished. Then, it would be possible to precisely calculate the PTR at any timepoint, even after ten minutes. As a surrogate, it is common practice to calculate the circulating volume of the recipient, and thereby estimate the percentage of donor RBCs as accurate as possible. However, the optimal method to assess the circulating volume in humans is by no means established. Several methods are described in literature to estimate circulating volume for clinical and research purposes, including but not limited to indocyanine green dilution and carbon mono-oxide rebreathing method[16–18]. Unfortunately, all these methods are estimates with different accuracies. For the purpose of determining RBC PTR, a method that allows an accurate estimation of the circulating volume is necessary.

Future directives

The implications of a higher PTR of fresh RBCs for clinical practice should be further investigated. It is not feasible to transfuse everyone with the freshest RBCs. Furthermore, in critically ill patients it is shown that transfusion of fresh RBCs did not result in improved clinical outcomes compared to longer stored RBCs[19–21]. Nonetheless, there might be specific patient populations that could benefit from an increased PTR. We postulate that patients with a chronic need for transfusion are a potential patient population that could benefit from fresh RBCs. A higher PTR could result in an increased interval between blood transfusion and thereby minimizing the long-term adverse effects of

blood transfusion. A randomized controlled trial testing this hypothesis should include long-term effects such as the development of transfusion iron overload.

Increased 2,3-DPG levels should theoretically result in increased oxygen offload in the peripheral tissues. It remains difficult to assess the additive value of increased 2,3-DPG levels on improving the oxygenation status in patients. Since it is not possible to measure oxygen delivery of RBCs to the tissues directly in patients, we need to use surrogate markers for tissue oxygenation. Several surrogate markers are used for research purposes including but not limited to near-infra red spectroscopy and mitochondrial oxygen content[22,23]. However, sufficient oxygen supply to the peripheral tissues is dependent on numerous other factors including cardiac output and arterial oxygen content. These factors are often compromised in patients, especially in those who are in need of a RBC transfusion. Possibly, a broader outcome than tissue oxygenation can be used, for instance clinical outcomes such as mortality.

Finally, this study functions as proof of concept study. It is the first study that was able to isolate biotinylated RBCs after transfusion on which analysis could be performed. This method is also applicable in clinical research. Recovery of the metabolic storage lesion may be different in patients suffering from systemic illness, such as septic patients. How RBCs recover in this sort of patient group might give insights on what part of the metabolism we should focus on during storage. If for instance RBCs are unable to recover their redox state in critically ill patients, it might be beneficial to preserve PPP activity, even if this is at the expense of other metabolic pathways.

Platelet labelling

In **chapter 7** we validated a novel method to label platelets with biotin, in order to assess their PTR as well as study their phenotype after transfusion, as an alternative for labelling using radioactive isotopes. We consider biotin labelled platelets to be a good alternative for radiolabelled platelets, as these can be even administered in vulnerable patient populations. However, we found that the procedural steps in our protocol result in a small increase in platelet activation. Since labelling with radioactive isotopes includes similar procedural steps platelet activation is hypothesized to be similar in labelling with radioactive isotopes[24]. However, to our knowledge, no data has been published about the effect of radioactive isotope labelling on platelet activation. Nonetheless, the current gold standard for assessing PTR of platelets is radioactive isotope labelling. Due to strict regulations on working with radioactive material, we were unable to assess

platelet activation of radioactive isotope labelled platelets. However, 111-In-oxine and biotin labelled platelets have a similar lifespan after transfusion in baboons[25]. These data support our assumption that platelet activation is similar between both labelling techniques.

An alternative technique that does not involve platelet manipulation prior to transfusion is using human leukocyte antigen (HLA) discrepancy. Here, the differences in HLA type between the donor and the recipient are used to distinguish donor from recipient PLTs. This method has several other limitations: 1) the majority of the transfused blood products are pooled products with five different donors and thus five different HLA patterns 2) when multiple platelet concentrates are administered within 10 days, it is difficult to distinguish the previous transfused product from the current product and 3) this approach cannot be used with autologous blood products since there is then no HLA mismatch. Therefore, we believe that despite higher platelet activation, biotinylated platelets should be the future gold standard in assessing platelet PTR.

