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Platelet transfusions reduce fibrinolysis but do not restore platelet function during trauma hemorrhage --Manuscript Draft--

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Meetings presented This study was presented at the 75th annual meeting of the American Association for the Surgery of Trauma, September 14-17, 2016, in Waikoloa, HI.

To: Dr E.E. Moore
Editor, Journal of Trauma and Acute Care Surgery.

Thank you for the opportunity to re-revise our manuscript and respond to the reviewer's comments. We acknowledge the valid concerns that have been raised, and have taken further steps to clarify our analysis strategy, provide additional information regarding the groups, and address the potential confounders relating to our observations. We have highlighted all changes in the manuscript text, and we include a point-by-point response to each of the comments below.

Yours sincerely

Dr Ross Davenport

Response to Reviewer:

I must echo the previous reviewer 2's comments about the confusing description of the groups. I acknowledge the efforts of the authors to improve this description but a diagram (as recommended by the reviewer) would do wonders to improve the clarity of the grouping.

We have included a flow diagram (figure 1) illustrating the inclusion and exclusion strategy, and have attempted to make the grouping clearer in the text.

I am also concerned (also echoing previous comments) that there are multiple confounders that can explain the findings, for which some form of adjustment is very much needed. I was unable to understand the authors' stated efforts to deal with the confounders (in which case the readers are likely to not understand as well). Assuming there are enough patients, survival analysis with time-varying covariates is a possibility using a propensity score (not for matching but as an adjustor for the confounders. A more elegant approach to the comparisons would be to model the interaction with platelet transfusion, but this is not a deal breaker. Also, comparing patients over time using a repeated measures approach would be useful and very elegant.

Thank you for these comments. We accept that there are confounders which could potentially contribute to our observations. Our analysis compares patients at a series of sampling points during resuscitation. Regarding our observations on trends in platelet aggregation, we have now included a table (table 2) outlining the characteristics of the groups being compared at each point. This demonstrates that there are no significant differences in injury characteristics or outcomes between the groups being compared, which we hope will mitigate some of the reviewer's concerns. The variation in time of sampling has been described in the results section and acknowledged in the limitations.

For the comparison of plasma proteins and viscoelastic measures, we have compared intervals rather than patients, and thus matching based on injury characteristics is not possible. This approach enabled us to adjust for concomitant FFP administration, a major confounder when considering interval changes in viscoelastic and plasma protein measurements. We have also conducted additional analyses regarding the relative duration of intervals, which show that this does not appear to represent a major confounder within our dataset. This is described in the results section and discussed in the limitations. Unfortunately, there are insufficient patient numbers for a propensity-score based approach.

The statistical tests described in Methods are inconsistent with the methods in Figures (possibly just the name, but possibly the tests are different?). Please provide consistent information for the statistical tests.

Thank you. We have corrected this omission in the text.

Minor: please replace the term parameter by variable or similar. Please include the study period in the Abstract. "Data" are not parametric/not parametric, "distributions" are.

We have made these changes in the abstract and manuscript text.

ABSTRACT

Introduction

Platelets play a critical role in hemostasis with aberrant function implicated in trauma-induced coagulopathy. However, the impact of massive transfusion protocols on platelet function during trauma hemorrhage is unknown. The aim of this study was to characterize the effects of platelet transfusion on platelet aggregation and fibrinolytic markers during hemostatic resuscitation.

Methods

Trauma patients enrolled into the prospective Activation of Coagulation and Inflammation in Trauma (ACIT) study between January 2008 and November 2015 who received at least four units of packed red blood cells (PRBCs) were included. Blood was drawn in the emergency department within 2 hours of injury and at intervals after every 4 units of PRBCs transfused. Platelet aggregation was assessed in whole blood with multiple electrode aggregometry. Plasma proteins were quantified by enzyme-linked immunosorbent assay.

Results

Of 161 patients who received 4 or more PRBCs as part of their initial resuscitation, 44 received 8-11 units and 28 received 12 units or more. At each time point during bleeding, platelet aggregation was similar in patients who had received a platelet transfusion compared to those who had only received other blood products ($p>0.05$ for all time points). Platelet transfusion during the 4PRBC intervals was associated with a decrease in maximum lysis on rotational thromboelastometry (start of interval 6% [2-12] vs end of interval 2% [0-5], $p=0.001$), an increase in plasminogen activator inhibitor-1 (PAI-1; start of interval: 35.9 +/-

14.9 vs end of interval: 66.7 +/- 22.0, p=0.007) and a decrease in tissue plasminogen activator (start of interval: 26.2 +/- 10.5 vs end of interval: 19.0 +/- 5.1, p=0.04). No statistically significant changes in these parameters occurred in intervals which did not contain platelets.

Conclusion

Current hemostatic resuscitation strategies do not appear to restore platelet aggregation during active hemorrhage. However, stored platelets may attenuate fibrinolysis by providing an additional source of PAI-1. Further investigation into the effects of early platelet transfusion on platelet function, haemostatic and clinical outcomes during bleeding are warranted.

Level of Evidence: Prospective observational study, level III

Key words: Platelets; transfusion; hemorrhage; resuscitation, fibrinolysis

TITLE PAGE

Title Platelet transfusions reduce fibrinolysis but do not restore platelet function during trauma hemorrhage.

Short Title Hemostatic effects of platelet transfusions

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Conflict of interest TEM International GmbH (Munich, Germany) provided ROTEM reagents and equipment on an unrestricted basis for the ACIT II study.

Meetings presented This study was presented at the 75th annual meeting of the American Association for the Surgery of Trauma, September 14th-17th, 2016, in Waikoloa, HI.

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MANUSCRIPT TEXT

Introduction

Hemorrhage is the principal cause of preventable death due to injury.¹ Contemporary damage-control resuscitation (DCR) is characterised by expedited hemorrhage control with simultaneous and empiric transfusion of blood and blood products in high ratios.² Maintaining hemostatic potential through early administration of plasma and platelets in high volumes alongside transfusion of packed red blood cells (PRBCs) minimises dilutional coagulopathy and is associated with improved outcomes.³ However, the effects of platelet transfusions remain unclear, while the provision of platelet transfusions can be challenging. Platelet function is known to be reduced in Trauma-Induced Coagulopathy (TIC),^{4,5} but the effects of platelet transfusion on coagulation during active bleeding are unknown. This knowledge gap has been highlighted by several contemporary major hemorrhage guidelines.^{6,7}

Platelets promote coagulation primarily by adhering to the endothelium or subendothelium to form aggregates at sites of vascular injury, and by providing a phospholipid platform to support thrombin generation.⁸ Haemostasis is further amplified through the release of reactive mediators from platelet storage granules into the plasma space,⁹ including clotting factors and mediators of fibrinolysis. In particular, platelets contain a large pool of plasminogen activator inhibitor-1 (PAI-1)¹⁰ and alpha-2 antiplasmin (A2AP),¹¹ which are hypothesised to represent an important mechanism through which platelets attenuate tissue plasminogen activator (tPA)-mediated fibrinolysis *in vitro*.^{12,13} Impaired platelet aggregation

is common after severe injury and associated with increased mortality, although the mechanisms of this remain unknown.¹⁴⁻¹⁶ Attempts to support platelet function through transfusion of allogeneic platelets during major haemorrhage therefore seems logical, but to date no studies have evaluated how platelet transfusions affect hemostasis during bleeding in trauma patients.

Our working hypothesis was that platelet transfusions exert their effects either through augmenting aggregation, promoting thrombin generation or by the release of granule contents into the plasma space. In this study, our objectives were to describe changes in whole blood platelet aggregation, rotational thromboelastometry, circulating levels of platelet-derived clotting factors, and plasma markers of fibrinolysis resulting from platelet transfusion. We compared interval changes in bleeding trauma patients receiving blood component therapy with and without platelets.

Methods

Study design and participants

Patients recruited into the ACIT II (UK CRN ID 5637) prospective, observational study at an urban major trauma centre in the United Kingdom between January 2008 and November 2015 were eligible for inclusion. Entry and exclusion criteria for ACIT II have been published in detail previously.¹⁷ The study was approved by the local Research Ethics Committee (reference 07/Q0603/29). For this study patients who received less than 4 units of packed red blood cells (PRBCs) were retrospectively excluded, to focus on bleeding patients who received platelet transfusions and to allow longitudinal analyses during bleeding. Assent

for patient recruitment was initially provided by an independent physician, with written informed consent obtained from a patient or relative as soon as possible after enrollment.

Major Hemorrhage Protocol

At our institution, activation criteria for the MHP are a systolic blood pressure <90mmHg, poor response to initial fluid resuscitation and/or clinical suspicion of active hemorrhage.

MHP activation occurs either from scene by the physician-led prehospital care team or upon arrival in the Emergency Department (ED) by the trauma team leader. Empiric transfusion with a FFP:Platelet:PRBC target ratio of 1:1:1 is initiated with 'pack A', which contains 4 units of PRBCs and 4 units of FFP. Universal donor PRBCs and thawed FFP are available in ED for immediate transfusion. If bleeding continues, 'pack B' and all subsequent packs provide 6 PRBC, 6 FFP, 2 pools of cryoprecipitate and 1 apheresis unit of platelets; the order of administration of these products is at the discretion of the clinical team. The MHP was updated to include tranexamic acid (TXA) administration in February 2011 and pre-hospital transfusion of up to four units of PRBCs in January 2014.

