Platelet transfusion improves clot formation and platelet function in severely thrombocytopenic haematology patients

Cecilia Karlström,1,2 Gunilla Gryfelt,3 Laurent Schmied,1 Stephan Meinke1 and Petter Höglund1,3
1Department of Medicine Huddinge, Center for Haematology and Regenerative Medicine (HERM), Karolinska Institutet, 2Medical Unit Haematology, Karolinska University Hospital, and 3Medical Unit Clinical Immunology and Transfusion Medicine, Karolinska University Hospital, Stockholm, Sweden

Received 4 February 2021; accepted for publication 23 August 2021
Correspondence: Cecilia Karlström and Petter Höglund, Center for Haematology and Regenerative Medicine (HERM), Department of Medicine Huddinge, Karolinska Institutet, Stockholm, Sweden.
E-mail: cecilia.karlstrom@ki.se; petter.hoglund@ki.se

Summary

Prophylactic platelet (PLT) transfusion is a common practice in severely thrombocytopenic patients that reduces mortality, but responses to platelet transfusions are variable and difficult to predict in individual patients. In this prospective study, we evaluated the outcome of PLT transfusions in 40 patients with haematological malignancies, linking corrected count increment (CCI) to clot formation and agonist-induced platelet activation after transfusion. The CCI was highly variable between patients and 34% showed no response (1-h CCI < 7.5). Short time since the last PLT transfusion and extended storage time of the PLT product were linked to poor transfusion response, while patient sex, C-reactive protein or the number of chemotherapy cycles prior to transfusion did not influence transfusion outcome. High CCI and good PLT responsiveness to agonist stimulation predicted efficient clot formation in rotational thromboelastometry, but transfusion did not restore poor PLT function in patients to the level of healthy controls. Our study provides new insights into factors affecting PLT transfusion outcome in haematology patients with severe thrombocytopenia, and suggests that the thrombocytopenic environment, or disease-associated factors, may hamper platelet responsiveness.

Keywords: platelet function, platelet transfusion, thrombocytopenia, acute myeloid leukaemia.

Introduction

Patients with haematological malignancies frequently develop thrombocytopenia as a consequence of their disease or following intensive chemotherapy. Bleeding complications are reduced by prophylactic platelet (PLT) transfusions,1,2 which represent standard care for these patients. However, platelets are fragile cell fragments and several factors, such as ongoing bleeding, fever, infection, splenomegaly and immunological barriers, especially anti-HLA antibodies, are known to limit PLT count increment.3,4 On the other hand, ABO compatibility, short storage time, platelet function in the concentrate and the administration of large doses increase the transfusion efficiency.5–6 For all these reasons, platelet transfusion outcome is notoriously unpredictable.

The outcome of a PLT transfusion is usually evaluated by measuring the PLT count increment after transfusion.7 This parameter gives an estimate of surviving PLTs after transfusion but reveals no direct information regarding how well PLT function or haemostasis have been improved after transfusion.

One problem studying this question is that many established methods to measure platelet function in the blood, for example whole-blood aggregometry, require platelet concentrations above those found in thrombocytopenic patients.8 Flow cytometry (FCM) can be used to measure platelet activation at the single-cell level also at low platelet concentrations. The use of FCM has revealed considerable variation in PLT function in different patient groups. For example, patients with acute myeloid leukaemia (AML), showed worse PLT function compared to patients with immune thrombocytopenia (ITP).9,10 In thrombocytopenic patients, FCM analysis suggested that poor PLT function predicts haemorrhagic events,11–13 and it was used to show that platelet transfusion leads to improved platelet function.14 Despite great potential for transfusion optimisation, FCM-based measurement of PLT function is currently not part of the clinical routine and there is no consensus on which parameters to assess.

Platelet transfusion outcome can also be assessed by measuring the effect on haemostasis, which depends on humoral factors, PLTs and other cell types. Rotational
thromboelastometry (Rotem) measures the coagulation capacity in whole blood. Rotem can thereby assess the contribution of platelets and other factors to clot formation. It is well established in intensive-care and trauma patients, but not in thrombocytopenic patients. In the latter, impaired clotting time and clot firmness, as well as clot lysis have been shown using Rotem instruments. Most studies showed that prophylactic PLT transfusion improved Rotem parameters in patients with low PLT counts, but studies investigating haematology patients with extremely low PLT counts, such as AML patients, are lacking.

