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Full Length Article

The effect of red blood cell transfusion on platelet function in critically ill patients

Maike E. van Hezel[a,b], Lisa van Manen[a,b], Margit Boshuizen[a,b], Marleen Straat[b], Iris M. De Cuyper[a], Boukje Beuger[a], Rienk Nieuwland[c], Michael W.T. Tanck[d], Dirk de Korte[a,e], Jaap Jan Zwaginga[f], Robin van Bruggen[a,1], Nicole P. Juffermans[b,*,1]

[a] Department of Blood Cell Research, Sanquin Research, Amsterdam, the Netherlands
[b] Department of Intensive Care Medicine, Amsterdam University Medical Center, location Academic Medical Center, Amsterdam, the Netherlands
[c] Laboratory of Experimental Clinical Chemistry, and Vesicle Observation Centre, Academic Medical Center of the University of Amsterdam, Amsterdam, the Netherlands
[d] Department of Clinical Epidemiology, Biostatistics and Bioinformatics (KEBB), Academic Medical Center of the University of Amsterdam, Amsterdam, the Netherlands
[e] Department of Product and Process Development, Sanquin Blood Bank, Amsterdam, the Netherlands
[f] Department of Immunohematology and Bloodtransfusion, Leiden University Medical Center, Leiden, the Netherlands

ABSTRACT

Introduction: Red blood cell (RBC) transfusion is associated with an increased risk of pro-thrombotic events, but the underlying mechanism is poorly understood. We hypothesized that RBC transfusion modulates platelet activity in critically ill patients with and without sepsis.
Methods: In a prospective cohort study, 37 critically ill patients receiving a single RBC unit to correct for anemia were sampled prior to and 1 h after transfusion. Platelet exposure of P-selectin, CD63 and binding of PAC-1 as well as formation of platelet-leukocyte complexes were measured by flow cytometry. The ability of plasma from critically ill patients to induce *ex vivo* platelet aggregation was assessed by flow cytometry after incubation with platelets from a healthy donor.
Results: RBC transfusion neither triggered the expression of platelet activation markers nor the formation of platelet-leukocyte complexes. Plasma from critically ill patients induced more spontaneous platelet aggregation prior to RBC transfusion compared to healthy controls, which was further augmented following RBC transfusion. Also collagen-induced platelet aggregation was already increased prior to RBC transfusion compared to healthy controls, and this response was unaffected by RBC transfusion. In contrast, ristocetin-induced platelet agglutination was decreased when compared to controls, suggesting impaired vWF-dependent platelet agglutination, even in the presence of high vWF levels. Following RBC transfusion, ristocetin-induced platelet agglutination further decreased. There were no differences between septic and non-septic recipients in all assays.
Conclusion: *Ex vivo* platelet aggregation is disturbed in the critically ill. Transfusion of a RBC unit may further increase the spontaneous platelet aggregatory response.

1. Introduction

Red blood cell (RBC) transfusions are often administrated to critically ill patients [1] to correct for anemia or to replace blood loss after acute bleeding. However, RBC transfusions are also associated with an increased risk for adverse effects, including thromboembolic events [2–9]. In patients with an acute coronary syndrome, RBC transfusion is associated with myocardial infarction [2–5]. In patients undergoing a surgical procedure, perioperative RBC transfusion is associated with stroke [6,7], contributing to mortality [10].

The underlying mechanisms by which RBC transfusion increases the risk of arterial thrombosis may not merely be a haematocrit-mediated platelet margination towards the vessel wall [11], but may be due to platelet activation as well. In an *in vitro* transfusion model, RBC products induced platelet activation and aggregation [12]. In a flow model, RBC enhanced platelet aggregation [13], *via* both physical and chemical effects [14–16]. Both extracellular vesicles (EVs) as well as cell free hemoglobin, released upon hemolysis, can increase nitric oxide scavenging, resulting in vasoconstriction [17] and platelet activation [18,19]. Also, adenosine diphosphate (ADP), released from activated/damaged RBCs, triggers platelet adhesion and aggregation *in vitro* [19]. Experimental models showed that both ADP and hemoglobin infusions in rats trigger platelet aggregation [19]. Besides arterial thromboembolic events, RBC transfusions are also associated with venous thromboembolic events. In a murine model, transfusion with murine RBCs resulted in an increased clot formation [20]. Furthermore, in a murine vascular thrombosis model, RBCs contribute to thrombus formation by mediating platelet adhesion to the intact endothelium [21].