Future directives

Our group is currently performing a trial in healthy volunteers (NTR 6493) to assess the safety and efficacy of biotinylated PLTs. Once proven safe and effective, biotinylated platelets will enable us to assess platelet clearance in numerous patient populations. Patient populations of special interest who often receive platelet transfusions are critically ill patients, patients with a haematological malignancy and patients undergoing large surgeries. Correlations between patient related factors, such as fever and underlying disease with post transfusion recovery of platelets should be assessed. These findings can help design targeted therapy strategies. Furthermore, biotin labeling can be used to assess the quality of new platelet products. For instance, the use of new platelet additive solutions, the use of cryo-stored platelets or the effects of new pathogen reduction therapies on PLT PTR.

In our method we assessed the labelling of PLTs using a single concentration of biotin. Previously, it was shown in mice, that two different densities of biotin can be used to label PLTs making it possible to distinguish different PLT populations[26]. In the near future, we plan to explore the labelling of PLTs using different concentrations of biotin, enabling us to perform similar experiments as described in **chapter 6,** but now using platelets. This methods provides possibilities to use internal controls by transfusing two different platelet populations into one recipient. The use of internal controls will reduce the noise of inter-donor and/or inter-recipient variability.

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CHAPTER 10

Nederlandse samenvatting

Achtergrond

Een mens heeft ongeveer 4-6 liter bloed door zijn of haar aderen stromen. Bloed bestaat grofweg uit twee componenten: een vloeistof component en een cellulaire component. De vloeistof component, ook wel bloedplasma genoemd, is ruim de helft van het totale volume van het bloed. In deze vloeistof zijn verschillende eiwitten opgelost met verschillende functies, waaronder het ondersteunen van de bloedstolling. De cellulaire component bestaat uit verschillende type bloedcellen, 99% daarvan zijn rode bloedcellen (RBCs). RBCs zijn verantwoordelijk voor het zuurstof transport naar alle organen. Daarnaast hebben we witte bloedcellen, de cellen die ons tegen bacteriën, virussen en schimmels in ons lichaam beschermen. Ten slotte zijn bloedplaatjes ook een belangrijke bloedcomponent, deze cellen heb je, naast de eiwitten in het plasma, nodig om het bloed te kunnen laten stollen als er ergens in het lichaam een bloeding is. Door ziekte, of bloedverlies door ongelukken of na een operatie kunnen we een tekort hebben van één of meerdere componenten in ons bloed. Eén van de mogelijke behandelingen van zo'n tekort is een bloedtransfusie. Om een bloedtransfusie mogelijk te maken, wordt een bloeddonatie bij de bloedbank over het algemeen gedoneerd bloed gescheiden in drie componenten welke resulteert in de meest voorkomende bloedproducten; RBC transfusie, bloedplaatjes transfusie en plasma transfusie.

Bloedtransfusie is een medische behandeling die levensreddend kan zijn. Echter, zoals elke medische behandeling, kent bloedtransfusie ook een risico op bijwerkingen zoals allergische reacties, bloed overdraagbare infecties en transfusie gerelateerde acute longschade. Deze bijwerkingen kunnen levensbedreigend zijn. Het is daarom belangrijk om bij elke patiënt de potentiële voordelen af te wegen tegen de potentiële nadelen.

In dit proefschrift ligt de focus op patiënten die zijn opgenomen op de intensive care. Dit zijn patiënten die een grote operatie hebben ondergaan waarna ze voor korte tijd gemonitord moeten worden en/of waarbij een onderliggende ziekte er toe leidt dat één of meerdere essentiële lichaamsfuncties bedreigd zijn, zoals de ademhaling, nierfunctie, leverfunctie en de bloedcirculatie. Door hun onderliggende ziektes, ontvangen veel van deze patiënten een transfusie ten tijde van een intensive care opname. Tegelijkertijd zijn deze patiënten ook gevoeliger voor het ontwikkelen van een ernstige bijwerking. In dit proefschrift werd onderzocht wat de afwegingen zijn op de intensive care om iemand een bloedproduct toe te dienen.

