Platelet function tests predict bleeding in patients with acute myeloid leukemia and thrombocytopenia

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Title: Platelet function tests predict bleeding in patients with acute myeloid leukaemia and thrombocytopenia

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Short title: Bleeding in AML

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Abstract

Bleeding is frequent among patients with thrombocytopenia and we studied whether *in vitro* platelet function predicts future bleeding in patients with acute myeloid leukemia (AML) and thrombocytopenia.

Adult AML patients with platelet count <50x10^9/L were included. Detailed bleeding history and blood samples were collected at inclusion and every seven days. We analyzed hematology and coagulation parameters. With flow cytometry we evaluated platelet activation (activated GPIIb/IIIa, P-selectin, and CD63 expression) and platelet aggregation. Agonists were thrombin-receptor activating peptide (TRAP) and collagen-related peptide (CRP-XL).

During 18 months, sixty participants were enrolled and followed for a total of 114 weeks. Bleeding occurred in 52 (46%) weeks and was not associated with clinical, hematology or coagulation parameters. Predictors of bleeding were assessed using multivariate logistic regression adjusted for platelet count, sex, age, and bleeding history and receiver operating curves were compared using c-index. Reduced TRAP-induced platelet aggregation had Odds ratio 3.00 (95% confidence interval 1.38-6.60) and reduced CRP-XL-induced platelet aggregation had Odds ratio 4.00 (95%-CI 1.70-9.20) for bleeding. Overall, C-index was 0.71 for the models including platelet aggregation results, 0.72 for activated GPIIb/IIIa-positive platelets after stimulation with TRAP, 0.68 for percent P-selectin positive platelets with TRAP and 0.63 for the platelet count. Among patients receiving no platelet transfusion, C-index was 0.83-0.87 for bleeding; highest for models using platelet aggregation. Change in platelet aggregation did not correlate with the number of platelet concentrates administered. In conclusion, platelet aggregation and platelet activation results predict bleeding better than platelet count alone among AML patients with thrombocytopenia.
Introduction

Bleeding is a frequent and potentially lethal complication of acute myeloid leukemia (AML). To prevent bleeding, prophylactic platelet transfusion is standard of care for patients with low platelet counts \(^1,2\). For decades, the platelet count has been the main trigger for platelet transfusions. However, the platelet count per se is poorly correlated with bleeding risk, and bleeding might occur at relatively high platelet counts and despite platelet transfusions \(^3-6\). In a large study of haematological patients, bleeding risk was equally high among patients with platelet count between 6 and 80 \(x10^9/L\); occurring in up to 25% of days with thrombocytopenia \(^3\).

Besides a sufficient number of platelets, functional platelets are required in order to achieve haemostasis upon vascular damage \(^7,8\). Flow cytometry has gained grounds for evaluation of platelet activation at a single-cell level and has revealed that reduced platelet activation capacity is observed among patients with AML \(^9-11\). However, no studies have evaluated the relationship between platelet function and bleeding episodes over time in patients with AML.

Due to methodological limitations, it has historically been challenging to study platelet aggregation in patients with thrombocytopenia. Light transmission aggregometry (LTA) is considered the gold standard for platelet function testing, but it is not recommended at platelet counts below 150 \(x10^9/L\) due to inaccurate measurements at low platelet count \(^12\). Recently, we presented a flow cytometric assay for testing \textit{in vitro} platelet aggregation independently of the patient platelet count \(^11\). Among patients with AML or myelodysplastic syndrome, high flow cytometric platelet aggregation results identified all patients without bleeding tendency. It is therefore meaningful to apply the platelet aggregation method for prospective studies of bleeding risk. Only few studies have investigated the potential of platelet activation for predicting bleeding phenotype in AML, and existing results are discordant \(^9,11,13\).
In the present study, we hypothesized that reduced platelet function in patients with AML and thrombocytopenia increases the frequency of bleeding. Thus, the aim was to evaluate the predictive value of in vitro platelet aggregation and activation for future bleeding episodes in patients with AML.

Methods

For the flow cytometry-based platelet aggregation assay, blood from healthy blood donors aged ≥ 18 years were used as controls. The donors were not allowed to take prescribed or over-the-counter drugs within 24 hours prior to sampling. Plasma from the same male AB RhD+ donor was used as matrix in all flow-cytometric platelet aggregation experiments.