While the variable response to PLT transfusion in haematological patients is well known, conclusive explanations for this variability remain elusive. We evaluated the outcome of PLT transfusions in 40 severely thrombocytopenic patients. In addition to PLT increment, we assessed PLT function by Rotem and FCM. Two aspects of platelet function in response to agonist stimulation were assessed by FCM: conformational change of the GPⅡb-Ⅲa complex, essential for platelet aggregation, and α granule secretion. Identifying factors controlling platelet function after transfusion will help clinicians to identify patients that would be expected to benefit the most from platelet transfusions, leading to more predictable transfusion outcome.

Materials and methods

Patient samples and PLT transfusion

The study was approved by the local ethics review board and all patients signed informed consent. Forty severely thrombocytopenic patients in the haematological inpatient care were recruited. This number was based on a small pilot study by our group together with published data on the use of FCM in thrombocytopenic patients. Whole blood was drawn from a central venous catheter or a subcutaneous venous port and collected in 5 ml/2 ml vacutainer tubes containing sodium citrate at three timepoints: within one hour (0 h), one hour (1 h) and 18–24 h (24 h) after transfusion. An additional sample was taken from the PLT concentrate after transfusion. Whole blood was drawn from 32 healthy donors and collected in vacutainer tubes containing sodium citrate at three timepoints: within one hour (0 h), one hour (1 h) and 18–24 h (24 h) after transfusion. An additional sample was taken from the PLT concentrate after transfusion. Whole blood was drawn from 32 healthy donors and collected in vacutainer tubes containing sodium citrate, serving for comparison in FCM analyses (n = 20) and in the experiments studying the effects of low PLT count on PLT activation (n = 12).

Most patients received one unit of pooled PLTs from eightuffy coats, pathogen-inactivated and ABO-matched. Three patients received two units and one patient received a PLT unit with a minor mismatch.

Fibrinogen analyses

Fibrinogen was analysed in frozen plasma of 38 patients and 11 healthy donors using the Fibrinogen Human ProcartaPlex Simplex Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and a MAGPIX reader (Bio-rad, Hercules, California, USA) following the manufacturers’ instructions. The fibrinogen levels in the patients are indicated as a ratio: patient values/median healthy donors.

Cell count, corrected count increment and percentage platelet recovery

Haematocrit and platelet count were measured in each sample. The corrected count increment (CCI) was calculated as follows: (PLT increment per µl) × (body surface area in m²)/(number of PLTs transfused × 10¹¹). Consistent with guidelines and previous studies, a CCI >7.5 × 10⁸/l was considered a successful transfusion outcome and a CCI <7.5 × 10⁸/l was considered a poor response. Percentage platelet recovery (PPR) was calculated as follows: PPR = (post-PLT – pre-PLT count) × (100 PPC), where PPC (percent predicted count) = No. PLTs transfused × 0.67 × 0.001 blood volume (ml).

Dilution of blood from healthy donors

To mimic thrombocytopenia, citrated blood from healthy donors was diluted with healthy-donor erythrocytes and plasma. The haematocrit in the different samples was kept low (mean of 0.27) to resemble the anaemia in haematological patients.

Flow cytometry analysis

The following antibodies were used: BD Bioscience (San Jose, CA, USA) anti-CD42a (GP9) BV421, anti-CD62P (GMP-140) APC and BioLegend (San Diego, CA, USA) PAC-1 FITC. Citrated whole-blood samples were stained in modified HEPES/Tyrode’s buffer. PLTs were activated with either adenosine diphosphate (ADP, Roche Diagnostics, Basel, Switzerland, 37 µM final concentration) or thrombin receptor-activating peptide (TRAP, Roche Diagnostics, Basel, Switzerland, 74 µM final concentration), two commonly used PLT agonists. No agonist was added to the negative control. Samples were incubated for 20 min at room temperature before fixing in 2% paraformaldehyde (PFA) for 10 min. Subsequently samples were diluted in phosphate-buffered saline (PBS) and analysed by FCM.

Single PLTs were gated based on expression of CD42a together with forward scatter (FSC) properties, and the median fluorescence intensity (MFI) of minimally 5 000 single PLTs was measured in each sample. Assessing PLT reactivity, MFI of anti-CD62P and PAC-1 antibodies on PLTs was measured. FCM data were analysed with FlowJo software (Treestar, Ashland, OR, USA).

Rotational thromboelastometry (Rotem)

Rotem was performed using a Rotem delta analyser (TEM International GmbH, Munich, Germany) within 4 h after

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collection of citrated whole blood. Clot time (CT), clot formation time (CFT), clot firmness at 10 (A10) and 20 (A20) minutes and maximum clot firmness (MCF) were measured after extrinsic pathway stimulation according to the Extem protocol. Reference intervals provided by the manufacturer were used.