Naast het toedienen van bloedproducten aan de juiste patiënten, is er ook ruimte om de kwaliteit van bloedproducten te verbeteren. De kwaliteit van een bloedtransfusie

kan op verschillende manieren verbeterd worden, bijvoorbeeld door de opbrengst na transfusie te verhogen, of de kans op bijwerkingen te verkleinen. Dit proefschrift richt zich op het verhogen van de opbrengst van RBCs na bloedtransfusie door de manier van het opslaan van RBCs te optimaliseren.

Ten slotte is het meten van de effectiviteit van een transfusie uitdagend. In dit proefschrift is een nieuwe manier onderzocht om de opbrengst en overleving van bloedplaatjes na transfusie te meten. De gouden standaard om opbrengst en overleving van bloedplaatjes na transfusie te meten is nu het labelen van bloedplaatjes met een radioactief label voorafgaand van transfusie. In dit proefschrift is een nieuwe methode ontwikkeld waarbij bloedplaatjes gelabeld worden met vitamine B8, ook wel bekend als biotine.

Transfusie van intensive care patiënten

In hoofdstuk 2 en 3 zijn enquêtes beschreven waarin de transfusiepraktijk van bloedende en niet bloedende kritiek zieke patiënten is beschreven. Hierin werden vragen gesteld over het toedienen van rode bloedcellen, bloedplaatjes en plasma. Daarnaast zijn er vragen gesteld over de beweegredenen die van belang zijn voor de beslissing voor het toedienen van bloedproducten, het toedienen van stollingsfactoren en de aanwezigheid van transfusie richtlijnen.

De belangrijkste bevinding uit deze enquêtes is dat rode bloed cel transfusie restrictief is in de meeste patiënten populaties met een mediane transfusiegrens van 7 g/dL. Er zijn een paar uitzonderingen: patiënten met een hartinfarct worden hebben een mediane transfusiegrens van 9 g/dL en patiënten die net een openhartoperatie hebben ondergaan een mediane transfusiegrens van 8 g/dL. De grootste spreiding in de transfusie praktijk wordt gezien in patiënten die aangesloten liggen aan de hart-long machine. Naast het gebruik van hemoglobine waardes werd ook aangeven dat andere factoren zoals lage bloeddruk en hoge hartslag een belangrijke rol speelden in de beslissing om iemand een zak RBCs toe te dienen. De transfusiepraktijk van bloedplaatjes en plasma is variabeler en minder restrictief dan RBC transfusie. Het merendeel van de artsen overweegt om in afwezigheid van een bloeding een vitamine K geïnduceerde stollingsstoornis te corrigeren met een plasma transfusie voor een geplande invasieve ingreep. Daarnaast werd in deze enquêtes een verband gezien tussen het moederspecialisme van de arts en de transfusiepraktijk. Artsen met het moederspecialisme anesthesie gaven laagdrempeliger aan iemand een bloedproduct dan artsen met het moederspecialisme interne geneeskunde.

Omdat enquêtes een beperkt inzicht geven hoe er daadwerkelijk bloed toegediend wordt op de intensive care is er in hoofdstuk 4 een prospectieve studie uitgevoerd op een intensive care unit in een academisch centrum. Deze studie dient als een proefstudie, zodat deze uiteindelijk internationaal uitgevoerd kan worden in veel verschillende soorten ziekenhuizen. In deze proefstudie zijn alle patiënten die in een specifieke week waren opgenomen op de intensive care dagelijks gevolgd. Demografische informatie, informatie over de achterliggende ziekte en laboratorium waarden werden dagelijks verzameld. De werklast is bijgehouden om de uitvoerbaarheid van de studie te beoordelen. In de studieperiode zijn 48 patiënten geïncludeerd. In totaal zijn er in 8 patiënten 30 transfusiemomenten gerapporteerd waaronder 17 RBC-transfusies, 11 bloedplaatjes transfusies en 2 plasma transfusies. De gemiddelde tijd per geïncludeerde patiënt was drie uur. Zoals verwacht, zijn dit niet genoeg patiënten om harde conclusies te trekken over de huidige transfusiepraktijk. Maar deze resultaten vormen wel een goede basis om deze studie wereldwijd op te zetten. Op dit moment loopt deze internationale studie nog en worden de eerste resultaten in 2022 verwacht.