Blood sampling

Blood was drawn for research purposes within 20 minutes of arrival in ED from either the femoral vein or antecubital fossa, alongside routine laboratory tests. Blood product use prior to this initial sample draw was recorded. Platelet counts were measured with a Coulter LH750 hematology analyzer (Beckman Coulter, CA, USA; normal range $150-400 \times 10^9/L$). Point-of-care arterial blood gas analysis was performed to determine base deficit (BD) and lactate concentration. In patients who received transfusion of blood products, further samples were obtained after every 4th unit of PRBC, up to and including the 12th unit. Samples were processed by a member of the research team within 45 minutes of collection.

Platelet Aggregation and Thromboelastometry

Platelet aggregation was assayed in whole blood with multiple electrode impedance aggregometry using the Multiplate™ analyzer (Roche pharmaceuticals, Sussex, UK). Samples were processed according to the manufacturer's instructions. Briefly, blood was collected into a 3ml vacutainer (Roche pharmaceuticals) containing >15µg/ml hirudin. Aggregation was measured in 300µl of blood over six minutes in response to 6.5µM adenosine diphosphate (ADP), 0.5mM Arachidonic acid (AA), 3.2µg/ml collagen and 32µM thrombin-receptor activating peptide-6 (TRAP; final concentrations given). Results are reported as area under the curve (AUC) in arbitrary units. Hypo- and hyperfunction were defined according to references ranges stated by the manufacturer (570-1130U for ADP, 710-1150U for AA, 720-1250U for collagen and 840-1280U for TRAP). Rotational thromboelastometry (ROTEM) was performed on citrated whole blood using a ROTEM™ delta instrument in the presence of tissue factor (EXTEM) and with the addition of cytochalasin D, an inhibitor of platelet aggregation (FIBTEM).

Clotting Factors and Fibrinolytic markers

For quantification of plasma coagulation protein levels, blood was drawn into 4.5ml glass vacutainer containing 3.2% sodium citrate (Becton, Dickinson and Company, Plymouth, UK) and centrifuged at 1,750g for ten minutes. The plasma component was then removed and re-centrifuged at the same speed and duration prior to storage at -80°C. Immediately after thawing levels of factor II (reference range 70-146iu/dL), factor V (66-114iu/dL), factor XIII (55-158iu/dL), D-dimer (0-440ng/mL), von Willebrand antigen (50-160iu/dL), soluble fibrin monomer complex (0-6µg/mL) and alpha-2 antiplasmin (A2AP; 68-136iu/dL) were quantified in the hospital laboratory with a Sysmex CS2100i automated analyzer (Sysmex,

UK) according to standard protocols; results are reported in international units. Levels of PAI-1 (4-43ng/mL), tPA (2-12ng/mL), plasmin-antiplasmin complex (PAP; 120-700µg/L) and prothrombin fragments 1+2 (PF1+2; 69-229pmol/L) were measured using sandwich enzyme-linked immunosorbent assay (ELISA; Asserchrom®, Diagnostica Stago).

Data Collection

Demographic data, injury characteristics and physiological **variables** were collected prospectively. Cessation of blood product transfusion was used as a surrogate of hemostasis for the purposes of this study. Daily follow-up was performed for the first 28 days of hospital stay or until death or discharge. Multiple Organ Dysfunction Syndrome (MODS) was defined as a Sequential Organ Failure Assessment score ≥ 6 .

Data analysis

Data were analysed separately to assess the effect of platelet transfusion on platelet aggregation, with a subgroup analysis to examine the changes in coagulation factors and ROTEM. First, to study the impact of platelet transfusion on platelet aggregation **over time during resuscitation**, patients were divided into two groups at each sampling point (4 unit PRBC, 8 unit PRBC, 12 unit PRBC): (1) those that received a platelet transfusion and (2) those that did not receive a platelet transfusion prior to each sampling point. To mitigate the potential effects of selection bias, we divided patients into those who received a massive transfusion (MT, defined as ≥ 10 PRBC units in 24 hours) and patients who required less than 10 PRBC units. Second, we divided all available 4 unit PRBC intervals into three groups depending on the blood products administered during this interval: PRBCs only; PRBCs and FFP; or PRBCs, FFP and platelets. **This approach was taken to isolate the effects of**

platelet transfusion on plasma protein levels and viscoelastic measurements from those of FFP. Interval changes were calculated by subtracting the value at the end of the interval from the value at the beginning of the interval.

Data analysis was performed using Excel v15.2 (Microsoft, CA) and Prism v6.0 (GraphPad software, CA). Normal-quantile plots were used to assess the distribution of continuous data.

Data from non-parametric distributions are reported as median with interquartile range (IQR) and were compared with the Mann-Whitney U-test, Kruskal-Wallis test with Dunn's post-test, **or Wilcoxon matched pairs test.** **Data from parametric distributions** are presented as mean +/- 95% confidence intervals and were compared with Student's **or paired T-test as applicable**, or one-way analysis of variance (ANOVA) with Tukey's post-hoc test for multiple comparisons. **Correlation was assessed with Spearman's coefficient.**

Categorical data are displayed as number and percentage and were compared with Fisher's exact test. A two-tailed p-value of <0.05 was considered significant for all comparisons.

Results

One-hundred sixty-one patients received at least 4 PRBCs and were included in the study (**figure 1**). Eighty-two patients received PRBC transfusion prior to baseline sampling, median 1 PRBC units (0-2). Patients who received platelets (n=107) **during the first 24 hours** had similar injury severity to those who did not receive a platelet transfusion (n=54) but were significantly more shocked, with longer length of hospital stay and increased rates of organ failure (Table 1). **A non-significant trend towards reduced mortality at 24 hours in patients receiving platelets was observed (platelets transfused: 8/107 (7%) vs no platelets transfused 9/54 (17%), p=0.10).** Median time from admission to platelet

transfusion was 90 (63-166) minutes and patients who required a platelet transfusion received significantly more blood and blood products (FFP, cryoprecipitate) during the first 24 hours (Table 1). The average ratio of platelets to PRBCs, assuming one apheresis unit to be equivalent to 6 units of PRBCs, was 0.3 between 0-4 PRBCs, 0.9 between 5-8 PRBCs and 1.2 between 9-12 PRBCs.

On admission to ED, more than one in four patients who required over 4 PRBC units had evidence of reduced platelet aggregation in response to agonist stimulation: ADP: 70 (42%), AA: 59 (36%), collagen: 38 (24%), TRAP: 38 (24%). Patients who received ≥ 10 PRBCs, (n=42) had lower platelet aggregation at admission compared to patients who received 4-9 PRBC units: ADP, **MT** 477 (158-790) **vs no MT** 681 (331-1007), p=0.016; AA, **MT** 635 (411-893) **vs no MT** 891 (591-1242), p=0.001; collagen, **MT** 747 (4678-993) **vs no MT** 940 (559-1262), p=0.033; TRAP, **MT** 1032 (650-1171) **vs no MT** 1277 (903-1584), p=0.006. Seventy-one patients had at least one additional sample taken for aggregometry during bleeding, after an average of 57 minutes (4 PRBC interval), 87 minutes (8 PRBC interval) and 130 minutes (12 PRBC interval) from admission. **Compared to patients who only underwent baseline sampling, these patients had a similar severity of injury (30 (22-38) vs (29 (18-40), p=0.10), depth of shock (8.9 (6.0-16.9) vs 7.4 (3.9-13mmol/L), p=0.13), and overall mortality (20/71 (28%) vs 23/90 (26%), p=0.72)**

In patients who received a massive transfusion and had samples during hemorrhage, platelet aggregation declined significantly during resuscitation (figure 2A-D) compared with admission. A similar pattern in aggregation was observed in patients who received more than four PRBC units but did not require a massive transfusion (supplemental figure 1A-D).

Comparing patients who had received platelets with those who had not received platelets prior to each sampling point, levels of platelet aggregation were similar ($p > 0.05$ at each sample time-point). **At each sampling point, groups were comparable in terms of injury severity and depth of shock, although patients who received platelets tended to have samples taken later after admission (table 2).** Aggregation in response to each of the agonists deteriorated at each sampling point during bleeding compared to baseline, and on average by 8 PRBC units had decreased from the admission value by 81% in patients who did not receive platelets and 78% in patients who had a platelet transfusion. Platelet count also declined during haemorrhage irrespective of platelet transfusion. After 8 PRBC units, the platelet count in those who received platelets was $97 (71-106) \times 10^9/L$ versus $109 (79-137) \times 10^9/L$ ($p=0.81$) in those who did not receive a platelet transfusion by this sample point.