Included were patients ≥ 18 years diagnosed with AML according to current guidelines and a platelet count <50 x10⁹/L. Exclusion criteria were surgery or treatment with platelet inhibitors within the last seven days. Patients were recruited from department of Hematology, Odense University Hospital during 18 months beginning September 2016. The AML was managed according to the institutional protocols during the study period. The institutional protocol was followed for management of prophylactic platelet transfusion. It stated that one pool of buffy coat derived platelets should be administered at platelet count<10x10⁹/L, while the trigger was <15x10⁹/L in patients with infection. Patients were followed for seven days after a study visit. Overall the patients were withdrawn from the study after four weeks (four periods), withdrawal of consent, discharge from hospital, or platelet count above 50 x10⁹/L on a study visit, whichever came first. Bleeding assessments from periods where patients were discharged before seven day follow-up time were not included. Upon inclusion and thereafter before starting a new follow-up period, a visit was made, where the patient was interviewed and blood samples were collected.
At each visit, patients were systematically interviewed about bleeding episodes within the past seven days, comprising information about bleeding episodes after the last visit and before the next follow-up period. Bleeding dates and bleeding symptoms were registered based on the interview, medical files and the patient self-recorded events as a supplement for the interview to verify that patient recalled all events at time of interview. Bleeding episodes were graded by authors HF and PJV based on World Health Organization and blinded for laboratory results. Based on interviews and the medical record, we also registered sex, familial history of bleeding disorders, previous immune thrombocytopenia, inclusion date, admission date, discharge date, and transfusions, surgery or infections within the past seven days. Infection was defined as temperature >38.0 degree Celsius and concurrent treatment with antibiotics. Platelet transfusions were defined as therapeutic when administered due to bleeding.

**Blood samples**

Blood was drawn from a peripheral vein using a 21-gauge needle into BD Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) with 3.2% (0.109 M) trisodium citrate or ethylenediaminetetraacetic acid (EDTA). Haemostasis parameters included fibrinogen activity (Clauss’ method, STA-fibrinogen), activated partial thromboplastin time (aPTT) (STA-PTT) and international normalized ratio (INR) (STA-SPA+) determined on citrate-anticoagulated plasma with reagents from Stago Diagnostica (Asnières-sur-Seine, France) on STA-R Evolution analyser (Diamond Diagnostics, Holliston, Massachusetts, USA). Hematology parameters were evaluated on EDTA-anticoagulated blood and included platelet count, immature platelet fraction (IPF), leucocyte count, hemoglobin on a Sysmex XN-9000 (Sysmex, Kobe, Japan).

*Flow cytometric platelet aggregation*
Platelet aggregation was tested as previously described and shown in Figure 1A. Briefly, platelets were isolated by centrifugation and resuspended in dilution buffer (NaCl 134 mM, KCl 2.9 mM, MgCl2 1mM, glucose 5.6 mM, and HEPES 20 mM, pH 7.4). Then, two fractions with platelet counts of 144 $\times 10^9$/L and 16 $\times 10^9$/L, respectively, were constructed by adjustment with dilution buffer; the two fractions were labeled with 0.05 µM calcein acetoxymethyl ester (AM) ultrapure grade (CAMU) or 1.1 µM calcein-AM Violet 450 (CV450), respectively (both from eBioscience, San Diego, CA, USA) and incubated in the dark for 15 min at 37 °C. For the platelet aggregation test, a platelet mix was made. The mix comprised 70 µL of thawed donor plasma and 35 µL of each of the CV450-labeled and CAMU-labeled platelet fraction mixed in a 1.5-mL Safe-Lock tube (Eppendorf, Hamburg, Germany). Then, 3.5 µL of agonist was added. Final concentrations of agonists were either 2.2 µg/mL collagen-related peptide (R. W. Farndale, University of Cambridge, UK) or 244 µM thrombin receptor-activating peptide (TRAP) (SFLLRN; JPT Peptide Technologies, Berlin, Germany). An unstimulated platelet mix was analyzed in parallel, and included all components except an agonist. To induce aggregation, tubes were shaken at 1000 r.p.m. for 5 min at 37 °C in an Eppendorf Thermomixer comfort (Eppendorf, Hamburg, Germany), and 25 µL was thereafter transferred to 225 µL fixation buffer (0.2% formaldehyde in PBS). Data acquisition was performed on a FACSCanto II flow cytometer and FACS DIVA software (Becton Dickinson) with no compensation. Dot plot quadrants (Q1–Q4) were set by use of the unstimulated platelet mix. Platelet aggregation was reported as the percentage of calcein-AM double-positive events (positive for both CV450 and CAMU) out of all CV450-positive events.