In surgical patients, RBC transfusion is associated with an increased risk of postoperative venous thromboembolic events [8,9]. Potential

* Corresponding author at: Intensive Care, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, the Netherlands.
E-mail address: n.p.juffermans@amsterdamumc.nl (N.P. Juffermans).
[1] These authors contributed equally.

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mechanisms include an increase in blood viscosity [2], a decrease in deformability of RBCs during storage [22] and the induction of shear stress by the release of potential pro-coagulant mediators [23]. EVs from RBC products have pro-coagulant properties by exposing phosphatidylserine (PS) [24] and can facilitate thrombin generation *in vitro* [25–27].

In critically ill patients, systemic inflammation is associated with enhanced coagulation and platelet activation [28,29]. Also, thromboembolic events are frequent in this patient population [30] and associated with poor outcome [31].

This study aimed to investigate the effect of RBC transfusion on platelet activation and aggregation in critically ill septic and non-septic patients. We hypothesized that RBC transfusion in critically ill patients may induce platelet activation, in particular when systemic inflammation is present. First, platelet surface expression markers were measured in critically ill patients before and after receiving a RBC transfusion. Second, the formation of complexes between platelets and leukocytes was measured [32,33]. Finally, the effect of plasma from the RBC recipients on the ability to form platelet aggregates was measured *ex vivo*, using platelets from healthy volunteers.

2. Material & methods

This prospective, observational cohort study adhered to the Declaration of Helsinki and was approved by the Medical Ethical Committee of the Academic Medical Center (trialregister.nl NTR 6596, NL61833.018.017). Written informed consent was obtained from all patients (or from their representatives) and healthy controls.

2.1. Patients

Critically ill patients receiving a single RBC transfusion because of anemia, when their hemoglobin level was 7 g/dL, were eligible for the study. Patients were excluded when they were actively bleeding or when they received multiple RBC units or a RBC, plasma or thrombocyte transfusion in the last 24 h prior to inclusion. Patients were subdivided into sepsis and non-sepsis at the time of the transfusion according to the SEPSIS-3 criteria [34]; which includes a Sequential Organ Failure Assessment (SOFA) score ≥ 2 in combination with a suspected or proven infection which was treated with antibiotics. Citrate-anticoagulated blood samples were collected from septic and non-septic patients from an indwelling, non-heparinized arterial catheter prior to and 1 h after administration of the RBC transfusion. Part of the whole blood samples was directly used for experiments, the rest was centrifuged (1500*g* for 20 min and 10,000*g* for 5 min) and the platelet poor plasma (PPP) was stored at −80 °C until use.

2.2. Whole blood platelet activation markers

Citrated whole blood was collected prior to and 1 h after the transfusion. Within 5 min after collection the blood was transferred to tubes containing Hepes buffer (pH 7.4, 132 mM NaCl, 6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1.2 mM K_2HPO_4, 20 mM Hepes, 5.5 mM glucose (Merck-Millipore, Burlington, MA) and 0.5% (w/v) human serum albumin (Brocacef, Maarsen, The Netherlands)) and the antibodies directed against activation markers. Platelet activation was measured before and after addition of the platelet agonist thrombin receptor activating peptide (TRAP-6; 15μM; Bachem, Bubendorf, Switzerland) by flow cytometry, combining antibodies against a general antigen (anti-CD61-APC; Dako, Cambridge, United Kingdom) and their activation markers: P-selectin (translocation of P-selectin; anti-CD62p-PE; Beckman Coulter, Marseille, France), CD63 (a lysosomal membrane glycoprotein; anti-CD63-PE; Beckman Coulter) and PAC1-binding (fibrinogen binding site exposed on the activated form of the glycoprotein (GP) IIb/IIIa receptor; anti-PAC1-FITC; BD Bioscience, San Jose, CA). After gentle mixing and incubation for 30 min at room temperature in the dark the samples (total volume 55 μL) were fixed with 2.5 mL 0.3% paraformaldehyde-containing Hepes buffer. Fixed samples were measured on flow cytometry (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ) to determine the number of antibodies that bind to platelets and evaluated by both the percentage (%) of positive platelets and as mean fluorescence intensity (MFI). The threshold was set at 1% of the isotype control.