We next assessed the efficacy of platelet transfusion on ROTEM **variables** in a subset of patients who had plasma coagulation protein levels measured during bleeding at each 4 unit PRBC interval ($n=89$). Overall, 115 interval samples were available for analysis - FFP was administered in 85 intervals of which 29 included at least one pool of platelets (Table 2). Compared to the main study cohort, this subgroup were more severely injured (ISS 34 [25-42] vs 29 [20-38], $p=0.03$) but had a similar degree of shock (BD 8.9 ± 1.3 mmol/L vs 9.8 ± 1.1 mmol/L, $p=0.31$), PRBC transfusion (8 [6-11] units vs 7 [5-10] units, $p=0.09$) and 28-day mortality (29 [33%] vs 40 [28%], $p=0.24$). The proportion of patients who received TXA prior to or during the interval was similar when PRBCs were given alone (33%), with FFP (38%) or with FFP and platelets (50%; $p=0.39$, chi-squared test). **Intervals which contained platelets and FFP were longer in duration compared to those containing FFP without platelets (84 (44-147) vs 46 (30-85) minutes, $p=0.05$).**

Comparing intervals in which platelets were transfused with those in which only FFP and PRBC were administered, there was no significant difference in viscoelastic **measurement of** clotting time, clot formation or clot strength (table 3). However, platelet transfusion was associated with a significant reduction in EXTEM maximum lysis (ML) (Figure 3A). Changes in circulating clotting factor levels were similar, irrespective of whether or not platelet transfusions were administered during a 4 unit PRBC interval (Table 3). Platelet transfusion during an interval was associated with a significant increase in circulating levels of PAI-1 compared to intervals in which PRBCs and FFP were administered (Figure 3B). Conversely, levels of soluble tPA decreased in intervals where platelets were transfused in combination with PRBCs and FFP (Figure 3C). PAP complex levels declined in intervals containing PRBCs and FFP regardless of whether platelets were transfused (Figure 3D). **No correlation was observed between interval duration and change in EXTEM ML ($r=0.13$, $p=0.27$), PAI-1 ($r=0.17$, $p=0.19$) or tPA ($r=-0.07$, $p=0.63$).** Overall, the only clear effects of platelet transfusions appeared to be a reduction in fibrinolysis with minimal effect on other functional aspects of hemostasis, including platelet aggregation.

Discussion

In this prospective single-centre cohort study, the strongest effect of platelet transfusion during trauma hemorrhage appears to be reduction of fibrinolysis without improvement in clot strength or clotting times. Administering platelets as part of a major haemorrhage protocol did not restore platelet aggregation or platelet count. Compared to transfusion of FFP and PRBC alone, platelets transfusions improved viscoelastic measures of fibrinolysis

with increased plasma PAI-1 levels and reduced circulating tPA. In cohort studies, high platelet:PRBC ratios appear to be associated with improved outcomes.¹⁸⁻²⁰ The reduction in early deaths due to exsanguination in the 1:1:1 arm of the PROPPR randomized control trial may due to the effects of early platelet administration rather than (or as well as) the higher doses of blood components. Reduction of hyperfibrinolysis] may be a key mechanism by which early platelet transfusion improves hemostasis in TIC.

Platelets contain a broad array of pro- and anti-coagulant factors which are released into the plasma space upon activation.⁹ Of particular relevance to the potential role of platelets in Acute Traumatic Coagulopathy (ATC) and TIC are inhibitors of fibrinolysis including PAI-1. Although the activity of platelet-derived PAI-1 has been questioned,²¹ recent evidence suggests that the active form is stored in large quantities within platelet alpha granules.¹⁰ In ATC, activated protein C-dependent fibrinolysis is thought to occur through inhibition of PAI-1, which allows tPA mediated plasminogen cleavage to proceed unchecked.²²⁻²⁴ Our findings suggest that transfusion of stored platelets may provide an important source of PAI-1, which forms an inactive complex with circulating tPA and hypothetically reduces the level of free tPA detectable in plasma.²⁵ FFP contains antiplasmin although platelet transfusion had an additive effect on reversal of functional fibrinolysis as demonstrated by a reduced maximum lysis. Although changes in PAP and D-dimer levels were not significantly different after platelet transfusions, this may be attributable to the long half-life of these biomarkers in the circulation. Both assays describe prior fibrinolytic activation rather than the dynamic changes in fibrinolysis during bleeding.²⁶ This apparent predominant effect of platelet transfusion is supported by *in vitro* evidence that platelet lysate attenuates tPA-dependent fibrinolysis¹² and raises the question of whether earlier administration of platelets could confer additional benefit particularly in patients with hyperfibrinolysis.

Trauma-induced platelet dysfunction on arrival to ED has been previously described by several authors,¹⁴⁻¹⁶ and it is plausible that the conditions which impair the function of endogenous platelets may have the same effect on stored platelets administered in transfusion. Whether trauma-induced platelet dysfunction is an intrinsic platelet defect or the result of circulating conditions is unknown and requires further study. With regards to platelet count, it is possible that the rate of platelet loss through ongoing bleeding exceeded the number of platelets being administered in our patient cohort. Alternatively, stored platelets may form microaggregates or be recruited to thrombus formation, and thus escape detection by the laboratory platelet count.²⁷ These hypotheses remain speculative however, and further investigation is required to determine how the post-injury intravascular milieu impacts upon the function of both stored and endogenous platelets.

There are a number of limitations to this study, principally as a result of its observational design. First, comparisons of interval changes in clotting factor levels and viscoelastic tests could in theory be influenced by variations in the rate of blood loss **and the time of sampling, which was slightly later after admission in patients receiving platelets.** Second, given the sample sizes for each interval we were unable to fully control for concomitant administration of FFP and cryoprecipitate. Third, we were not able to evaluate the effect of timing of platelet transfusion as part of our MHP. In particular, whether ‘up front’ platelet transfusion, as administered in the 1:1:1 arm of the PROPPR trial, conferred additional hemostatic benefits could not be addressed in this study due to the design of the MHP at our institution. Platelets are delivered in the second MHP pack and therefore later in the clinical episode. Further work is required to assess other possible sources for the delayed rise in PAI-1 e.g. reperfusion injury of hypoperfused endothelium. Fourth, we did not

directly measure thrombin generation potential but used PF 1+2 fragments in plasma as a surrogate. Platelet transfusion has been advocated as a method to support thrombin generation²⁸ although we found no significant difference in PF 1+2 fragments between groups. Fifth, we did not perform any functional coagulation tests on the apheresis platelet units themselves as this forms part of an ongoing study. Finally, we did not undertake a detailed evaluation of the impact of platelet transfusion on clinical outcomes although these have previously been described in larger multi-centre studies.^{3,18} Our novel findings demonstrating a potential role for platelets in reducing fibrinolytic activity as measured by laboratory assays, and requires clinical validation in larger trauma trials.

In conclusion, this study suggests that platelet transfusions given in standard doses as part of a MHP primarily reduce fibrinolysis but do not preserve either platelet aggregation or platelet count during bleeding. Platelets administered to bleeding trauma patients appear to have anti-fibrinolytic effects over and above FFP through inhibition of tPA-mediated fibrinolysis by providing an additional dose of PAI-1. Further work **in larger cohorts** is required to ascertain the impact of early platelet delivery during DCR, the role of platelet-derived clotting factors on reversing fibrinolysis and potential improvements in outcomes from trauma hemorrhage. Whether earlier platelet transfusion has a particular hemostatic benefit in all, or selected bleeding patients remains an important question and merits further investigation.

Author Contributions

PV, SG, KB and RD designed the study. PV, SG and LSG collected and analyzed data. PV, SG, KB, LSG, LG and RD wrote and edited the manuscript. All authors have read and approved the final version.

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Figure Legends

Figure 1: Flow diagram of patient inclusion and blood sampling. Patients had either aggregometry, plasma protein measurement, or both performed at the indicated sampling points. PRBC, packed red blood cells.

Figure 2: Trends in platelet aggregation during bleeding in patients requiring massive transfusion. A, Adenosine diphosphate (ADP). B, Arachidonic acid (AA). C, Collagen. D, thrombin receptor activating peptide (TRAP). Box-whisker plots depict median and interquartile range with 10th-90th percentiles. Dashed lines denote normal range. $p > 0.05$ at each time point comparing platelets transfused with no platelets transfused (Mann-Whitney U-test). AU, arbitrary units. Massive transfusion defined as ≥ 10 red cell units in 24 hours.

Figure 3: Interval changes in mediators and markers of fibrinolysis during bleeding. A, EXTEM maximum lysis (ML). B, Plasminogen Activator Inhibitor-1 (PAI-1). C, tissue Plasminogen Activator (tPA). D, Plasmin-antiplasmin complex (PAP). Tukey box plots (A and D); * $p < 0.05$, ** $p < 0.01$, Wilcoxon matched pairs test. Bars denote mean with 95% confidence interval; * $p < 0.05$, ** $p < 0.01$, paired T-test (B and C). n.s., not significant.

Supplemental Figure 1: Trends in platelet aggregation during bleeding in patients requiring less than 10 red cell units in 24 hours. A, Adenosine diphosphate (ADP). B, Arachidonic acid (AA). C, Collagen. D, thrombin receptor activating peptide (TRAP). Box-whisker plots depict median and interquartile range with 10th-90th percentiles. Dashed lines denote normal range. $p > 0.05$ at each time point comparing platelets transfused with no platelets transfused (Mann-Whitney U-test). AU, arbitrary units.