Platelet activation

In vitro platelet activation was tested in 2.5 µL whole blood incubated for 10 min at room temperature with the following fluorophore-conjugated mAbs in PBS: phycoerythrin (PE)-conjugated glycoprotein (GP) Ib (CD42b, clone HIP1, dilution factor in final sample [DF] 78),
allophycocyanin (APC)-conjugated P-selectin (CD62p, clone Psel.KO2.3, DF 325) (both eBioscience), PE–cyanine 7 (Cy7) granulophysin (CD63, clone H5C6, DF 13), and fluorescein isothiocyanate (FITC)-conjugated PAC-1 (DF 13) (both Becton Dickinson Bioscience, San Jose, CA, USA), which only binds to activated GPIIb–IIIa. In addition, either agonist or PBS was added to a final volume on 31.5 µL. Agonists were TRAP (10 µM) and collagen-related peptide (1.1 µg/mL). A negative sample was constructed with mAbs against CD63, CD42b, and PAC1, and with an anti-P-selectin matched APC-conjugated isotype control (eBioscience). For inhibition of specific binding of PAC1 and CD63 mAbs, EDTA (10 mM) was added. Data acquisition was performed on a FACSCanto II flow cytometer and data were analyzed using KALUZA software 1.3 (Beckman Coulter, Brea, CA, USA). Platelets were gated based on GPIbα expression and scatter pattern. The results were expressed as mean fluorescence intensities (MFIs) or the percentage of platelets positive for P-selectin, CD63 and/or PAC1 as compared with the negative sample.

**Ethics**

All patients gave written informed consent. The study was approved by the Regional Scientific Ethical Committees of Southern Denmark (s- 20160081) and the Danish Data Protection Agency (2008-58-0035) and was conducted in accordance with the guidelines of the Declaration of Helsinki.

**Statistics**

The sample size calculation was based on the estimated TRAP-induced platelet aggregation on 40% ±11 (mean ±SD) in AML patients without bleeding and 25% lower platelet aggregation in AML.
patients with bleeding \textsuperscript{11}. With an anticipation of bleeding within one week in 33\% of patients, power =0.9, \( \alpha=0.05 \), 60 participants were required.

For demographic data and univariate analyses, categorical variables were presented as numbers and percentages. To evaluate the association with bleeding, between group comparisons of continuous data were made with t-test and Wilcoxon Rank-sum test, as appropriate. Chi-square test was conducted to compare groups of categorical variables. We identified whether patient characteristics or platelet function (aggregation or activation) were associated with bleeding for subsequent introduction into logistic regression models.

Logistic regression analysis was done to identify whether platelet function (aggregation or activation) was associated with bleeding within seven days and clustering based on patient identification was used to account for the same individual appearing in more than one follow-up period. For logistic regression, platelet activation and aggregation were assessed on a continuous scale, while for main analyses platelet aggregation results were dichotomized according to previously established cut-offs for bleeding risk assessment: 38\% for TRAP- and 40\% for collagen-induced platelet aggregation \textsuperscript{11}. In multivariate analyses, age, sex, platelet count and bleeding episodes within the past 7 days (yes/no) were included as fixed factors. Measurements of platelet function were evaluated in separate models. Results were reported as odds ratios (OR) with 95\% confidence intervals (95\% CI). Observations with missing values were excluded from the analyses. Receiver operating characteristic (ROC) curves were made from multivariate models and the predictive values of the applied models were compared using C-indices and test of the equality of ROC was used to compare models with and without platelet function markers.

Secondary multivariate logistic regression analyses were conducted and included separate analyses for those platelet function markers that were associated with bleeding in the overall analyses. A
secondary analysis was done because platelet transfusion was expected to modify the effect, but for ethical reasons could not be withheld. Therefore, we did separate analyses of the results in the subgroup of patients who either did not receive platelet transfusion or bled before administration of platelet transfusion during the seven days follow-up – hereafter named the "no platelet transfusion group". We also did stratified analyses to determine the models in the subsample having infection during follow-up. Moreover, we examined extended follow-up time to determine the association between baseline markers and bleeding within 14 days follow-up. Finally, we tested the association with bleeding WHO grade ≥2 by grouping WHO grade 1 bleeds with those with no bleeding or excluding data with WHO grade 1 bleeds.

Patients with severe thrombocytopenia comprise a high bleeding risk population, and received prophylactic platelet transfusions according to guidelines18. To evaluate whether other markers identified this high bleeding risk subgroup, we did logistic regression to evaluate if baseline markers identified patients who received prophylactic platelet transfusion the following seven days follow-up. Results of platelet activation and aggregation were blinded to the attending clinicians during the study.

We used Pearson’s correlation to evaluate the relationship between platelet aggregation results and the blast count in the most recent bone marrow examination, and between the change in platelet aggregation and change in platelet count during the first seven day follow-up period. ANOVA with posthoc Tukey test was used for pairwise comparison of platelet aggregation among groups with different AML Fab types.