2.3. Circulating platelet-leukocyte complexes

Platelet-leukocyte complexes (PLC) are defined as monocytes, granulocytes or lymphocytes that are bound to platelets. Citrate-anticoagulated whole blood was collected prior to and 1 h after the transfusion, and within 5 min after collection the blood was added to tubes containing Hepes and antibodies. The PLC were measured by using a platelet marker (anti-CD61-PerCP; BD Bioscience) in combination with a marker for monocytes (anti-CD14-PE; eBioscience, San Diego, CA), granulocytes (anti-CD66b-FITC; Beckman Coulter) or lymphocytes (anti-CD4-PE; eBioscience). After gentle mixing and incubation for 30 min at room temperature in the dark, the samples (total volume 55 μL) were fixed with 0.5 mL 0.3% paraformaldehyde-containing Hepes buffer. After 60 min of fixation, red blood cells were lysed by addition of distilled water [35] and samples were measured by flow cytometry. Leukocytes were identified by the characteristic side scatter and the pan leukocyte marker (anti-CD45-APC; BD Bioscience). Samples were analyzed by percentage CD61 positive cells within the population of monocytes, granulocytes and lymphocytes. The threshold of platelet-leukocyte binding was set at 1% of the isotype control.

2.4. Platelet aggregation test

The capacity of RBC transfusion to trigger platelet aggregation was assessed following *ex vivo* incubation of platelets from healthy volunteers with citrate plasma from critically ill patients collected both *before* and *after* transfusion, measured by flow cytometry [36–40]. This assay was chosen because it required less volume sample and is faster to perform than the light transmission aggregometry. First, heparinized blood from healthy volunteers (blood group O) was centrifuged for 15min at 210*g* and platelet-rich plasma (PRP) was collected. PRP was divided in two suspensions and stained with anti-CD31-APC (BD Bioscience) or anti-CD31-FITC (BD Bioscience). After incubation the stained populations were washed twice with sequestrine buffer (17.5 mM Na_2HPO_4, 8.9 mM Na_2EDTA, 154 mM $NaCl_2$, pH 6.9 containing 0.1% (w/v bovine albumin) (Sigma-Aldrich)) by 5 min at 2250*g* centrifugation and resuspended to a concentration of 40×10^6/mL in Hepes buffer. The differently labeled platelets from healthy volunteers were 1:1 mixed and subsequently incubated with 50% citrate plasma from transfused recipients and 20 μM PPACK (D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; Calbiochem, Darmstadt, Germany), while shaking at 700 rpm at 37 °C. After 25 min incubation 3 mM $CaCl_2$ was added and incubated extra for 5min as done before [36,40]. Platelet aggregation was induced using different stimuli. After taking the baseline sample, the platelet suspensions were on a shaker and activated with 10 μg/mL collagen (Agro-Bio, STAGO; Asnieres, France), 100 ng/mL phorbol myristate acetate (PMA, Sigma-Aldrich; St Louis, MO) or 1.5 mg/mL ristocetin (Biopool, Trinity Biotech Plc, Bray, Co Wicklow, Ireland). Here, PMA was used because of the number of events measured on the flow cytometry was found to be higher compared to TRAP. This enhanced the gating and the assay's sensitivity. At different time points samples were taken stopping the reaction by addition to a 9× volume of 0.5% (v/v) formaldehyde (Polysciences Inc., Warrington, NJ, USA, methanol-free) in PBS. The collagen and PMA stimulation were measured over a time frame of 5 min and the ristocetin over 1min. Fixed samples were measured by flow cytometry (LSR + HTS, BD Bioscience) within 60 min. For analysis a quadrant was set in box plot of the control samples without adding plasma, the

percentage of double-colored platelets were used for measurement of platelet aggregation (described more in detail in [36]). Data are analyzed as Area Under the Curve (AUC). Von Willebrand Factor antigen (vWF:Ag) levels in the plasma were measured by ELISA with antibodies from DAKO (Copenhagen, Denmark).

2.5. Statistical analysis

Data were expressed as mean and SD (when normally distributed) or as median with interquartile range (IQR, when non-normally distributed). Comparisons between before and after transfusion were tested using the paired *t*-test (when data were normally distributed) or Wilcoxon Signed Ranks (when data were not normally distributed). For the comparisons between septic and non-septic patients the post transfusion change *versus* baseline (delta) was determined and tested by Mann Whitney *U* test (when data were not normally distributed). Analyses were done by IBM SPSS Statistics version 24. p values < 0.05 were considered statistically significant.