Table 1: Characteristics of the aggregometry cohort. Values in parentheses are interquartile range for continuous variables and percentages for categorical variables. P-values compare platelets transfused vs no platelets transfused (Mann-Whitney U-test or Fisher's exact test). ISS, Injury severity score; AIS, abbreviated injury score; INR,

international normalized ratio; PRBC, packed red blood cells; FFP, fresh frozen plasma. ICU, intensive care unit.

Table 2: Characteristics of patients with samples during bleeding. Patients were divided into two groups based on whether or not platelets were transfused prior to the sample being taken. Values are median with interquartile range unless stated. P-values compare platelets transfused vs no platelets transfused at each sampling time point (Mann-Whitney U-test).

¹Values are n (%).

Table 3: Interval changes in viscoelastic variables and plasma proteins during four-PRBC intervals. Values are mean with 95% confidence intervals and compared with one-way ANOVA unless specified. * $p < 0.05$, RBC+FFP+platelets vs RBC+FFP, one-way ANOVA with Tukey's multiple comparisons test. ¹EXTEM value unless specified ²Values are median with interquartile range. p-values derived from Kruskal-Wallis test with Dunn's post-test correction.

MANUSCRIPT TEXT

Introduction

Hemorrhage is the principal cause of preventable death due to injury.¹ Contemporary damage-control resuscitation (DCR) is characterised by expedited hemorrhage control with simultaneous and empiric transfusion of blood and blood products in high ratios.² Maintaining hemostatic potential through early administration of plasma and platelets in high volumes alongside transfusion of packed red blood cells (PRBCs) minimises dilutional coagulopathy and is associated with improved outcomes.³ However, the effects of platelet transfusions remain unclear, while the provision of platelet transfusions can be challenging. Platelet function is known to be reduced in Trauma-Induced Coagulopathy (TIC),^{4,5} but the effects of platelet transfusion on coagulation during active bleeding are unknown. This knowledge gap has been highlighted by several contemporary major hemorrhage guidelines.^{6,7}

Platelets promote coagulation primarily by adhering to the endothelium or subendothelium to form aggregates at sites of vascular injury, and by providing a phospholipid platform to support thrombin generation.⁸ Haemostasis is further amplified through the release of reactive mediators from platelet storage granules into the plasma space,⁹ including clotting factors and mediators of fibrinolysis. In particular, platelets contain a large pool of plasminogen activator inhibitor-1 (PAI-1)¹⁰ and alpha-2 antiplasmin (A2AP),¹¹ which are hypothesised to represent an important mechanism through which platelets attenuate tissue plasminogen activator (tPA)-mediated fibrinolysis *in vitro*.^{12,13} Impaired platelet aggregation

1 is common after severe injury and associated with increased mortality, although the
2 mechanisms of this remain unknown.¹⁴⁻¹⁶ Attempts to support platelet function through
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4 transfusion of allogeneic platelets during major haemorrhage therefore seems logical, but to
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6 date no studies have evaluated how platelet transfusions affect hemostasis during bleeding in
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8 trauma patients.
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14 Our working hypothesis was that platelet transfusions exert their effects either through
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16 augmenting aggregation, promoting thrombin generation or by the release of granule contents
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18 into the plasma space. In this study, our objectives were to describe changes in whole blood
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20 platelet aggregation, rotational thromboelastometry, circulating levels of platelet-derived
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22 clotting factors, and plasma markers of fibrinolysis resulting from platelet transfusion. We
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24 compared interval changes in bleeding trauma patients receiving blood component therapy
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26 with and without platelets.
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31 32 33 34 **Methods**

35 36 37 38 39 *Study design and participants*

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41 Patients recruited into the ACIT II (UK CRN ID 5637) prospective, observational study at an
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43 urban major trauma centre in the United Kingdom between January 2008 and November
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45 2015 were eligible for inclusion. Entry and exclusion criteria for ACIT II have been
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47 published in detail previously.¹⁷ The study was approved by the local Research Ethics
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49 Committee (reference 07/Q0603/29). For this study patients who received less than 4 units of
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51 packed red blood cells (PRBCs) were retrospectively excluded, to focus on bleeding patients
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53 who received platelet transfusions and to allow longitudinal analyses during bleeding. Assent
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1 for patient recruitment was initially provided by an independent physician, with written
2 informed consent obtained from a patient or relative as soon as possible after enrollment.
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6 7 *Major Hemorrhage Protocol*

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9 At our institution, activation criteria for the MHP are a systolic blood pressure <90mmHg,
10 poor response to initial fluid resuscitation and/or clinical suspicion of active hemorrhage.
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13 MHP activation occurs either from scene by the physician-led prehospital care team or upon
14 arrival in the Emergency Department (ED) by the trauma team leader. Empiric transfusion
15 with a FFP:Platelet:PRBC target ratio of 1:1:1 is initiated with ‘pack A’, which contains 4
16 units of PRBCs and 4 units of FFP. Universal donor PRBCs and thawed FFP are available in
17 ED for immediate transfusion. If bleeding continues, ‘pack B’ and all subsequent packs
18 provide 6 PRBC, 6 FFP, 2 pools of cryoprecipitate and 1 apheresis unit of platelets; the order
19 of administration of these products is at the discretion of the clinical team. The MHP was
20 updated to include tranexamic acid (TXA) administration in February 2011 and pre-hospital
21 transfusion of up to four units of PRBCs in January 2014.
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39 *Blood sampling*

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41 Blood was drawn for research purposes within 20 minutes of arrival in ED from either the
42 femoral vein or antecubital fossa, alongside routine laboratory tests. Blood product use prior
43 to this initial sample draw was recorded. Platelet counts were measured with a Coulter
44 LH750 hematology analyzer (Beckman Coulter, CA, USA; normal range $150-400 \times 10^9/L$).
45
46 Point-of-care arterial blood gas analysis was performed to determine base deficit (BD) and
47 lactate concentration. In patients who received transfusion of blood products, further samples
48 were obtained after every 4th unit of PRBC, up to and including the 12th unit. Samples were
49 processed by a member of the research team within 45 minutes of collection.
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2 *Platelet Aggregation and Thromboelastometry*
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4 Platelet aggregation was assayed in whole blood with multiple electrode impedance
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7 aggregometry using the Multiplate™ analyzer (Roche pharmaceuticals, Sussex, UK).
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9 Samples were processed according to the manufacturer's instructions. Briefly, blood was
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12 collected into a 3ml vacutainer (Roche pharmaceuticals) containing >15µg/ml hirudin.
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14 Aggregation was measured in 300µl of blood over six minutes in response to 6.5µM
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17 adenosine diphosphate (ADP), 0.5mM Arachidonic acid (AA), 3.2µg/ml collagen and 32µM
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20 thrombin-receptor activating peptide-6 (TRAP; final concentrations given). Results are
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23 reported as area under the curve (AUC) in arbitrary units. Hypo- and hyperfunction were
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25 defined according to references ranges stated by the manufacturer (570-1130U for ADP, 710-
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27 1150U for AA, 720-1250U for collagen and 840-1280U for TRAP). Rotational
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30 thromboelastometry (ROTEM) was performed on citrated whole blood using a ROTEM™
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33 delta instrument in the presence of tissue factor (EXTEM) and with the addition of
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36 cytochalasin D, an inhibitor of platelet aggregation (FIBTEM).
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39 *Clotting Factors and Fibrinolytic markers*
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42 For quantification of plasma coagulation protein levels, blood was drawn into 4.5ml glass
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45 vacutainer containing 3.2% sodium citrate (Becton, Dickinson and Company, Plymouth, UK)
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48 and centrifuged at 1,750g for ten minutes. The plasma component was then removed and re-
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51 centrifuged at the same speed and duration prior to storage at -80°C. Immediately after
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54 thawing levels of factor II (reference range 70-146iu/dL), factor V (66-114iu/dL), factor XIII
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57 (55-158iu/dL), D-dimer (0-440ng/mL), von Willebrand antigen (50-160iu/dL), soluble fibrin
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60 monomer complex (0-6µg/mL) and alpha-2 antiplasmin (A2AP; 68-136iu/dL) were
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65 quantified in the hospital laboratory with a Sysmex CS2100i automated analyzer (Sysmex,

1 UK) according to standard protocols; results are reported in international units. Levels of
2 PAI-1 (4-43ng/mL), tPA (2-12ng/mL), plasmin-antiplasmin complex (PAP; 120-700µg/L)
3
4 and prothrombin fragments 1+2 (PF1+2; 69-229pmol/L) were measured using sandwich
5
6 enzyme-linked immunosorbent assay (ELISA; Asserchrom®, Diagnostica Stago).
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10 11 *Data Collection*

12 Demographic data, injury characteristics and physiological variables were collected
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14 prospectively. Cessation of blood product transfusion was used as a surrogate of hemostasis
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16 for the purposes of this study. Daily follow-up was performed for the first 28 days of hospital
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18 stay or until death or discharge. Multiple Organ Dysfunction Syndrome (MODS) was
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20 defined as a Sequential Organ Failure Assessment score ≥ 6 .
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29 *Data analysis*

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34 Data were analysed separately to assess the effect of platelet transfusion on platelet
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36 aggregation, with a subgroup analysis to examine the changes in coagulation factors and
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38 ROTEM. First, to study the impact of platelet transfusion on platelet aggregation over time
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40 during resuscitation, patients were divided into two groups at each sampling point (4 unit
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42 PRBC, 8 unit PRBC, 12 unit PRBC): (1) those that received a platelet transfusion and (2)
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44 those that did not receive a platelet transfusion prior to each sampling point. To mitigate the
45
46 potential effects of selection bias, we divided patients into those who received a massive
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48 transfusion (MT, defined as ≥ 10 PRBC units in 24 hours) and patients who required less than
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50 10 PRBC units. Second, we divided all available 4 unit PRBC intervals into three groups
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52 depending on the blood products administered during this interval: PRBCs only; PRBCs and
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54 FFP; or PRBCs, FFP and platelets. This approach was taken to isolate the effects of platelet
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transfusion on plasma protein levels and viscoelastic measurements from those of FFP.