Statistical analyses were conducted with Stata software package (Stata 13.1; StataCorp, College Station, TX, USA).
Data Sharing Statement

For original data, please contact the corresponding author (email: pernille.vinholt@rsyd.dk). Only deidentified individual participant data are shared.

Results

Patient characteristics and bleeding symptoms

We enrolled 60 participants for follow-up. As some patients met the criteria for inclusion more than once during the 18 month period, we included overall 51 unique patients with AML with a median age of 70 years (range 19-85) and 27 (53%) were males. Two patients had acute promyelocytic leukaemia, nine had relapse of AML, and seven had AML secondary to other haematological conditions including chronic myeloproliferative neoplasm (n=3), chronic lymphocytic leukaemia (n=2) or myelodysplastic syndrome (n=2). One patient reported familial bleeding episodes, while none had previous immune thrombocytopenia.

A total of 114 weeks of follow-up, ranging one to six weeks per participant was evaluated. Bleeding episodes were observed in 52 weeks (45%). The most prominent bleeding symptoms were epistaxis (n=15), cutaneous bleeding (n=13), haematuria (n=6), bleeding from invasive sites (n=4), gastrointestinal bleeding (n=4), oral bleeding (n=4), haemoptysis (n=4), and menorrhagia (n=2). No intracranial bleeding episodes were observed.

A total of 65% of the patients experienced bleeding. The most prominent bleeding severity being WHO grade 1 (27 weeks), WHO grade 2 (22 weeks) and WHO grade 3 (3 weeks). In weeks with bleeding episodes, patients had median two days with bleeding of any grade (IQR): 1-3 days.

Platelet transfusions were administered in 88 (77%) weeks of follow-up. Thereof n=12 were
administered therapeutically due to bleeding. The median number of platelet concentrates administered per week was 2 (range 0-12).

Risk factors for bleeding within the following seven days

We included all 114 periods of ‘seven days follow-up’ in the overall analysis. Clinical and routine biochemical characteristics were not associated with bleeding episodes during the following seven days follow-up time (Table 1). The platelet count, IPF, leukocyte count, hemoglobin, APTT, INR, or fibrinogen activity were not associated with bleeding within seven days follow-up.

A representative figure of TRAP-induced platelet aggregation in patients with AML is shown in Figure 1. Overall, both TRAP- and CRP-induced platelet aggregation were significantly lower before weeks with than weeks without bleeding (Figure 1C). There was no difference in platelet aggregation in unstimulated samples collected before weeks with bleeding compared with weeks without bleeding, i.e. no difference in spontaneous aggregation was observed. Platelet activation was evaluated by measuring the activation capacity of the fibrinogen receptor and platelet granule release. We found that the percentage of activated GPIIb/IIIa and P-selectin-positive platelets following stimulation with TRAP were significantly lower prior to weeks with bleeding than weeks without bleeding (Table 2). Results of other platelet activation markers and forward scattered light (FSC), as a measure of platelet size, did not differ in samples collected prior to weeks with versus without bleeding. Further, expression levels (MFIs) of platelet activation markers reflecting the amount of receptors per platelet were not associated with bleeding (Supplemental Table 1).
Odds ratios for bleeding within the subsequent seven days are shown in Figure 2. Platelet aggregation (both agonists), activated GPIIb/IIIa after TRAP stimulation and P-selectin-positive platelets after TRAP stimulation were independently associated with bleeding episodes in univariate and multivariate analyses. Odds ratio for bleeding was 3.00 (95% CI 1.38-6.60) for collagen-related peptide-induced platelet aggregation and 4.00 (95% CI 1.70-9.20) for TRAP-induced platelet aggregation in multivariate analyses. In adjusted analyses, the platelet count was also independently associated with bleeding during the seven days follow-up, while sex, age or bleeding history was not independently associated with bleeding. Further, we found that platelet aggregation and platelet activation results were associated with when including all parameters on a continuous scale and were also associated with bleeding within the first seven days follow-up time with similar risk estimates, except for TRAP-induced platelet aggregation (data not shown).

In Figure 3, ROC curves are depicted to compare the prediction of models for bleeding. In the overall population, the best model included platelet aggregation results or percentage of activated GPIIb/IIIa-positive platelets as covariates had C-indices of 0.71-0.72, while the model including percentage of P-selectin positive platelets or platelet count had C-indices of 0.68 and 0.63, respectively.