2.6. Power statement

The sample size of the study was based on the primary outcome (trialregister.nl NTR 6596, NL61833.018.017). For the present outcomes, the sample size used (17 *vs* 20) will have 80% power to detect an effect size of 0.95 using a *t*-test with a two-sided significance level of 0.05.

3. Results

In total 37 patients were included, of which 17 patients were septic at the time of the transfusion. Due to logistical problems, measurement of activation markers was not done in 2 patients and PLC measurements were missing in 4 patients. The baseline characteristics are presented in Table 1. Non-septic patients were more often male. There were no differences in age, referral specialty, hemoglobin level (pre- and post transfusion) and storage time of the RBC between the septic and non-septic group. The platelet count did not differ before and after RBC transfusion.

3.1. The effect of RBC transfusion on whole blood platelet activation markers and platelet-leukocyte complexes (PLC)

Prior to RBC transfusion, platelets had a markedly high expression of CD63 even without additional stimulation with TRAP. RBC transfusion did not induce a change in the percentage and the mean fluorescence intensity (MFI) of platelets expressing P-selectin, CD63 or binding of PAC1 compared to pre-transfusion levels. These results were found with and without additional stimulation with TRAP (Fig. 1), with the exception of a slight decrease of TRAP-induced binding of PAC1 after RBC transfusion (23.2% [9.6–44.6] *vs* 19.7% [7.0–42.1], $p < 0.05$). Also, after RBC transfusion, the formation of platelet-monocyte, platelet-granulocyte and platelet-lymphocyte complexes did not differ compared to pre-transfusion levels. The platelet cell surface activation markers and the number of PLC post transfusion did not differ between septic and non-septic recipients of a RBC transfusion (Fig. 2).

3.2. The effect of RBC transfusion on the ability of recipients to induce ex vivo platelet aggregation

In the functional platelet aggregation assay, the capacity of plasma from the critically ill patients to induce *ex vivo* spontaneous platelet aggregation was already increased prior to RBC transfusion when compared to plasma from the healthy controls (AUC 69 [46–87] *vs* 44 [30–52], $p < 0.001$, Fig. 3). The capacity of patient plasma to induce platelet aggregates was further augmented following RBC transfusion when compared to pre-transfusion levels (AUC 71 [53–90] *vs* 69 [46–86], $p < 0.05$, Fig. 3). Also, collagen-induced platelet aggregation was increased in the presence of plasma from the critically ill patients when compared to control plasma (AUC 19 [15–25] *vs* 12 [11–14], $p < 0.001$, Fig. 4a), but this aggregation was not further augmented following RBC transfusion (AUC 18 [14–26]). In the PMA-induced platelet aggregation, there were no differences between plasma from the critically ill patients and healthy controls, and transfusion did not affect the PMA-induced platelet aggregation capacity of the plasma samples. In contrast, ristocetin-induced platelet agglutination was decreased in plasma from the critically ill patients compared to those of healthy controls (AUC 9 [6–12] *vs* 11 [9–14], $p < 0.05$, Fig. 4c), suggesting that vWF dependent platelet agglutination is impaired. This response was further decreased after RBC transfusion when compared

Table 1
Critically ill patient characteristics.

	All patients (n = 37)	Septic patients (n = 17)	Non-septic patients (n = 20)	p value
Age (years)	61 [55–71]	63 [56–71]	60 [53–66]	0.357
Sex				
Male, n (%)	22 (59)	7 (41)	15 (75)	0.037
Specialty, n (%)				0.355
Cardiology	7 (19)	2 (12)	5 (25)	
Cardiothoracic surgery	9 (24)	3 (18)	6 (30)	
Internal medicine	9 (24)	5 (29)	4 (20)	
Neurology	1 (3)	0 (0)	1 (5)	
Surgery	8 (22)	6 (35)	2 (10)	
Traumatology	3 (8)	1 (6)	2 (10)	
SOFA on transfusion day	7 [5–11]	8 [5–11]	7 [5–9]	0.341
RBC storage time, days	13 [6–22]	12 [4–22]	16 [6–23]	0.407
Hemoglobin (mmol/L)				
Pre transfusion	4.1 [3.9–4.5]	4.0 [3.8–4.4]	4.3 [4.1–4.6]	0.209
Post transfusion	5.0 [4.6–5.3]	5.0 [4.5–5.3]	5.0 [4.6–5.2]	0.821
Platelet count ($*10^9$/L)				
Pre transfusion	156 [106–205]	174 [130–276]	134 [104–199]	0.297
Post transfusion	183 [104–241]	207 [129–256]	150 [103 − 211]	0.266
Leukocytes ($*10^9$/L)				
Pre transfusion	14.2 [9.5–17.9]	15.0 [9.9–18.9]	13.1 [10.7–17.0]	0.684
Post transfusion	15.3 [9.5–16.7]	15.4 [9.6–19.1]	15.3 [9.3–16.0]	0.330
Hospital mortality, n (%)	12 (32)	8 (47)	4 (20)	0.080