Interval changes were calculated by subtracting the value at the end of the interval from the value at the beginning of the interval.

Data analysis was performed using Excel v15.2 (Microsoft, CA) and Prism v6.0 (GraphPad software, CA). Normal-quantile plots were used to assess the distribution of continuous data. Data from non-parametric distributions are reported as median with interquartile range (IQR) and were compared with the Mann-Whitney U-test, Kruskal-Wallis test with Dunn's post-test, or Wilcoxon matched pairs test. Data from parametric distributions are presented as mean +/- 95% confidence intervals and were compared with Student's or paired T-test as applicable, or one-way analysis of variance (ANOVA) with Tukey's post-hoc test for multiple comparisons. Correlation was assessed with Spearman's coefficient. Categorical data are displayed as number and percentage and were compared with Fisher's exact test. A two-tailed p-value of <0.05 was considered significant for all comparisons.

Results

One-hundred sixty-one patients received at least 4 PRBCs and were included in the study (figure 1). Eighty-two patients received PRBC transfusion prior to baseline sampling, median 1 PRBC units (0-2). Patients who received platelets (n=107) during the first 24 hours had similar injury severity to those who did not receive a platelet transfusion (n=54) but were significantly more shocked, with longer length of hospital stay and increased rates of organ failure (Table 1). A non-significant trend towards reduced mortality at 24 hours in patients receiving platelets was observed (platelets transfused: 8/107 (7%) vs no platelets transfused 9/54 (17%), p=0.10). Median time from admission to platelet transfusion was 90 (63-166)

1 minutes and patients who required a platelet transfusion received significantly more blood
2 and blood products (FFP, cryoprecipitate) during the first 24 hours (Table 1). The average
3 ratio of platelets to PRBCs, assuming one apheresis unit to be equivalent to 6 units of
4 PRBCs, was 0.3 between 0-4 PRBCs, 0.9 between 5-8 PRBCs and 1.2 between 9-12 PRBCs.
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14 On admission to ED, more than one in four patients who required over 4 PRBC units had
15 evidence of reduced platelet aggregation in response to agonist stimulation: ADP: 70 (42%),
16 AA: 59 (36%), collagen: 38 (24%), TRAP: 38 (24%). Patients who received ≥ 10 PRBCs,
17 (n=42) had lower platelet aggregation at admission compared to patients who received 4-9
18 PRBC units: ADP, MT 477 (158-790) vs no MT 681 (331-1007), p=0.016; AA, MT 635
19 (411-893) vs no MT 891 (591-1242), p=0.001; collagen, MT 747 (4678-993) vs no MT 940
20 (559-1262), p=0.033; TRAP, MT 1032 (650-1171) vs no MT 1277 (903-1584), p=0.006.
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22 Seventy-one patients had at least one additional sample taken for aggregometry during
23 bleeding, after an average of 57 minutes (4 PRBC interval), 87 minutes (8 PRBC interval)
24 and 130 minutes (12 PRBC interval) from admission. Compared to patients who only
25 underwent baseline sampling, these patients had a similar severity of injury (30 (22-38) vs
26 (29 (18-40), p=0.10), depth of shock (8.9 (6.0-16.9) vs 7.4 (3.9-13mmol/L), p=0.13), and
27 overall mortality (20/71 (28%) vs 23/90 (26%), p=0.72)
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50 In patients who received a massive transfusion and had samples during hemorrhage, platelet
51 aggregation declined significantly during resuscitation (figure 2A-D) compared with
52 admission. A similar pattern in aggregation was observed in patients who received more than
53 four PRBC units but did not require a massive transfusion (supplemental figure 1A-D).
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55 Comparing patients who had received platelets with those who had not received platelets
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1 prior to each sampling point, levels of platelet aggregation were similar ($p > 0.05$ at each
2 sample time-point). At each sampling point, groups were comparable in terms of injury
3 severity and depth of shock, although patients who received platelets tended to have samples
4 taken later after admission (table 2). Aggregation in response to each of the agonists
5 deteriorated at each sampling point during bleeding compared to baseline, and on average by
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7 8 PRBC units had decreased from the admission value by 81% in patients who did not
8 receive platelets and 78% in patients who had a platelet transfusion. Platelet count also
9 declined during haemorrhage irrespective of platelet transfusion. After 8 PRBC units, the
10 platelet count in those who received platelets was $97 (71-106) \times 10^9/L$ versus $109 (79-137)$
11 $\times 10^9/L$ ($p=0.81$) in those who did not receive a platelet transfusion by this sample point.
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29 We next assessed the efficacy of platelet transfusion on ROTEM variables in a subset of
30 patients who had plasma coagulation protein levels measured during bleeding at each 4 unit
31 PRBC interval ($n=89$). Overall, 115 interval samples were available for analysis - FFP was
32 administered in 85 intervals of which 29 included at least one pool of platelets (Table 2).
33 Compared to the main study cohort, this subgroup were more severely injured (ISS 34 [25-
34 42] vs 29 [20-38], $p=0.03$) but had a similar degree of shock (BD 8.9 ± 1.3 mmol/L vs 9.8
35 ± 1.1 mmol/L, $p=0.31$), PRBC transfusion (8 [6-11] units vs 7 [5-10] units, $p=0.09$) and 28-
36 day mortality (29 [33%] vs 40 [28%], $p=0.24$). The proportion of patients who received TXA
37 prior to or during the interval was similar when PRBCs were given alone (33%), with FFP
38 (38%) or with FFP and platelets (50%; $p=0.39$, chi-squared test). Intervals which contained
39 platelets and FFP were longer in duration compared to those containing FFP without platelets
40 (84 (44-147) vs 46 (30-85) minutes, $p=0.05$).
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1 Comparing intervals in which platelets were transfused with those in which only FFP and
2 PRBC were administered, there was no significant difference in viscoelastic measurement of
3 clotting time, clot formation or clot strength (Table 3). However, platelet transfusion was
4 associated with a significant reduction in EXTEM maximum lysis (ML) (Table 3 and Figure
5 3A). Changes in circulating clotting factor levels were similar, irrespective of whether or not
6 platelet transfusions were administered during a 4 unit PRBC interval (Table 3). Platelet
7 transfusion during an interval was associated with a significant increase in circulating levels
8 of PAI-1 compared to intervals in which PRBCs and FFP were administered (Figure 3B).
9 Conversely, levels of soluble tPA decreased in intervals where platelets were transfused in
10 combination with PRBCs and FFP (Figure 3C). PAP complex levels declined in intervals
11 containing PRBCs and FFP regardless of whether platelets were transfused (Figure 3D). No
12 correlation was observed between interval duration and change in EXTEM ML ($r=0.13$,
13 $p=0.27$), PAI-1 ($r=0.17$, $p=0.19$) or tPA ($r=-0.07$, $p=0.63$). Overall, the only clear effects of
14 platelet transfusions appeared to be a reduction in fibrinolysis with minimal effect on other
15 functional aspects of hemostasis, including platelet aggregation.
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40 Discussion