**The association with bleeding in subgroups** We examined the “no platelet transfusion” subgroup, which comprised 43 weeks follow-up and bleeding occurred in 21 weeks. In this subgroup, samples taken before weeks with bleeding had significantly lower platelet count, and lower platelet function in terms of lower platelet aggregation after stimulation with TRAP or collagen-related peptide, and lower percentage P-selectin positive platelets after TRAP stimulation. All these variables, except the platelet count, were independently associated with bleeding in multivariate logistic regression in this subgroup. Among patients with “no platelet transfusion”, the model including platelet aggregation (with TRAP or collagen-related peptide), and the model including percentage of P-
selectin positive platelets following TRAP stimulation as covariates were good for bleeding risk assessment (C-index 0.85-0.91) (Figure 3).

We also evaluated the bleeding risk assessment in weeks with infection (n=82). In multivariate analyses, all platelet function markers remained independently associated with bleeding while platelet count was not. The presence of infection had little effect on the C-indices as compared to the overall analyses (Figure 3).

When evaluating the multivariate analyses for the association with WHO grade ≥2 bleeding, we found that all platelet function markers were independently associated with bleeding in the overall dataset if WHO grade 1 bleeds were excluded from analyses, while none of the platelet function markers were associated with bleeding when WHO grade 1 bleeding was grouped with “no bleeding” (data not shown).

For 33 of our cases we had extended results from 14 days follow-up. We found that none of the identified risk markers for bleeding within seven days were associated with bleeding within 14 days in the overall dataset. When focusing on patients who did not receive platelet transfusions, 8 out of 10 patients bled within 14 days, and in this group all patients with low TRAP – or collagen induced platelet aggregation at baseline experienced bleeding.

**Markers of prophylactic platelet transfusion**

Platelet transfusion was associated both with platelet count with OR=0.95 (95% CI 0.92-0.98) per 1x10^9/L increase up to 50 x10^9/L, but also TRAP-induced platelet aggregation, OR=0.96 (95% CI 0.93-0.99) per % increase; collagen-related peptide-induced platelet aggregation, OR=0.97 (95% CI 0.94-0.99) per % increase and the percentage P-selectin-positive platelets after TRAP stimulation OR=0.92 (95% CI 0.88-0.97) per % increase predicted whether prophylactic platelet transfusion was given within the following 7 days.
We found no difference in platelet aggregation when comparing results from patients with relapsed AML versus newly diagnosed AML, e.g. median platelet aggregation 35% (IQR 23-54) versus 42% (IQR 30-50) with TRAP or when comparing AML related to transformation from other myeloid cancers or not, median platelet aggregation 40% (IQR 33-47) versus 42% (IQR 28-55) with TRAP. Further, there was no correlation between platelet aggregation and the percentages of blasts in their most recent bone marrow examinations, $r=-0.06$, $p=0.65$ and $r=-0.16$, $p=0.21$ for TRAP- and collagen-related peptide induced platelet aggregation. Finally, we found no difference in platelet aggregation among different AML Fab-types. In total, 33 patients had at least two sets of blood collections one week apart. Regardless of bleeding, the platelet aggregation (Figure 4) and platelet activation results (Supplemental Table 2) did not significantly change during the first seven days of follow-up. On the individual level, some experienced a change in platelet aggregation during the week (Figure 4), but the change in platelet aggregation did not correlate with the number of platelet concentrates administered within the intermediate week ($r=-0.2$, $p=0.25$). Further, the change in platelet count was not correlated with the change in platelet aggregation, with the exception of TRAP-induced platelet aggregation among patients who did not receive platelet transfusion ($r=0.59$, $p=0.03$; Figure 4b).

**Discussion**

Our study shows that reduced *in vitro* platelet aggregation and platelet activation predict clinical relevant bleeding episodes among patients with AML with thrombocytopenia independently of platelet count. In samples taken one week apart, platelet transfusion in the intermediate period did not significantly enhance *in vitro* platelet function.
There is a need for new predictive markers of bleeding risk in AML patients with thrombocytopenia because bleeding appears frequently\textsuperscript{1,2}. Moreover, the platelet count is the main trigger for prophylactic platelet transfusions, and does per se not adequately predict bleeding since a significant percentage of bleeding episodes occur at relatively high platelet counts\textsuperscript{3,5}. In the present prospective study, we found independent predictors of bleeding within the following 7 days; comprising low platelet aggregation response and reduced platelet activation capacity measured using the percentage of activated GPIIb/IIIa and P-selectin positive platelets after TRAP stimulation. As demonstrated with ROC curves, good prediction of bleeding was obtained when concomitant testing of platelet count and platelet function was conducted. It was particularly prominent in the subgroup in which prophylactic platelet transfusions were not administered. This subgroup include patients where bleeding was not suspected based on current bleeding risk assessment procedures. Nonetheless, bleeding was observed in 50\% - emphasizing the need for better markers. For ethical reasons, platelet transfusion could not be withheld in patients with severe thrombocytopenia as they comprise a high bleeding risk population\textsuperscript{18}. We showed that platelet function markers, (platelet aggregation after TRAP or collagen-related peptide or the percentage of P-selectin positive platelets after TRAP stimulation) were associated with administration of prophylactic platelet transfusions during the next week. This finding indicates that platelet function markers also identify this high risk group. Hence, the platelet function results were useful for bleeding risk assessment with C-index of up to 0.91 for models including platelet aggregation results. In comparison the widely used “HAS-BLED” score which is established for prediction of bleeding in atrial fibrillation has a C-index on 0.65-0.69 for clinically relevant bleeding among patients receiving anticoagulation treatment\textsuperscript{19,20}.