Het opslaan van rode bloedcellen in een basisch opslagmedium

In hoofdstuk 5 is er een literatuursamenvatting gemaakt over de metabole veranderingen van RBCs over de tijd. Er is onderscheid gemaakt tussen normale veroudering in het lichaam, veroudering tijdens de opslag na bloeddonatie, en metabole veranderingen nadat een RBC-eenheid weer getransfundeerd is. Er bestaan meerder overeenkomsten tussen veroudering in het lichaam en veroudering tijdens opslag. Glycolyse en pentose fosfaat route, twee belangrijke metabole routes voor RBC-overleving en functioneren, worden geremd tijdens veroudering. Deze verandering vindt in sterkere mate ook plaats tijdens de opslag van rode bloedcellen. In hoeverre dit gebeurt hangt af van het bewaarmedium waarin de rode bloedcellen bewaard worden. In bewaarmedia met een meer basische pH kunnen de glycolyse en de pentose fosfaat route langer actief blijven. In enkele studies is beschreven dat de glycolyse 72 uur na transfusie weer hersteld is. Hoe dat voor andere metabole routes zoals de pentose fosfaat route is, is niet duidelijk.

In hoofdstuk 6 is de effectiviteit van rode cellen getest die in een basisch opslagmedium zijn bewaard. In een gerandomiseerde studie werd een nieuw opslagmedium PAGGGM (phosphate-adenine-glucose-guanosine-gluconate-mannitol) vergeleken met de huidige standaard SAGM (saline-adenine-glucose-mannitol). Gezonde vrijwilligers ontvingen twee kleine RBC concentraten tegelijkertijd: 2 dagen en 35 dagen opgeslagen RBCs. Op basis van loting ontving de helft RBCs opgeslagen in SAGM en de andere helft in PAGGGM. Om de RBCs te kunnen vervolgen na transfusie werden ze gelabeld met

biotine. In deze studie vinden we dat de RBCs opgeslagen in PAGGGM niet langer in het lichaam overleven dan de RBCs die opgeslagen zijn in SAGM. De metabole status is wel beter in PAGGGM RBCs. De glycolyse en pentose fosfaat route is na transfusie actiever dan in de SAGM RBCs. Echter, na een dag zijn de verschillen tussen de twee groepen verdwenen.

Er is wel een verschil gevonden in de overleving tussen de 2 dagen en 35 dagen opgeslagen RBCs. Tussen 2 en 7 dagen na transfusie is de overleving van de 2 dagen oude RBCs significant hoger dan de 35 dagen oude RBCs. Daarnaast hebben 2 dagen oude RBCs een beter metabool profiel dan de 35 dagen oude RBCs. Ook hier is de glycolyse en pentose fosfaat route tot 1 dag na transfusie actiever.

Het labelen van bloedplaatjes met biotine

De huidige gouden standaard voor het labelen van bloedplaatjes is een radioactief label zoals Indium-111 en Chromium-51. Omdat dit niet is toegestaan in kwetsbare patiënten groepen zoals minderjarige en kritiek zieke patiënten op de intensive care is een veilig alternatief wenselijk. In hoofdstuk 7 exploreren we een alternatieve techniek met biotine, ook wel bekend als vitamine B8. Met behulp van biotine kunnen getransfundeerde bloedplaatjes na transfusie uit de circulatie geïsoleerd worden, iets wat niet mogelijk is met radioactieve labels.