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44 In this prospective single-centre cohort study, the strongest effect of platelet transfusion
45 during trauma hemorrhage appears to be reduction of fibrinolysis without improvement in
46 clot strength or clotting times. Administering platelets as part of a major haemorrhage
47 protocol did not restore platelet aggregation or platelet count. Compared to transfusion of
48 FFP and PRBC alone, platelets transfusions improved viscoelastic measures of fibrinolysis
49 with increased plasma PAI-1 levels and reduced circulating tPA. In cohort studies, high
50 platelet:PRBC ratios appear to be associated with improved outcomes.¹⁸⁻²⁰ The reduction in
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1 early deaths due to exsanguination in the 1:1:1 arm of the PROPPR randomized control trial
2 may due to the effects of early platelet administration rather than (or as well as) the higher
3 doses of blood components. Reduction of hyperfibrinolysis] may be a key mechanism by
4 which early platelet transfusion improves hemostasis in TIC.
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11 Platelets contain a broad array of pro- and anti-coagulant factors which are released into the
12 plasma space upon activation.⁹ Of particular relevance to the potential role of platelets in
13 Acute Traumatic Coagulopathy (ATC) and TIC are inhibitors of fibrinolysis including PAI-1.
14 Although the activity of platelet-derived PAI-1 has been questioned,²¹ recent evidence
15 suggests that the active form is stored in large quantities within platelet alpha granules.¹⁰ In
16 ATC, activated protein C-dependent fibrinolysis is thought to occur through inhibition of
17 PAI-1, which allows tPA mediated plasminogen cleavage to proceed unchecked.²²⁻²⁴ Our
18 findings suggest that transfusion of stored platelets may provide an important source of PAI-
19 1, which forms an inactive complex with circulating tPA and hypothetically reduces the level
20 of free tPA detectable in plasma.²⁵ FFP contains antiplasmin although platelet transfusion had
21 an additive effect on reversal of functional fibrinolysis as demonstrated by a reduced
22 maximum lysis. Although changes in PAP and D-dimer levels were not significantly different
23 after platelet transfusions, this may be attributable to the long half-life of these biomarkers in
24 the circulation. Both assays describe prior fibrinolytic activation rather than the dynamic
25 changes in fibrinolysis during bleeding.²⁶ This apparent predominant effect of platelet
26 transfusion is supported by *in vitro* evidence that platelet lysate attenuates tPA-dependent
27 fibrinolysis¹² and raises the question of whether earlier administration of platelets could
28 confer additional benefit particularly in patients with hyperfibrinolysis.
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1 Trauma-induced platelet dysfunction on arrival to ED has been previously described by
2 several authors,¹⁴⁻¹⁶ and it is plausible that the conditions which impair the function of
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4 endogenous platelets may have the same effect on stored platelets administered in
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6 transfusion. Whether trauma-induced platelet dysfunction is an intrinsic platelet defect or the
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8 result of circulating conditions is unknown and requires further study. With regards to
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10 platelet count, it is possible that the rate of platelet loss through ongoing bleeding exceeded
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12 the number of platelets being administered in our patient cohort. Alternatively, stored
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14 platelets may form microaggregates or be recruited to thrombus formation, and thus escape
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16 detection by the laboratory platelet count.²⁷ These hypotheses remain speculative however,
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18 and further investigation is required to determine how the post-injury intravascular milieu
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20 impacts upon the function of both stored and endogenous platelets.
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29 There are a number of limitations to this study, principally as a result of its observational
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31 design. First, comparisons of interval changes in clotting factor levels and viscoelastic tests
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33 could in theory be influenced by variations in the rate of blood loss and the time of sampling,
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35 which was slightly later after admission in patients receiving platelets. Second, given the
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37 sample sizes for each interval we were unable to fully control for concomitant administration
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39 of FFP and cryoprecipitate. Third, we were not able to evaluate the effect of timing of platelet
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41 transfusion as part of our MHP. In particular, whether ‘up front’ platelet transfusion, as
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43 administered in the 1:1:1 arm of the PROPPR trial, conferred additional hemostatic benefits
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45 could not be addressed in this study due to the design of the MHP at our institution. Platelets
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47 are delivered in the second MHP pack and therefore later in the clinical episode. Further
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49 work is required to assess other possible sources for the delayed rise in PAI-1 e.g. reperfusion
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51 injury of hypoperfused endothelium. Fourth, we did not directly measure thrombin
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53 generation potential but used PF 1+2 fragments in plasma as a surrogate. Platelet transfusion
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1 has been advocated as a method to support thrombin generation²⁸ although we found no
2 significant difference in PF 1+2 fragments between groups. Fifth, we did not perform any
3 functional coagulation tests on the apheresis platelet units themselves as this forms part of an
4 ongoing study. Finally, we did not undertake a detailed evaluation of the impact of platelet
5 transfusion on clinical outcomes although these have previously been described in larger
6 multi-centre studies.^{3,18} Our novel findings demonstrating a potential role for platelets in
7 reducing fibrinolytic activity as measured by laboratory assays, and requires clinical
8 validation in larger trauma trials.
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22 In conclusion, this study suggests that platelet transfusions given in standard doses as part of
23 a MHP primarily reduce fibrinolysis but do not preserve either platelet aggregation or platelet
24 count during bleeding. Platelets administered to bleeding trauma patients appear to have anti-
25 fibrinolytic effects over and above FFP through inhibition of tPA-mediated fibrinolysis by
26 providing an additional dose of PAI-1. Further work in larger cohorts is required to ascertain
27 the impact of early platelet delivery during DCR, the role of platelet-derived clotting factors
28 on reversing fibrinolysis and potential improvements in outcomes from trauma hemorrhage.
29 Whether earlier platelet transfusion has a particular hemostatic benefit in all, or selected
30 bleeding patients remains an important question and merits further investigation.
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46 **Author Contributions**

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51 PV, SG, KB and RD designed the study. PV, SG and LSG collected and analyzed data. PV,
52 SG, KB, LSG, LG and RD wrote and edited the manuscript. All authors have read and
53 approved the final version.
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Figure Legends

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Figure 1: Flow diagram of patient inclusion and blood sampling. Patients had either aggregometry, plasma protein measurement, or both performed at the indicated sampling points. PRBC, packed red blood cells.

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Figure 2: Trends in platelet aggregation during bleeding in patients requiring massive transfusion. A, Adenosine diphosphate (ADP). B, Arachidonic acid (AA). C, Collagen. D, thrombin receptor activating peptide (TRAP). Box-whisker plots depict median and interquartile

1 range with 10th-90th percentiles. Dashed lines denote normal range. p>0.05 at each time point
2 comparing platelets transfused with no platelets transfused (Mann-Whitney U-test). AU, arbitrary
3 units. Massive transfusion defined as ≥10 red cell units in 24 hours.
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9 **Figure 3: Interval changes in mediators and markers of fibrinolysis during bleeding. A,**
10 EXTEM maximum lysis (ML). B, Plasminogen Activator Inhibitor-1 (PAI-1). C, tissue
11 Plasminogen Activator (tPA). D, Plasmin-antiplasmin complex (PAP). Tukey box plots (A and
12 D); * p<0.05, **p<0.01, Wilcoxon matched pairs test. Bars denote mean with 95% confidence
13 interval; * p<0.05, ** p<0.01, paired T-test (B and C). n.s., not significant.
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24 **Supplemental Figure 1: Trends in platelet aggregation during bleeding in patients requiring**
25 **less than 10 red cell units in 24 hours. A,** Adenosine diphosphate (ADP). B, Arachidonic acid
26 (AA). C, Collagen. D, thrombin receptor activating peptide (TRAP). Box-whisker plots depict
27 median and interquartile range with 10th-90th percentiles. Dashed lines denote normal range.
28 p>0.05 at each time point comparing platelets transfused with no platelets transfused (Mann-
29 Whitney U-test). AU, arbitrary units.
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41 **Table 1: Characteristics of the aggregometry cohort.** Values in parentheses are
42 interquartile range for continuous variables and percentages for categorical variables. P-
43 values compare platelets transfused vs no platelets transfused (Mann-Whitney U-test or
44 Fisher's exact test). ISS, Injury severity score; AIS, abbreviated injury score; INR,
45 international normalized ratio; PRBC, packed red blood cells; FFP, fresh frozen plasma. ICU,
46 intensive care unit.
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Table 2: Characteristics of patients with samples during bleeding. Patients were divided into two groups based on whether or not platelets were transfused prior to the sample being taken. Values are median with interquartile range unless stated. P-values compare platelets transfused vs no platelets transfused at each sampling time point (Mann-Whitney U-test).

¹Values are n (%).

Table 3: Interval changes in viscoelastic variables and plasma proteins during four-PRBC intervals. Values are mean with 95% confidence intervals and compared with one-way ANOVA unless specified. *p<0.05, RBC+FFP+platelets vs RBC+FFP, one-way ANOVA with Tukey's multiple comparisons test. ¹EXTEM value unless specified ²Values are median with interquartile range. p-values derived from Kruskal-Wallis test with Dunn's post-test correction.

Table 1: Characteristics of the study cohort.