SOFA: Sequential Organ Failure Assessment, RBC: red blood cell. Data are expressed as median with interquartile range [IQR].

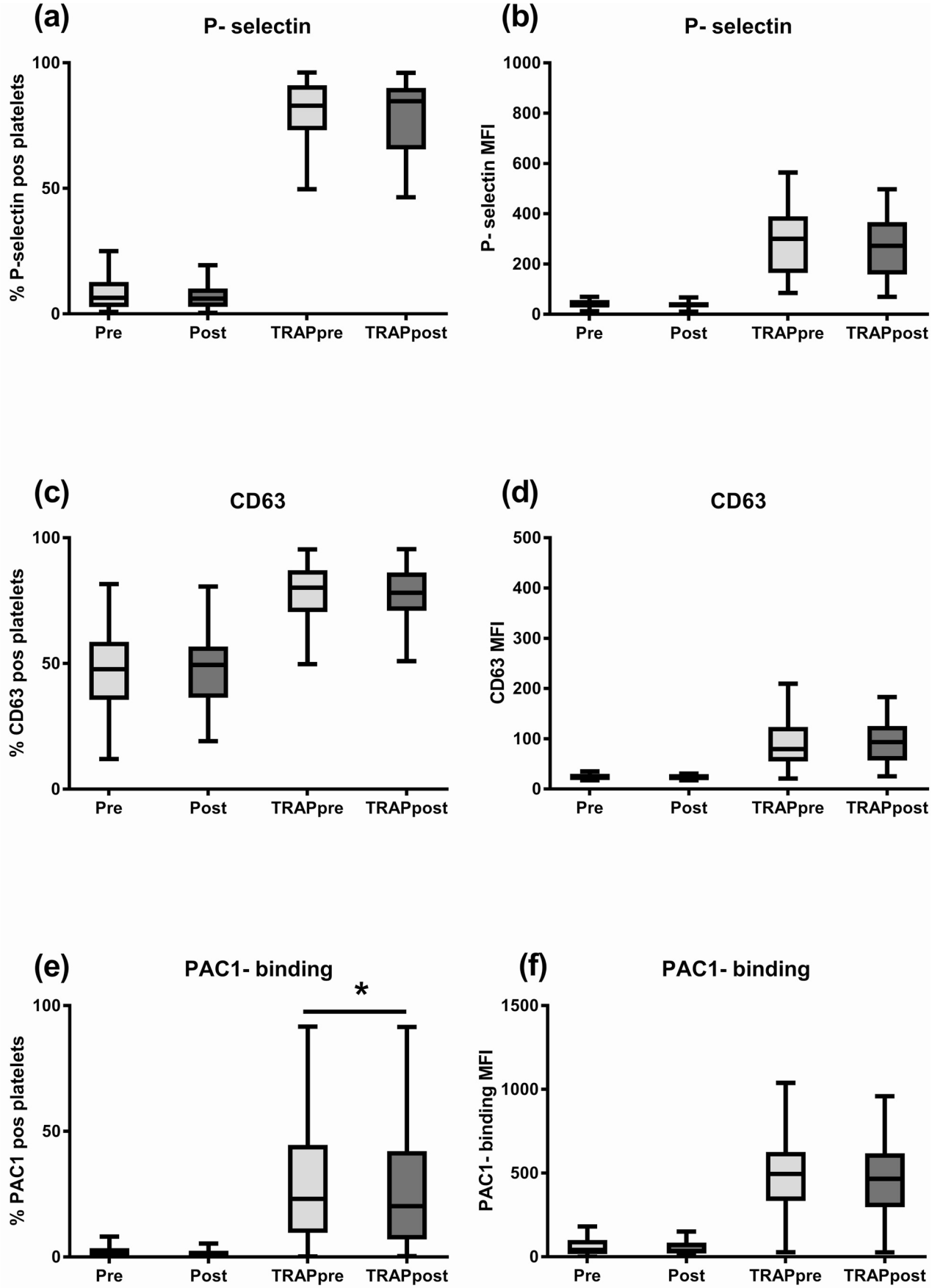

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Fig. 1. Flow cytometric determination of surface expression markers on resting and TRAP-stimulated platelets from critically ill patients who received a RBC transfusion. (a) Percentage (%) platelets expressing P-selectin and (b) mean fluorescence intensity (MFI) of the antibody binding P-selectin; (c) % platelets expressing CD63 and (d) MFI of the antibody binding CD63; (f) % of platelet binding PAC1 and (e) MFI of the antibody binding PAC1. Data are median [IQR]. *p < 0.05, **p < 0.005, ***p < 0.001.

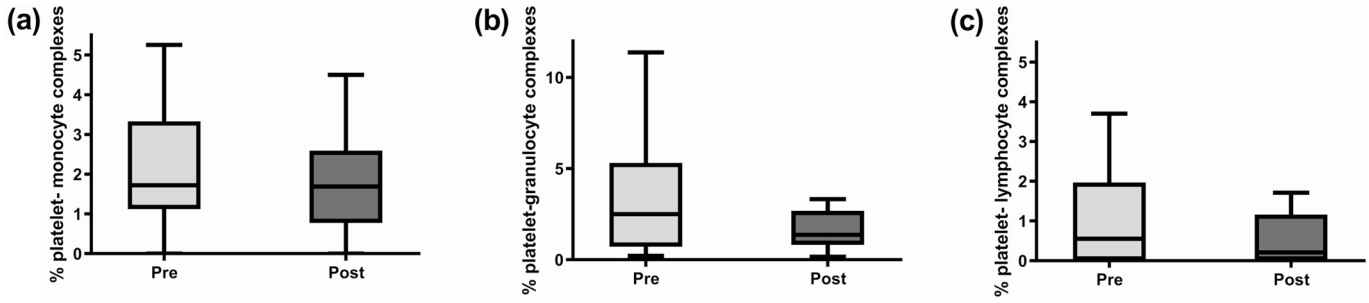

Fig. 2. Flow cytometric determination of platelet-leukocyte complexes in critically ill patients who received a RBC transfusion. (a) Percentage (%) of platelet-monocyte complexes, (b) % of platelet-lymphocyte complexes, (c) % of platelet-granulocyte complexes. Data are median [IQR].