**Data collection and statistics**

The patients’ electronic medical records were manually scanned for diagnosis, height, weight, bleeding, blood parameters, fever, antibiotic or antifungal treatment, previous cytostatic treatment, and complications related to the treatment or the transfusion. IBM SPSS Statistics 26.0.0 for Windows (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Data were compared by non-parametric tests: the Mann–Whitney U-test for unrelated groups, the Friedman test for several related samples and the Wilcoxon signed ranks test for two related samples. Correlation was tested for with Spearman rank correlation. Significance was defined as $P < 0.05$.

Figures were prepared using GraphPad (Prism 8, GraphPad Software Inc., San Diego, California, USA).

**Results**

**Patient and transfusion characteristics**

AML was the most common diagnosis, representing 75% of the whole cohort (Table I). Of all patients, 43% ($n = 17$) had undergone one intensive chemotherapy cycle and 35% ($n = 14$) received four cycles or more; 15% ($n = 6$) had received allogeneic stem cell transplantation. HLA class I antibodies were detected in 8% ($n = 3$) of 38 analysed patients.

| Table I. Patient characteristics. |
|----------------------------------|
| Number of patients | 40 |
| Sex (male/female) | 26/14 |
| Age (years), median (range) | 62 (31–80) |
| Diagnosis | |
| Acute myeloid leukaemia | 30 |
| Myelodysplastic syndrome | 4 |
| Myeloma | 3 |
| B-cell acute lymphoblastic leukaemia | 1 |
| Diffuse large B-cell lymphoma | 1 |
| Mantle cell lymphoma | 1 |
| Previous allogeneic stem cell transplantation | 6 |
| Chemotherapy, number of courses prior to transfusion | |
| 1 | 17 |
| 2–3 | 6 |
| 4–5 | 8 |
| >5 | 9 |
| HLA class I antibodies (Y/N/Unknown) | 3/35/2 |
| Number of PLT transfusions prior to study, mean (range) | 22 (1–109) |

The transfusion response was highly variable: 35% of transfusions showed a 1-h CCI below the threshold for a successful response (Table II; Fig 1A). Neither the 1-h CCI nor the 24-h CCI correlated with sex, age, C-reactive protein (CRP) or the number of cytostatic treatment cycles (Fig S1A–D and data not shown). In line with previous studies, we found that storage time correlated negatively with the 1-h CCI, but not with the 24-h CCI (Fig 1B; Fig S1E). In addition, duration since last PLT transfusion correlated positively with both the 1-h CCI and 24-h CCI (Fig 1C; Fig S1F).

**PLT transfusion improves clot formation time and maximum clot firmness**

Rotem measurements were performed before PLT transfusion (0h), 1 h (1h) and 18–24 h (24h) after transfusion (Fig 2A gives one example). CT, indicating the start of clot formation, did not differ at either timepoint after compared to before transfusion (Fig 2B). This is expected since CT reflects the contribution of coagulation factors and is not PLT-dependent. CFT, in contrast, was significantly shorter at 1 h and 24 h post transfusion (Fig 2C). A10, A20 (Fig S2A) and MCF (Fig 2D) were likewise significantly increased after transfusion. Importantly, the improvement was still seen at 24 h (Fig 2D), suggesting a long-lasting effect.

To investigate the relationship between PLT counts and clot formation, PLT count was plotted against CFT and MCF using all existing data points before and after transfusion. PLT counts correlated to both MCF and CFT (Fig 2E, F), suggesting that Rotem analysis is indeed susceptible to small differences in PLT counts. The mean haematocrit was 0.22 at all timepoints and did not correlate to MCF (Fig S2B). To test if an increased PLT count after transfusion improved the clot formation in the individual patient, CCI was plotted against the difference in MCF before and after transfusion. The increase in MCF after 1 h ($\Delta$MCF1h) showed a trend towards a correlation to 1-h CCI but was not significant (Fig S2C). In contrast, the increase in MCF after 24 h ($\Delta$MCF24h) correlated to 24-h CCI (Fig S2D).