Patients with infection might experience even higher bleeding risk, which also is the rationale behind a higher platelet count trigger for platelet transfusions in this situation. When evaluating the
subsample where infection is present during follow-up, the platelet markers remained associated with bleeding. However, when determining the association between the platelet function markers and WHO grade \( \geq 2 \) bleeds, all platelet function markers were independently associated with bleeding in the overall dataset if WHO grade 1 bleeds were excluded from analyses, while none of the platelet function markers were associated with bleeding if WHO grade 1 bleeding was defined and grouped with “no bleeding”. One interpretation could be that impaired platelet function determines whether bleeding occur, but do not determine the size of lesion and therefore not determine the extent of bleeding.

In vivo, platelets become activated upon endothelial damage. When activated, platelets release their granules and the GPIIb/IIIa receptor changes conformation to an activated state \(^7,8\). It leads to platelet aggregation when the activated GPIIb/IIIa receptor binds ligands in plasma, thereby linking platelets \(^7,8\). Thus, the low platelet aggregation response in AML patients might be mechanistically linked to the observed reduced activation capacity of the GPIIb/IIIa receptor. While it indicates that patients with AML have a platelet signaling defect, the cause remains to be determined.

One study of 20 patients with AML reported that a high expression level of activated GPIIb/IIIa and P-selectin on platelets in samples with either no agonist or TRAP were associated with a positive bleeding history \(^9\). In contrast, others found that reduced platelet function defined using TRAP-induced platelet expression levels of P-selectin and CD63 was associated with bleeding at time of diagnosis of AML (n=50), while platelet activation markers did not predict bleeding within 28 days in multivariate analysis \(^13\). In the latter study, the long follow-up time on 28 days might explain the discrepancy with the present study where the follow up time was seven days. Further, and in contrast to our study, the patient population was not limited to patients with low platelet counts in the study of Leinoe EB et al \(^13\).
The important notion that protease activated receptor-1 (PAR-1) is relevant for bleeding risk in thrombocytopenia is not limited to patients with AML. Thus, reduced TRAP-induced platelet activation (P-selectin- and activated GPIIb-IIIa–positive platelets) has been associated with bleeding phenotype in a study among patients with immune thrombocytopenia 21.

In a previous study, we used the flow cytometry based platelet aggregation method to show that reduced platelet aggregation was associated with past bleeding episodes among thrombocytopenic patients with AML or myelodysplastic syndrome 11. It shall be noted that the concept of this method differs from other existing aggregation methods. It is based on identification of differently dyed platelets that form double-positive events when aggregating, while classic LTA measures a decrease in turbidity during aggregate formation. Further, the platelet number in the sample is fixed and the results are thus independent of the patient platelet count in the present approach 22. It makes this flow cytometric approach ideal for measurement of platelet aggregation among patients with thrombocytopenia.

Theoretically, the handling of platelets when performing the test could lead to altered platelet function. Nonetheless, we have previously found a low inter-individual variation in results among healthy persons which indicates that handling does not introduce random errors 11. Further, the CV% for repeated preparations from the same blood sample was 1% for TRAP and 3% for collagen-related peptide which emphasizes the robustness of the method and points to the test being very specific for platelet function 11. The good test performance is an important prerequisite for its use in clinical decision making 11.

In the present study, we used the lowest agonist concentration that resulted in maximal platelet aggregation in the recent validation study 11, however, the agonist concentration is higher than normally used in LTA. It could be related to the methodology, and it is not explained by the
handling procedure as results from tests with isolated platelets agreed with results from platelet-rich plasma samples with normal platelet counts as typically used for LTA studies. Importantly, when evaluating the test performance by assessment of the effect of antiplatelet drugs on platelet aggregation in spiked samples from healthy individuals, dose-response curves agree well with results from LTA, which emphasizes that the test’s results specifically reflects the platelet function.