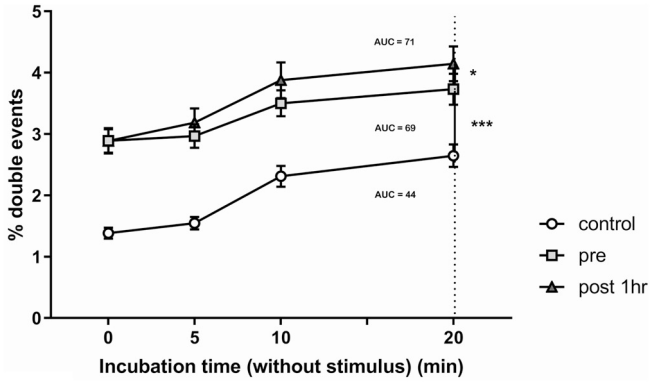

Fig. 3. Flow cytometric determination of spontaneous platelet aggregation of platelets from healthy with plasma from critically ill patients before or after a RBC transfusion. Controls were incubated with plasma from healthy controls. Platelet aggregation was measured by flow cytometry, as determined by the percentage of double events. Time is incubation time. Data are Area Under the Curve (AUC).

to pre-transfusion levels (AUC 9 [6–11] *vs* AUC 9 [6–12], p < 0.05, Fig. 4c). Of note, compared to controls, the levels of vWF:Ag were increased in plasma from the critically ill patients (109% [46–204] *vs* 477% [170–1825], p < 0.001). However, there were no differences in vWF:Ag between pre- and post-transfusion levels. In all assays used, the effect of the RBC transfusion on platelet aggregation response was not different between septic and non-septic patients.