	All patients (n=161)	Platelets transfused (n=107)	No platelets transfused (n=54)	p value
<i>Patient characteristics</i>				
Male gender	112 (70)	73 (68)	39 (72)	0.72
Age, years	37 (24-57)	33 (24-51)	42 (28-59)	0.04
Injury Severity Score	29 (20-38)	29 (20-41)	29 (17-38)	0.68
ISS>15	138 (86)	95 (89)	43 (80)	0.04
Base Deficit, mmol/L	7.9 (4.0-15.1)	9.0 (5.1-16.5)	6.0 (2.7-9.6)	0.006
Blunt Mechanism	120 (75)	30 (28)	11 (20)	0.34
AIS Head \geq 3	54 (34)	36 (34)	18 (33)	1.0
<i>Baseline Laboratory results</i>				
INR>1.2	71 (44)	53 (50)	18 (33)	0.06
EXTEM CA5 \leq 37mm	64 (40)	45 (42)	19 (35)	0.50
<i>Fluids prior to baseline sampling</i>				
Crystalloid, ml	250 (0-600)	200 (0-735)	400 (0-500)	0.45
Packed red blood cells, units	0 (0-2)	1 (0-2)	0 (0-2)	0.31
Tranexamic acid	83 (52)	60 (56)	23 (43)	0.13
<i>Fluids at 24 hours</i>				
Crystalloid, ml	3575 (2225-5050)	3700 (2455-5200)	3200 (2000-5000)	0.11
Colloid, ml	0 (0-1500)	250 (0-1750)	0 (0-1000)	0.18
PRBC, units	7 (5-10)	8 (6-11)	5 (4-6)	<0.001
FFP, units	5 (4-8)	7 (4-10)	4 (0-4)	<0.001
Cryoprecipitate, pools	2 (0-2)	2 (2-3)	0 (0-0)	<0.001
Platelets, apheresis units	1 (0-2)	1 (1-2)	0 (0-0)	<0.001
\geq 10 PRBC units	42 (26)	38 (36)	4 (7)	<0.001
<i>Outcome</i>				
Length of stay, days ¹	30 (14-50)	33 (16-56)	24 (13-39)	0.02
ICU length of stay, days ¹	6 (1-16)	8 (2-18)	3 (0-12)	0.03
Infection	84 (52)	61 (56)	31 (54)	1.0
MODS	114 (71)	85 (79)	29 (53)	<0.001
24-hour mortality	17 (11)	8 (7)	9 (17)	0.10
28-day mortality	40 (25)	25 (23)	15 (28)	0.56

Values in parentheses are interquartile range for continuous variables and percentages for categorical variables. Injury Severity Score (ISS); AIS, Abbreviated Injury Score; INR, International Normalized Ratio; PRBCs, Packed Red Blood Cells; FFP, Fresh Frozen Plasma; ICU, Intensive Care Unit; MODS, Multiple Organ Dysfunction Syndrome. Comparison between platelets transfused vs no platelets transfused (Mann-Whitney U-test or Fisher's exact test). ¹Survivors only.

Table 2: Characteristics of patients with samples during bleeding.

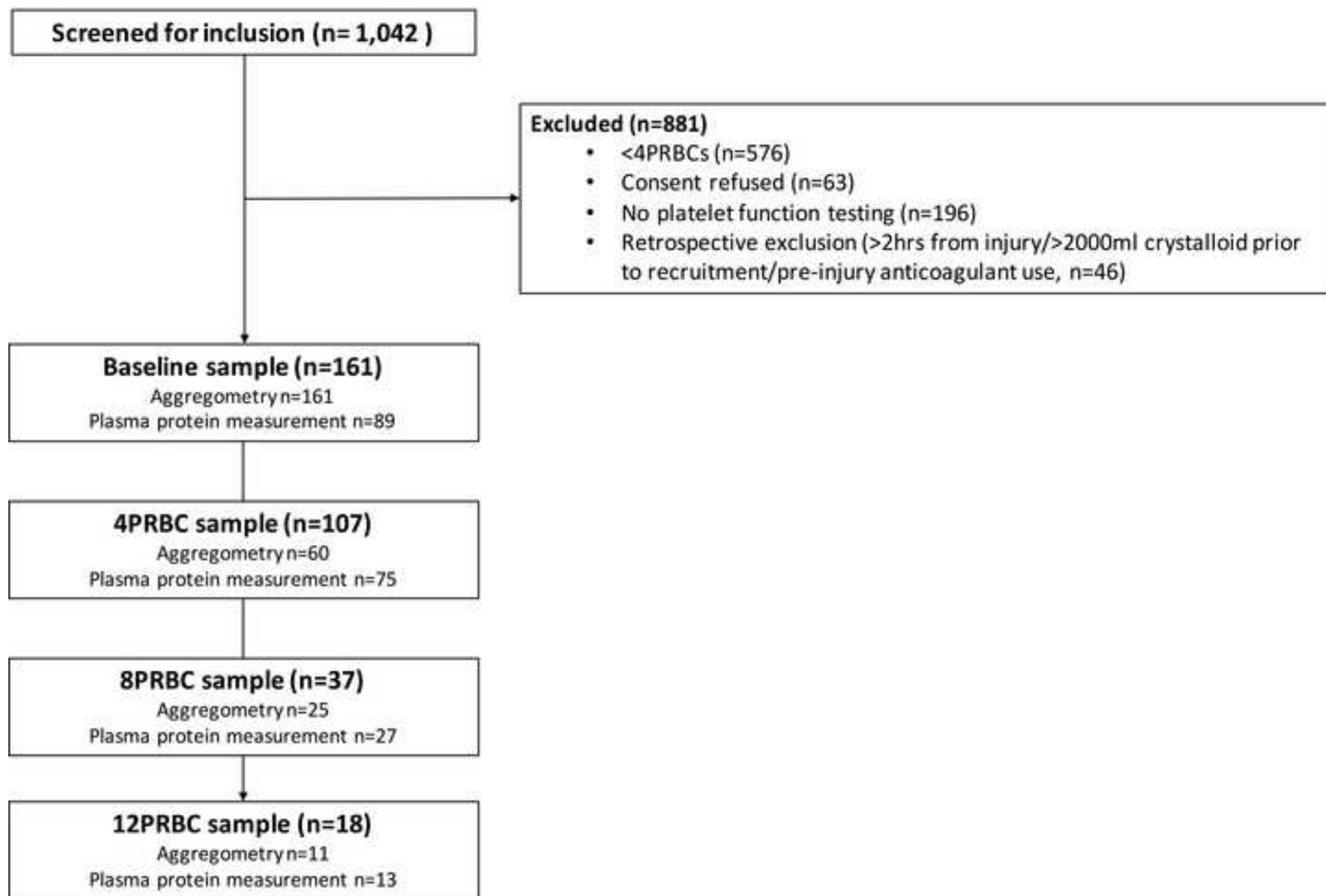
	4PRBC			8PRBC			12PRBC		
	No platelets transfused (n=88)	Platelets transfused (n=19)	p	No platelets transfused (n=17)	Platelets transfused (n=20)	p	No platelets transfused (n=3)	Platelets transfused (n=15)	p
ISS	34 (25-42)	27 (20-36)	0.259	36 (29-41)	27 (25-38)	0.273	34 (16-50)	29 (26-48)	0.99
Base Deficit, mmol/L	8.3 (5.1-15.0)	7.5 (5.5-10.9)	0.642	9.2 (4.7-17.2)	9.2 (6.2-15.3)	0.946	10.0 (8.9-10.6)	15.1 (8.0-21.0)	0.296
Time from admission to sample, minutes	57 (36-81)	70 (51-144)	0.148	63 (53-147)	115 (77-223)	0.051	48 (45-89)	131 (98-207)	0.014
PRBCs in 24 hours, units	7 (4-9)	8 (6-11)	0.536	12 (8-18)	10 (8-16)	0.596	19 (15-25)	16 (12-25)	0.686
Mortality ¹	29 (33)	5 (26)	0.787	9 (53)	7 (35)	0.331	1 (33)	8 (53)	1.00

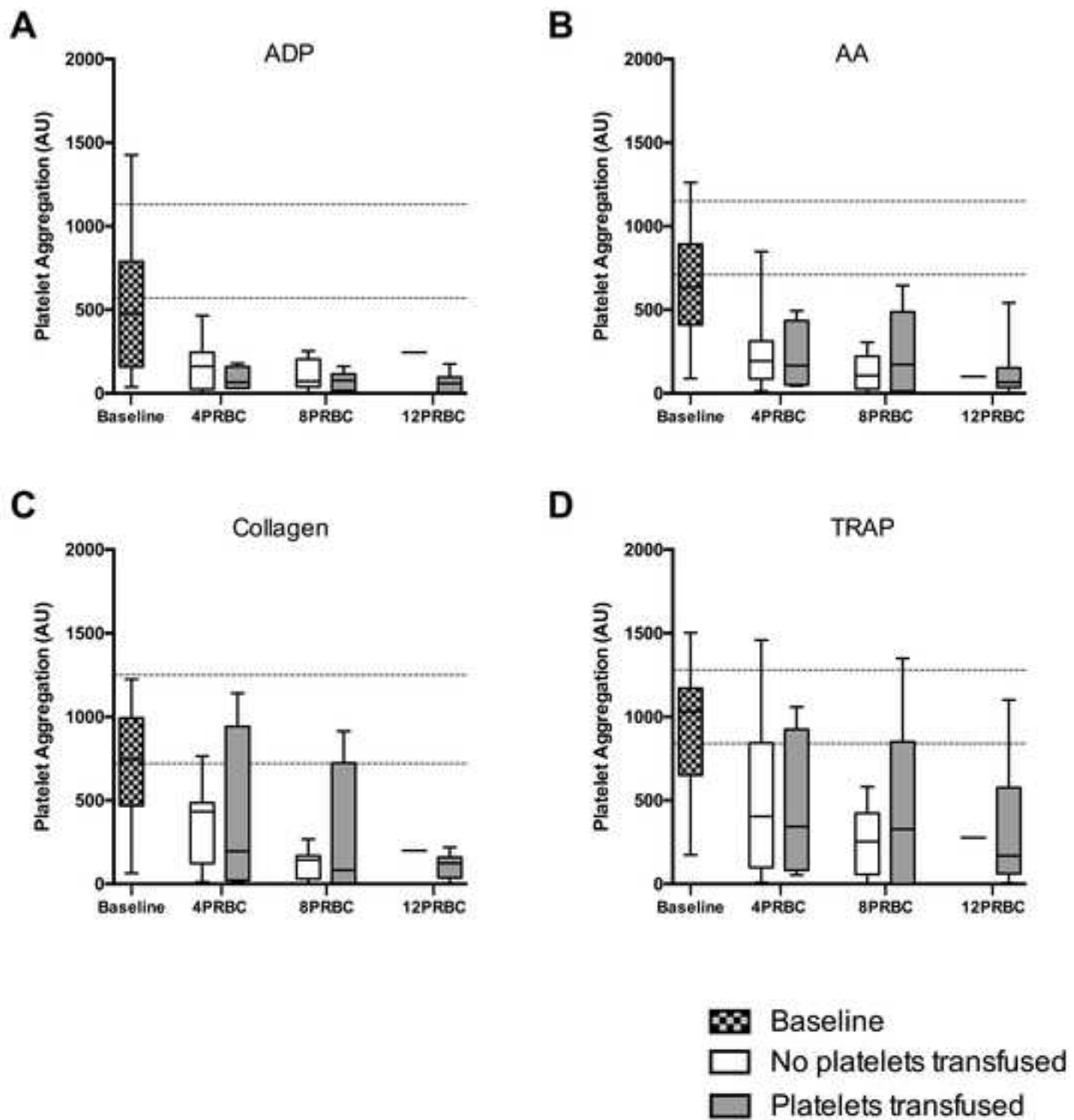
Patients were divided into two groups at each sampling point based on whether or not platelets were transfused prior to the sample being taken. Values are median with interquartile range unless stated. P-values compare platelets vs no platelets at each sampling point (Mann-Whitney U-test). ISS, Injury Severity Score. PRBCs, packed red blood cells. ¹Values are n (%)