There have been speculations whether patient platelet function is stable over time. In particular, it is currently unknown to which extent platelet transfusions alter the overall platelet function in vivo. The in vitro platelet function deteriorates over time in stored platelet concentrates, and it may be speculated that administration of platelets with poor function do not sufficiently protect against bleeding. In the present study, blood samples were drawn one week apart in 33 patient cases, and we showed that the overall platelet aggregation and platelet activation capacity was relatively stable over time. Moreover there was, to a lesser extent, a relationship between the change in platelet count and the change in platelet aggregation. It suggests that regeneration of thrombopoiesis leading to increase in platelet count do not fully restore the platelet function within the observation time. It supports that evaluation of platelet function adds independent important information. While individual differences were observed, the change in platelet count and function were not significantly different in patient who received platelet transfusions. Further, no correlation between changes in platelet aggregation and numbers of platelet transfusions administered was observed. It does, however, not exclude a short passing effect on in vivo platelet function. The finding is consistent with a recent study which showed that platelet function in children with ITP is consistent over time. It implies the advantage that frequent testing of platelet function might not be required if platelet function tests are to be used for decision making in clinical practice.
Although a limitation to the present study is that it is not powered to do stratified analysis, the strong associations between \textit{in vitro} platelet function and bleeding in patients who did not receive platelet transfusions raises the possibility that assessment of platelet function might be clinical useful for bleeding risk stratification. Future studies are needed to reveal the potential of platelet function tests as triggers for prophylactic platelet transfusions.

Conclusion

\textit{In vitro} platelet aggregation and platelet activation predicted in vivo bleeding within seven days in patients with AML and thrombocytopenia – particularly, among patients who do not meet the platelet count trigger for prophylactic platelet transfusion. Platelet transfusion did not lead to changes in the \textit{in vitro} platelet function.

Authorship

PJV, CN, HF conceived the studies, analysed and interpreted the data, and wrote the manuscript. CN, SS and GHK performed the research and contributed to the manuscript.

The authors state that they have no conflict of interest and declare no competing financial interests.

References


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Figure legend

**Figure 1** Description of the platelet aggregation assay and illustration of platelet aggregation in patients with acute myeloid leukaemia and platelet count < 50 x10^9/L

**A)** The figure shows the steps when performing the flow cytometric platelet aggregation assay, independent of patient platelet count.

**B)** Representative results from patients with acute myeloid leukaemia and platelet count <50 x10^9/L. The top panel is an unstimulated sample, while middle and lower panel depict results from samples stimulated with thrombin-receptor stimulating peptide from two different patients. Left panel of plots reflect forward and side scatter of light, and right panel figures are quadrant plots illustrating platelet expression of the fluorescent dye Calcein-AM (Q4) and Calcein AM Violet 450 (Q1). Data from unstimulated sample (upper panel) show that two differently labeled fractions of platelets are mixed. In the middle and lower panel, double positive events (Q2) reflect aggregated platelets. Aggregated platelets are shown in blue.

**C)** Boxplots illustrating flow cytometry-based platelet aggregation with thrombin-receptor stimulating peptide (TRAP), collagen-related peptide or unstimulated samples from patients who do or do not bleed within 7 days follow-up, n= 60 participants followed for 114 weeks. Horizontal lines reflect median, boxes are interquartile range and whiskers are 2.5-97.5th percentiles. Circles are single outlying measurements. Groups comprise weeks with bleeding (n=52) and without bleeding (n=62) and were compared with Wilcoxon rank sum test, p<0.05 was considered significant.
Figure 2 Odds ratios for bleeding within one week among patients with acute myeloid leukaemia and platelet count <50 x10⁹/L

Black square represent odds ratios and bars are 95% confidence intervals for bleeding within one week in the overall group (n=114 weeks). A) Odds ratios (ORs) based on univariate logistic regression. ORs for markers that are significantly associated with bleeding and ORs for bleeding for fixed factors used in multivariate the models are shown. B) Results from multivariate logistic regression for bleeding in the overall group adjusted for platelet count, sex, age and bleeding history. CRP-XL: collagen-related peptide.

Figure 3 ROC curves of prediction models for bleeding

Models are based on multivariate logistic regression, adjusted for sex, age, bleeding history (within the past one week) and platelet count. The upper panels are models including data for the overall group (n=114 weeks) and the lower panel reflect models for data in the “no platelet transfusion” group comprising weeks without platelet transfusion or weeks where bleeds occurred before platelet transfusion during follow-up (n=43 weeks). Results are C-indices. We tested equality of ROC by comparing each model to the model without platelet function markers (left graph) and p<0.05 was considered statistically significant and marked with *.