4. Discussion

This study investigated the effect of a single RBC transfusion on platelet function in critically ill patients. The main conclusions are (1) a single unit of RBC transfusion does not induce expression of platelet activation markers or formation of platelet-leukocytes complexes. Furthermore, (2) the ability of critically ill patients to induce *ex vivo* platelet aggregation is disturbed compared to controls, and (3) RBC transfusion further augmented the spontaneous platelet aggregation response.

In this study, RBC transfusion did not induce expression of platelet activation markers in critically ill patients. Of note, we found a reduction in the percentage of platelets binding PAC1 after TRAP stimulation, which may indicate a reduced number of reactive platelets. However, MFI did not differ between groups. Thereby, we think this is a modest effect. Also, RBC transfusion did not result in the formation of platelet-leukocyte complexes in critically ill recipients, when compared to pre-transfusion values. Whether this relates to the pre-transfusion activation status remains to be determined, as we could not perform measurements in healthy controls receiving an RBC transfusion.

There are several explanations for the observed modest effect of the RBC transfusion on the activation markers and formation of complexes. First, to a certain extent, platelets of critically ill patients may already be activated [41–43]. Activated platelets can attach to the blood vessel wall, rendering them no longer measurable in our assays [44]. Also, unstimulated platelets in this cohort partially expressed CD63, indicating some activation. However, there were still robust responses in platelet activation after exogenous activation with TRAP, this would not be the case if platelets were preactivated. Second, the study could be underpowered to find a difference in activation markers. If not

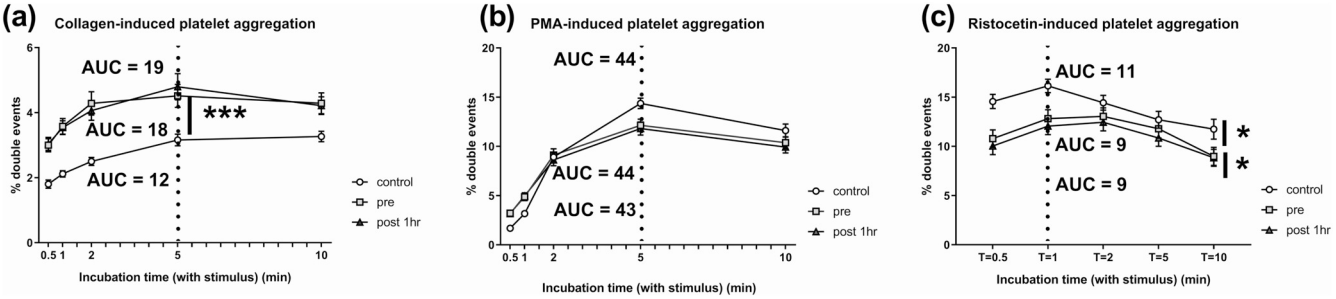

Fig. 4. Flow cytometric determination of collagen-, PMA-, or ristocetin-induced platelet agglutination of platelets from healthy volunteers with plasma from critically ill patients before or after a RBC transfusion. Aggregation was measured by flow cytometry as percentage of double events with collagen stimulation (a), with PMA stimulation (b) and with ristocetin stimulation (c). Time is incubation with stimulus. Data are Area Under the Curve (AUC).

underpowered, the effects of RBC transfusion on platelet aggregation in this study may occur independent of expression of activation markers.

We show that the capacity of plasma of critically ill patients to induce platelet aggregation is disturbed when compared to healthy controls. The capacity to induce spontaneous aggregation was increased in plasma from the critically ill patients compared to controls. We hypothesize that this is related to an underlying inflammatory status. In line with this, *in vitro* platelet aggregation is increased in the presence of lipopolysaccharide (LPS) [45]. It has previously been demonstrated that plasma from septic patients increases the aggregation of platelets from healthy volunteers *ex vivo* [41]. We built on these findings in this study, showing that both spontaneous as well as collagen-induced aggregation capacity of plasma is enhanced in critically ill patients when compared to controls. We hypothesize that several inflammatory conditions, including sepsis and trauma, may cause activation of the vascular endothelium [46–48] with disruption of the vessel wall and exposure of collagen [49,50].