We hebben een reproduceerbare methode ontwikkeld in een gesloten systeem zodat er geen besmetting met bacteriën kan optreden tijdens bewerkingsprocedure. Het biotine label zelf heeft geen effect op de kwaliteit van bloedplaatjes. Wel leiden de bewerkingstappen, die nodig zijn voor het labelen, tot enige activatie. Ondanks de kleine toename in activatie, voldoet een gebiotinyleerd bloedplaatjes product aan alle kwaliteitseisen van de Nederlandse bloedbank, inclusief de mate van Annexine V en CD62P expressie, wat belangrijke markers van bloedplaatjes activatie zijn. Daarnaast zijn de gebiotinyleerde bloedplaatjes nog in staat om te activeren na toediening van een stimulus. Dit geeft aan dat de gebiotinyleerde bloedplaatjes nog wel functioneel zijn en kunnen bijdragen aan de bloedstolling. Gebiotinyleerde bloedplaatjes zouden daarom in de toekomst gebruikt kunnen worden om de opbrengst en overleving van bloedplaatjes te meten, wat van belang is voor het onderzoeken van nieuwe bloedplatjes producten en de effectiviteit van bloedplaatjes transfusie in kwetsbare patiënten.

Appendices

Publication list

Curriculum Vitae

PhD Portfolio

Publication list

First authorships

de Bruin S, Peters AL, Wijnberge M, *et al.* Storage of red blood cells in the alkaline storage solution PAGGGM improves metabolism after transfusion but has no effect on post transfusion recovery. submitted

de Bruin S, Eggermont D, Vlaar APJ. Transfusion practices in the bleeding critically ill: an international survey- TRACE2 survey. Tranfusion 2021, in press

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van Oort PMP*, **de Bruin S***, Weda H, et al. Exhaled Breath Metabolomics for the Diagnosis of Pneumonia in Intubated and Mechanically-Ventilated Intensive Care Unit (ICU)-Patients. Int J Mol Sci. 2017;18(2):1-14. doi:10.3390/ijms18020449 **Both authors contributed equally*

Coauthorships

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Collaborator COVID-Biobank consortium

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Collaborator ESICM transfusion taskforce consortium

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Curriculum Vitae

Sanne de Bruin was born on the 29th of August 1990 in Haarlem, the Netherlands. She grew up in Lisse where she completed pre-university education (VWO) in 2009. Directly after high school she applied to medical school for which she was not accepted. To enhance her mathematical skills she studied Econometrics and Operational Research at the Vrije Universiteit in Amsterdam. After she successfully finished her first year and met the father of her daughter, she successfully applied for the second time for medical school. In 2010 she started medical school at the University of Amsterdam. Here she obtained her master's degree with honors in 2017. After a scientific internship on the intensive care, she decided that obtaining a PhD degree would be the next goal after her medical degree.

In 2017 she started as PhD student at Sanquin Research and Landsteiner Laboratory in Amsterdam and at the department of Intensive Care Medicine at the Amsterdam University Medical Centers, location AMC. The results of this research are described in this thesis. The progress of her thesis was delayed due to the COVID-19 pandemic. During the COVID-19 pandemic Sanne started with her post-doc on COVID-19 research. Currently she is working as a physician on the department of internal medicine in the Sint Antonius hospital in Nieuwegein.

PHD portfolio

Name PhD-student:	Sanne de Bruin
PhD-period:	2017 - 2022
Promotor:	prof. dr. A.P.J. Vlaar
Co-promotores:	dr. R. van Bruggen
	dr. D. de Korte

	Year	ECTs
General courses		
Sanquin science course	2017	1
Practical biostatistics	2017	1,4
BROK	2017	1,5
Escience	2017	0,6
Crash cours: basic chemistry, biochemistry and molecular biology	2017	0,2
Mass spectometry, proteomics and protein research	2018	2,1
Advanced biostatistics	2018	2,1
Oral presentation in english	2018	0,7
Data analysis in Matlab	2019	0,8
Bioinformatics	2019	1,1
Module: personal development (Sanquin)	2019	0,4
Clinical Epidemiology: Evaluation of Medical Tests	2019	0,9
Scientific Writing in English	2019	1,5
Annual Informed consent training	2017-2019	0,5
Presentations		
Het effect van opslagduur en opslag medium op de post transfusie klaring en metabool herstel van rode bloed cellen – eerste resultaten Oral presentation at the "Sanquin avond", bijscholingsavond voor artsen en transfusie experts	2018	0,5
Het effect van opslagduur en opslag medium op de post transfusie klaring en metabool herstel van rode bloed cellen – eerste resultaten Poster presentation at the annual congress of the Dutch Society for Blood Transfusion NVB, Ede, The Netherland	2019	0,5
Biotinyleren van trombocyten voor transfusie doeleinden - een nieuwe methode van het labelen van trombocyten in een gesloten systeem Oral presentation at the annual congress of the Dutch Society for Blood Transfusion NVB, Ede, The Netherlands	2019	0,5
Biotinylation of platelets for transfusion purposes- a novel method to label platelets in a closed system Oral presentation at the annual congress of the International Society of Blood Transfusion ISBT, Basel, Switzerland	2019	0,5