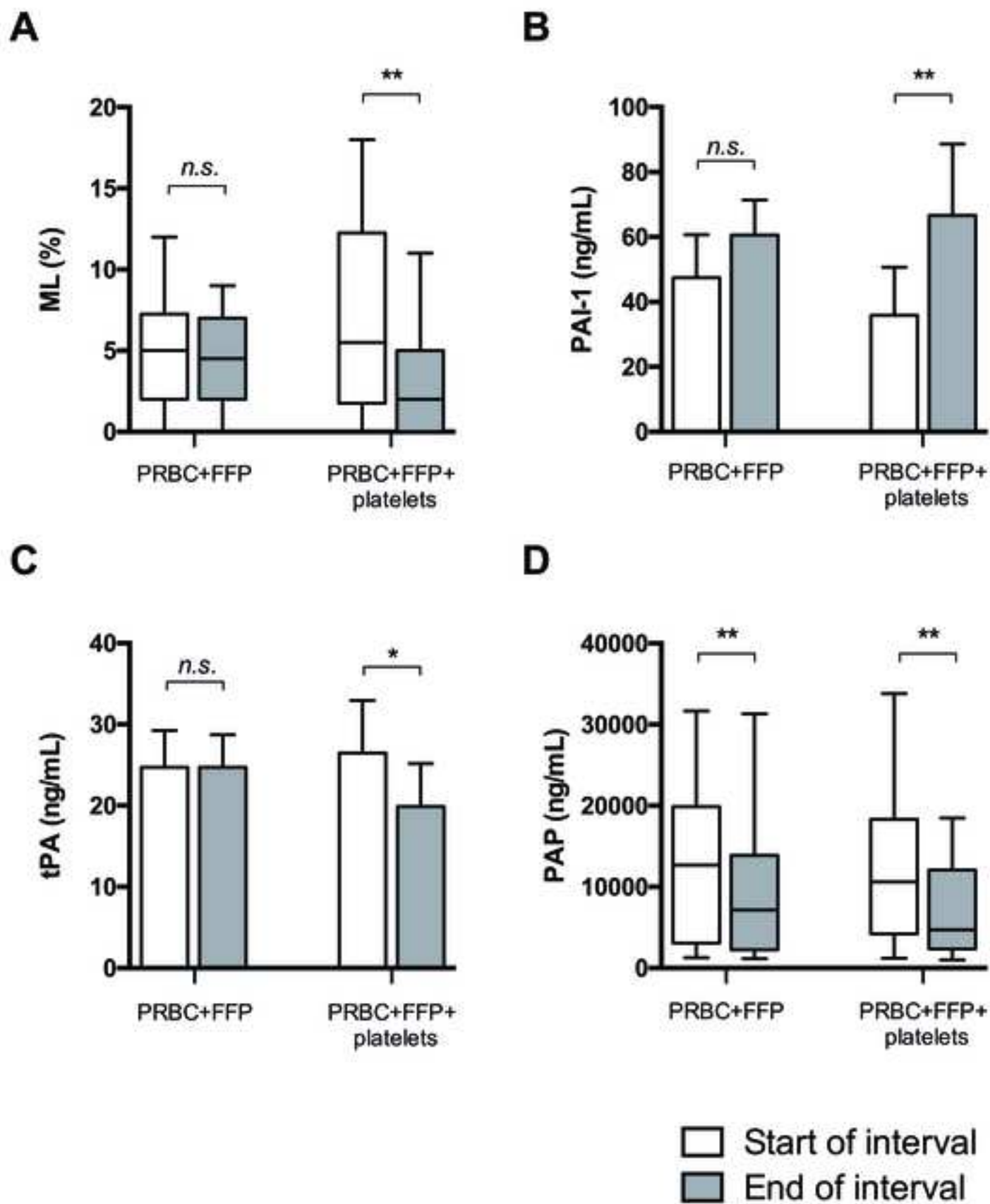
Table 3: Changes in ROTEM parameters and coagulation plasma proteins during four-PRBC intervals.

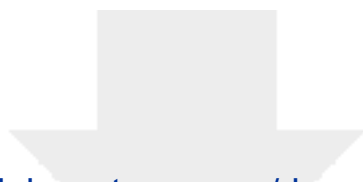
	RBCs only (n=30)	RBC + FFP (n=56)	RBC + FFP + Platelets (n=29)	p value
<i>ROTEM parameters¹</i>				
Clotting Time, seconds	15 (3-28)	-1 (-10 – 7)	-14 (-33 – 5)	0.014
Clot Formation Time, seconds	59 (20-98)	17 (-4 – 37)	-4 (-50 – 42)	0.039
Clot Amplitude at 5 minutes, mm	-7 (-11 – -4)	-4 (-6 – -2)	-2 (-7 – 3)	0.102
Maximum Clot Firmness, mm	-6 (-10 – -3)	-1 (-4 – 2)	-1 (-6 – 4)	0.085
EXTEM-FIBTEM Maximum Clot Firmness, mm	-2 (-7 – 3)	-1 (-4 – 2)	-3 (-8 – 2)	0.743
Maximum Lysis, % ²	-1 (-2 – 2)	-1 (-2 – 2)	-3 (-7 – 0)*	0.044
<i>Coagulation factors</i>				
Factor II, IU/dL	-11 (-19 – -2)	-4 (-9 – 1)	-3 (-9 – 2)	0.246
Factor V, IU/dL	-9 (-18 – -1)	-1 (-7 – 4)	-3 (-10 – 4)	0.239
Factor XIII, IU/dL	-13 (-20 – -6)	5 (-2 – 12)	8 (-3 – 19)	0.002
Von Willebrand Antigen, IU/dL	-64 (-94 – -35)	-49 (-72 – -25)	-47 (-92 – -2)	0.725
<i>Thrombin generation and clot formation</i>				
Prothrombin fragments 1+2, nmol/L	-3 (-5 – -1)	-2 (-3 – 0)	-1 (-4 – 2)	0.313
Soluble fibrin monomer complex ² , µg/L	10 (-34 – 44)	-31 (-70 – 7)	-13 (-76 – 37)	0.158
<i>Mediators and markers of fibrinolysis</i>				
Antithrombin, IU/dL	-12 (-21 – -3)	0 (-6 – 5)	-4 (-14 – 6)	0.082
Plasminogen-activator inhibitor-1, ng/ml	12 (2 – 23)	10 (-2 – 23)	31 (11-50)*	0.022
Tissue plasminogen activator, ng/mL	6 (1 – 12)	0 (-3 – 4)	-7 (-16 – 2)	0.013
Alpha-2 antiplasmin	-12 (-24 – 0)	0 (-10 – 8)	2 (-12 – 17)	0.207
D-dimer ² , ng/mL	-9115 (-32493 – 17875)	-3244 (-14969 – 2768)	-3011 (-14661 – 631)	0.878
Plasmin-antiplasmin complex ² , µg/L	-3719 (-6654 – -784)	-2631 (-4460 – -802)	-3680 (-5820 – -1539)	0.737

Values are mean with 95% confidence intervals and compared with one-way ANOVA unless specified. *p<0.05, RBC+FFP+platelets vs RBC+FFP, one-way ANOVA with Tukey's multiple comparisons test. ¹EXTEM value unless specified ²Values are median with interquartile range. p-values derived from Kruskal-Wallis test with Dunn's post-test correction.









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Supplemental Data File (.doc, .tif, pdf, etc.)

Supplemental figure 1.tif