| Table II. Transfusion data. |
|-----------------------------|
| PLT* count before transfusion ($\times 10^9$ cells/l) | 6.3 (1–34)† |
| PLT* count 1 h after transfusion ($\times 10^9$ cells/l) | 20.9 (1–41)† |
| PLT* count 18–24 h after transfusion ($\times 10^9$ cells/l) | 13.4 (1–39)† |
| 1-h CCI‡, all transfusions | 10.4 (0–24.9)† |
| 18–24-h CCI‡, all transfusions | 5.1 (–2.9–23.3)† |
| PPR§, 1 h (%) | 39.5 (0–99.9) |
| PPR§, 18–24 h (%) | 19.3 (–9.9–91.2) |
| Transfusions with 1-h CCI >7.5 | 65% ($n = 26$) |
| Transfusions with 1-h CCI <7.5 | 35% ($n = 14$) |

*Platelet.
†Mean values (range).
‡Corrected count increment.
§Percentage platelet recovery.
In addition to platelets, fibrinogen levels are known to contribute to clot formation in Rotem. Relative fibrinogen levels in our patients at the time of transfusion was associated to 0-h MCF (Fig S3A), supporting this notion. However, we could not identify a link between the 1-h CCI and fibrinogen (Fig S3B), suggesting that fibrinogen and platelet transfusions may control clot formation independently. Taken together, these data show that in thrombocytopenic patients even a small increase of PLT count improves coagulation, for minimally 24 h.

Platelet transfusion increased platelet baseline activation and activation capacity

We next investigated if PLT activation capacity was affected by transfusion. FCM was used to detect upregulation of the activation markers CD62P and PAC-1 after PLT stimulation. Following stimulation with TRAP, expression of both CD62P and PAC-1 was significantly higher after 1 h as compared to 0 h, and for PAC-1 this difference remained at 24 h (Fig 3A, C). Upon ADP activation, the expression of CD62P was significantly higher both after 1 h and 24 h, while expression of PAC-1 showed no difference at 1 h but was significantly higher at 24 h compared to 0 h (Fig 3B, D). Despite the improvement after transfusion, the induction of CD62P and PAC-1 was still much lower in patients compared to healthy controls (Fig 3A–D).

We next tested if the upregulation of activation markers correlated to PLT count. When plotted against each other, PLT count showed a significant correlation to the MFI of CD62P and PAC-1 after TRAP or ADP stimulation (Fig 4A, B; Fig S4A, B), indicating an effect of donor PLTs in the samples after transfusion. This finding was supported by the strong correlation between CD62P upregulation in patient PLTs at 1 h and PLTs in the corresponding concentrates (Fig 4C). The responsiveness of transfused PLTs might therefore predict PLT responsiveness after transfusion. An improved PLT responsiveness might translate into clot formation, as suggested by the link between the activation capacity (MFI of CD62P and PAC-1) and both CFT and MCF (Fig S5).

Low platelet concentration impairs platelet function

The patients’ PLTs showed a strikingly poor responsiveness both before and after transfusion, compared to those of healthy donors (Fig 3; Fig S4). We asked if thrombocytopenia as such compromises PLT function, e.g. as a result of reduced paracrine activation of PLTs, or if a disease-induced inhibitory environment counteracts PLT activation. We diluted blood from healthy donors, simulating thrombocytopenia in vitro. In these samples we observed a correlation between the PLT count and CD62P induction upon TRAP stimulation (Fig 4D), whereas in the ADP-activated samples, significantly reduced PLT function was only seen at the lowest PLT concentrations (Fig 4E). The diluted samples from the healthy controls were much more responsive compared to patient samples with similar PLT counts (Fig 4D, E). We conclude that PLT activation is affected by thrombocytopenia per se, while there are likely additional factors involved reducing our patient’s PLT function.
Fig 2. Data from Rotem analyses before and after transfusion in 37 patients (three patients excluded due to technical issues). (A) Representative Rotem graphics before and 1 h after transfusion in one patient. Rotem EXTEM reference intervals are shown to the right. (B–D) CT, CFT, MCF before (0 h), 1 h after transfusion and 24 h after transfusion. (E, F) Correlation between CFT and total PLT count; MCF and total PLT count. Error bars show mean and standard deviation (SD). Grey areas indicate normal reference values. ns, non-significant **, P ≤ 0.01; *** P ≤ 0.001. [Colour figure can be viewed at wileyonlinelibrary.com]
In this study, we evaluated the effect of PLT transfusions in severely thrombocytopenic haematology patients. We found that PLT transfusion outcomes varied between patients but on average improved clot formation, even in patients with very low PLT counts and small PLT count increments. Efficient clot formation was predicted by good PLT responsiveness to agonist stimulation, but transfusion did not restore PLT function to the same degree as in healthy controls. The time to the last PLT transfusion correlated to the outcome, with a poor response in patients with frequent transfusions.