Notably, we found that platelet aggregation results vary depending on the stimulus used. In this study, plasma-induced platelet agglutination mediated by vWF was decreased in critically ill patients when compared to healthy controls. This was not due to low levels of vWF, as the level of vWF antigen was higher in critically ill patients when compared to controls, as found before [51]. An explanation may be stress-induced GPIb shedding, because ristocetin binds to GPIb as part of the vWF-receptor complex and spontaneous receptor shedding is frequently observed under stress [52]. Alternatively, the presence of abnormal vWF variants may have resulted in decreased vWF-mediated platelet agglutination. However, we think this is unlikely, because the binding in patients was overall low. We do not have an explanation for our finding that vWF-mediated platelet agglutination is decreased. Of note, the decrease in vWF mediated platelet agglutination was modest and may not reflect clinical significance.

RBC transfusion further aggravated the patient plasma induced disturbances in platelet aggregation capacity in critically ill recipients. Our findings confirm *in vitro* data, in which RBCs increased platelet aggregation after stimulation with weak agonists ADP and arachidonic acid [53]. In patients with acute coronary syndrome, RBC transfusion resulted in an increase in plasma-induced platelet aggregation after stimulation with ADP and TRAP compared with pre-transfusion levels, measured by light transmission aggregometry (LTA) [54]. In addition, in patients with chemotherapy-induced anemia, RBC transfusion was associated with increased fibrin formation with impaired clot strength, measured by thromboelastography, which was more present in the patients who received fresh RBCs [55]. The clinical relevance of this finding is unknown. Notably, critically ill patients often receive RBC transfusions [1] while having low platelet counts with a tendency to bleed. Theoretically, our results may suggest that RBC transfusion increases the bleeding risk in these patients by further derangement of platelet aggregation. On the other hand, our data could also suggest that in patients who need RBC transfusions in the intensive care unit, an active approach to detect (micro) thrombotic events should be employed. Of note, however, we did not find a decrease in platelet counts after RBC transfusion, so even the plasma from the critically patients gives an increased aggregation of platelets from a healthy donor, the effect on the platelets of the critically patients is unclear.

It has been suggested previously that the underlying inflammatory condition may influence the effects of the RBC transfusion. *In vitro* under flow, both endotoxin and TNF-a can promote the adherence of donor RBCs to the endothelium [56,57]. Although we could not see differences between septic and non-septic patients, the number of patients we studied was limited and might have been too small to detect differences.

Our study had several limitations. As previously mentioned, activated platelets might sequestrate from the circulation and in this respect not show up in our platelet activation or PLC assay. For logistical reasons we had to fix the samples, which might affect the measurements. Also we were not able to perform aggregation assays or studies in flow models with the platelets derived from the patients themselves. Furthermore, we did not investigate a control group in the platelet activation and PLC assay. In addition, blood samples from patients and controls differed, as arterial samples were used in the patient population and venous samples were used in the controls. Notwithstanding this, the method of processing was the same and patients served as their own control by comparing pre- and post-transfusion measurements. General limitations were that our study design is limited by time points and the moderate number of patients studied. In this respect, critically ill patients in particular are notably heterogeneous. Moreover, only the effect of a single RBC transfusion episode was monitored, the lack of effect may be due to the fact that a single RBC is under the detection limit of RBC effects on circulating platelets based on the volume of circulating blood. However, studying transfusion of one RBC unit was deliberately chosen since a single RBC transfusion to correct anemia is common practice in the ICU [58]. A final limitation is that we cannot exclude that some of the effects is a result of volume loading instead of a specific effect of RBC transfusion.

In conclusion, this study found that RBC transfusion in critically ill patients is associated with mixed patient plasma induced effects on platelet aggregation. Future studies are needed to investigate which factors mediate the enhanced platelet aggregation after RBC transfusion, and if there are possibilities to eliminate this effect by adjustment of processing methods [59].

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Author contributions

M.E.v.H., N.P.J. and R.v.B. designed the investigation; M.E.v.H., L.v.M, M.B., M.S., I.M.D.C. and B.B. performed the research; M.E.v.H. and M.W.T. analyzed the data; N.P. and R.v.B. supervised the conduct of the study; and M.E.v.H. wrote the paper. All authors read and corrected the paper.

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Declaration of competing interest

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

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