Transfusion practice in the non-bleeding critically ill patient; results from the international TRACE survey Poster presentation at the annual congress of the European Society for Intensive Care Medicine ESICM, Berlin, Germany	2019	0,5
Effect of Storage Time and Additive Solution on Post-transfusion Clearance of Red Blood Cells Poster presentation at the annual congress of the American Association of Blood Banks AABB, San Antonio, United States of America	2019	0,5
Biotinylation of platelets for transfusion purposes- a novel method to label platelets in a closed system Poster presentation at the annual congress of the American Association of Blood Banks AABB, San Antonio, United States of America	2019	0,5
Transfusion strategies in non-bleeding critically ill adults Invited speaker at the annual congres of the European Society for Intensive Care Medicine ESICM	2020	0,5
Prolonged storage time and storage in PAGGGM of red blood cells have a negative effect on post transfusion in vivo survival but does not influence pentose phosphate activity after transfusion Oral presentation at the annual congres of the International Society of Blood Transfusion ISBT, online conference	2020	0,5
Transfusion practice in the bleeding critically ill: an international online survey - the TRACE -2 survey Poster presentation at the annual congres of the International Society of Blood Transfusion ISBT, online conference	2020	0,5
Case reviewed - Transfusion update Invited speaker at the annual congres of the European Society for Intensive Care Medicine ESICM	2021	0,5
Grants		
AUF/ Spinoza Fonds, AMC	2019	
Travel grant AI&II, AMC	2019	
Conferences		
Attendance annual congres of the Durch Society for Blood Transfusion NVB, Ede, The Netherlands	2017	0,5
Attendance annual congres of the Durch Society for Blood Transfusion NVB, Ede, The Netherlands	2018	0,5
Attendance annual congres of the European Society for Intensive Care Medicine ESICM, Paris, France	2018	0,75
Attendance annual congres of the Durch Society for Blood Transfusion NVB, Ede, The Netherlands	2019	0,75
Attendace annual congres of the International Society of Blood Transfusion ISBT, Basel, Switzerland	2019	0,75
Attendance annual congres of the European Society for Intensive Care Medicine ESICM, Berlin, Germany	2019	0,75

Attendance annual congres of the American Association of Blood Banks AABB, San Antonio, United States of America	2019	0,75
Teaching		
Bachelor student, medical natural sciences, Vrije Universiteit Amsterdam	2018	1
Research internship (Medicine), University of Amsterdam	2019	1
Research internship (Medicine), University of Amsterdam	2019	1
Research internship (Master of Pathology, Immunology and Hematology), Ismailia blood transfusion Services Center Egypt	2019	1
Other activities		
Screen service clinical studies intensive care unit AMC	2017-2019	24
Intensive Care Research Meeting (weekly)	2017-2021	12
Intensive Care Journal Club (monthly)	2017-2021	4
Laboratory of Experimental Intensive Care and Anesthesiology (LEICA) research meeting (weekly)	2017-2021	12
Sanquin research meeting (weekly)	2017-2019	12
Product Proces Ontwikkeling research meeting (monthly)	2017-2019	4

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Dit proefschrift is tot stand gekomen met hulp van meer mensen dan ik kan opnoemen. Op deze manier wil ik iedereen bedanken die mij heeft geholpen op wat voor manier dan ook. Dat kan op vele manieren geweest zijn, bijvoorbeeld technische of logistieke ondersteuning, een kritische blik, een tissue aangeven of samen koffie (of iets anders) drinken. Een aantal mensen wil ik in het bijzonder noemen.

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