Our data show that Rotem parameters correlate with PLT count, confirming the relationship between thromboelastographic measurements and PLT counts seen previously. The variation between patients could be explained by the fact that clot formation measured in Rotem also depends on fibrinogen. Increased fibrinogen levels could thus compensate for reduced platelet numbers. Higher fibrinogen levels indeed correlated to higher MCF in the samples before transfusion. However, fibrinogen levels did not correlate to CCI, suggesting that improved clot formation after platelet transfusion was not directly related to high fibrinogen levels. Of note, platelet concentrates contain donor plasma, but this should...
have little impact on the patients’ fibrinogen levels, considering the small transfusion volume.

Transfusions were given prophylactically and only few of the patients had an ongoing bleeding; therefore the effect of PLT transfusion on bleeding was not evaluated. Opheim et al. found a correlation between thromboelastography (TEG) measurements in thrombocytopenic patients and grade 2 bleedings, suggesting that small changes in TEG may predict bleeding. In light of those data, we believe that the improvement in Rotem parameters indeed represents a degree of bleeding protection. This is further supported by the reduced bleeding seen after prophylactic PLT transfusions in a large randomised clinical trial.2

Correlating PLT count to bleeding risk is of clinical relevance not only during leukaemia treatment but also in diagnostic settings. Many invasive procedures are often only performed at PLT counts above certain levels, frequently set arbitrarily and based on weak scientific evidence. For example, a PLT count of $>80 \times 10^9/\text{L}$ is recommended for the setting of an epidural catheter, a decision based on safety arguments.31,32 In our study, Rotem measures correlated in an almost linear manner in a PLT count interval of $0 - 20 \times 10^9/\text{L}$, after which the curve took on a plateau-like shape. It is possible that the early plateau phase reflects a limitation of the Rotem method in assessing the contribution of platelets to coagulation, but it is still striking to note that only very small improvements in platelet count have such unexpectedly large effects on homeostasis. Larger studies will be needed to test to which extent Rotem measurements would be a more sensitive predictor for bleeding than PLT counts.

Fig 4. Correlation between PLT activation measured by flow cytometry and total PLT count in whole blood and serial dilutions from healthy controls (black squares) and the patients (black dots). Presented statistics for A and B are only including patient values. (A, B) Correlation between MFI of CD62P and PAC-1 after stimulation with TRAP and total PLT count. (C) Correlation between MFI of CD62P in the PLT concentrate and in the patients at 1 h after transfusion. (D, E) Correlation of PLT count and PLT activation after TRAP and ADP stimulation in a serial dilution of whole blood from healthy controls.
FCM has been evaluated in several previous studies as a method to assess PLT function in thrombocytopenic patients.\textsuperscript{14,33,34} We found that activation measured by FCM was, at least partly, dependent on PLT count in the sample. This was irrespective of whether the sample was thrombocytopenic from the start or rendered thrombocytopenic by dilution. Other studies have shown similar results, even when using PLT-rich plasma or isolated PLTs.\textsuperscript{11,35} Boknås \textit{et al}. showed PLT count-dependent effects when PAR1-AP (TRAP) was used to activate PLTs, but not when using ADP. This result is reminiscent of our results when TRAP but not ADP-mediated activation showed a lower response in diluted PLTs. Interestingly, Boknås \textit{et al}. found that the poor effect of PAR1-AP was reversed when samples were pre-incubated with apyrase, concluding that this effect might be due to reduced purinergic signalling in the thrombocytopenic sample.\textsuperscript{13} However, other studies found no impact of low PLT counts on agonist-induced CD62P expression, even upon activation with TRAP.\textsuperscript{11} Similar to our data, Boknås \textit{et al}. also concluded that CD62P was a more stable marker at low PLT counts than PAC-1. However, Kander \textit{et al}. saw no change in expression of CD62P after transfusion; with ADP stimulation a small decrease of PAC-1 binding was instead detected after transfusion.\textsuperscript{19} Our results suggest the importance of interpreting PLT function in FCM with great care when it comes to correlating it to PLT function in vivo. With the limitations of FCM in mind, PLT activation was severely reduced in all our patient samples, compared to healthy controls, confirming previous findings in AML patients.\textsuperscript{9}

Our in vitro dilution data argue against the hypothesis that PLT function in AML patients is reduced only as a result of low PLT counts. The leukaemic environment, leukaemia treatment, or dysregulated cytokine production may disrupt normal PLT function, which could also explain the impaired response of transfused PLTs of healthy donors compared to those of healthy controls. However, an alternative, but not mutually exclusive, explanation for poor PLT function after transfusion could be functional impairment developed during PLT production and storage. We found that TRAP stimulation is less efficient in stored PLTs compared to PLTs from healthy blood. Irrespective of the reasons, further studies are needed to explore PLT deficiency in transfused patients. For AML and other patient groups, it would be highly desirable to study their functional deficiencies in more detail and to uncover the underlying mechanisms reducing PLT activity.

Our data also suggests that beside PLT counts, PLT function should be evaluated. For example, Batman \textit{et al}. showed that low PLT reactivity correlated with bleeding.\textsuperscript{12} Based on this, and the data in our study, we conclude that the assessment of PLT function beside its bare number may provide a novel approach for a personalised transfusion medicine. Our data also show a correlation between the response capacity of the PLTs in the concentrate and in the patient PLTs after transfusion. This is perhaps not surprising but emphasises the importance of optimising platelet production routines to secure the most efficient platelet function in the platelet units. Rotem analysis can assess both the contribution of PLTs as well as fibrinogen levels to the aggregation capacity of blood. It might therefore be favourable to include e.g. Rotem tests in the decision tree of PLT transfusion recommendations.

Our study provides novel insights into the regulation of PLT function in haematological patients with severe thrombocytopenia as well as the impact of healthy-donor PLT transfusion on overall PLT function. Of note, our study included mainly AML patients and can therefore not be generalised to all conditions of thrombocytopenia. In fact, we consider it likely that disease-specific factors contribute to the functional phenotypes we observe, and larger studies including thrombocytopenic patients with additional diagnoses are necessary to deepen the knowledge and to generalise our findings.

**Acknowledgements**

We would like to acknowledge Iyadh Douagi and the MedH Flow Cytometry core facility (Karolinska Institutet), supported by KI/Region Stockholm, for providing cell analysis services and expert assistance. We also express our thanks to all personnel at the Karolinska University Hospital Blood bank as well as the nurses and other colleagues at the Hematology ward, and not the least the patients included in the study. All coworkers at the center for Hematology and Regenerative Medicine (HERM) are acknowledged for continuous support. This study was supported by grants to PH from the Swedish Cancer Society (2018/689), Radiumhemmets Forskningsfonder (181133), Cancer och Allergifonden, Aroseniusfonden, the Karolinska Institute Strategic Research Area in Stem Cells and Regenerative Medicine (StratRegen), and Region Stockholm ALF grant FoUI-952144. CK has been supported by a Research Residency grant from Karolinska Institutet-Region Stockholm and a grant from Martin Rinds stiftelse.

**Author contributions**

CK, PH and SM designed the research study. CK, GG, LS and SM performed the research. CK, SM, LS and PH analysed the data. CK drafted the manuscript. PH, SM and LS provided critical review.

**Conflicts of interest**

The authors declare to have no potential conflicts of interest regarding the present work.

**Supporting Information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.
Fig S1. CCl in relation to patient parameters. (A) 1-h CCl in relation to patient age (ns). (B) 1-h CCl in relation to sex (male/female, ns). (C) 1-h CCl in relation to C-reactive protein (CRP; ns). (D) 1-h CCl in relation to number of cytostatic courses prior to transfusion (ns). (E) 18–24-h CCI in relation to storage time (days) of the PLT concentrate (ns). (F) 18–24-h CCI in relation to time (days) since last PLT transfusion.

Fig S2. Rotem parameters. (A) A10 and A20 before (0h), 1 h after transfusion and 24 h after transfusion. (B) Correlation of HCT and MCF. (C) Correlation of AMCF1h and 1-h CCI. (D) Correlation of AMCF24h and 1-h CCI.

Fig S3. Fibrinogen and effect on MCF and 1-h CCI. Fibrinogen are expressed as relative levels compared to healthy controls as described in Materials and methods. (A) Correlation between MCF at 0 h and fibrinogen. (B) Correlation between 1-h CCI and fibrinogen.

Fig S4. (A,B) Correlation between MFI of CD62P and PAC-1 after stimulation with ADP and total PLT count.

Fig S5. Correlation of Rotem parameters and activation capacity after activation with TRAP. (A) Correlation of CFT and MFI of CD62P. (B) Correlation of MCF and MFI of CD62P. (C) Correlation of CFT and MFI of PAC-1. (D) Correlation of MCF and MFI of PAC-1.

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