Figure 4 Time course of platelet aggregation among patients with acute myeloid leukaemia and platelet count <50 x10⁹/L

(A) Platelet aggregation at time of inclusion (day 0) and after 7 (n=33) and 14 days (n=14), respectively. Graphs show measurements in patients with acute myeloid leukaemia. Measurements
are depicted with dots and for each patient connected with lines for illustration of time course on the
individual level. Measurements are made with seven days interval.

No statistically significant change from baseline was found in between group comparisons at day 7
and day 14; evaluated with ranksum test. P-value <0.05 was considered significant.

(B) Scatter plots; Each dot or triangle reflect the corresponding change in platelet count and the
change in platelet aggregation from day 0 to day 7. Grey tringles reflect weeks with platelet
transfusions administered within the day 0-7 interval, while black circles are weeks without platelet
transfusion.

Table 1 Descriptive characteristics of patients with acute myeloid leukaemia and platelet
count <50 x10^9/L with 114 weeks follow-up for bleeding episodes

Table 2 Platelet characteristics and platelet activation results among patients with acute
myeloid leukaemia and platelet count <50 x10^9/L with 114 weeks follow-up for bleeding
episodes.

Supplemental Table 1 Platelet characteristics and platelet activation results expressed as
mean fluorescence intensities among patients with acute myeloid leukaemia and platelet count
<50 x10^9/L with 114 weeks follow-up for bleeding episodes

Supplemental Table 2 Evaluation of the time course of platelet activation results

Platelet activation was compared using Wilcoxon signed rank test. No statistically significant
change was found with p-value <0.05 considered significant.
Table 1

<table>
<thead>
<tr>
<th>Clinical information</th>
<th>Weeks with bleeding episodes (n=52)</th>
<th>Weeks with no bleeding episodes (n=62)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, y (IQR)</td>
<td>70 (55-73)</td>
<td>70 (59-75)</td>
<td>0.30</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>25 (48)</td>
<td>39 (63)</td>
<td>0.08</td>
</tr>
<tr>
<td>Infection within the previous 7 days, n (%)</td>
<td>45 (88)</td>
<td>50 (81)</td>
<td>0.20</td>
</tr>
<tr>
<td>Platelet transfusion within the previous 7 days, n(%)</td>
<td>42 (44)</td>
<td>53 (56)</td>
<td>0.62</td>
</tr>
<tr>
<td>Concurrent chemotherapy, n (%)</td>
<td>17 (27)</td>
<td>19 (37)</td>
<td>0.20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Laboratory results</th>
<th>Reference range</th>
<th>Weeks with bleeding episodes (n=52)</th>
<th>Weeks with no bleeding episodes (n=62)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count (x10^9/L), median (IQR)</td>
<td>F: 145-350 M: 165-400</td>
<td>19 (10-26)</td>
<td>23 (15-35)</td>
<td>0.05</td>
</tr>
<tr>
<td>Leukocyte count (x10^9/L), median (IQR)</td>
<td>3.5-8.8</td>
<td>0.49 (0.13-1.4)</td>
<td>0.55 (0.22-0.86)</td>
<td>0.98</td>
</tr>
<tr>
<td>Hemoglobin (mmol/L), mean (SD)</td>
<td>F: 7.3-9.5 M: 8.3-10.5</td>
<td>5.3 (0.81)</td>
<td>5.3 (0.60)</td>
<td>0.51</td>
</tr>
<tr>
<td>Immature platelet fraction, median (IQR)</td>
<td>2-4</td>
<td>3 (1-7)</td>
<td>2 (1-5)</td>
<td>0.17</td>
</tr>
<tr>
<td>APTT (seconds), mean (SD)</td>
<td>27-40</td>
<td>37 (6.4)</td>
<td>37 (5.0)</td>
<td>0.35</td>
</tr>
<tr>
<td>INR, mean (SD)</td>
<td>0.9-1.1</td>
<td>1.1 (0.15)</td>
<td>1.0 (0.15)</td>
<td>0.22</td>
</tr>
<tr>
<td>Fibrinogen activity (µmol/L), median (IQR)</td>
<td>5.5-11.5</td>
<td>14.9 (12.4-17.0)</td>
<td>14.3 (11.9-17.5)</td>
<td>0.83</td>
</tr>
</tbody>
</table>

IQR, interquartile range; SD, standard deviation; APTT, activated partial thromboplastin time; INR, international normalized ratio. Data represent characteristics for all follow-up periods for the 60 participants and shows differences in baseline characteristics before follow-up divided based on whether bleeding occurred within the seven days follow-up period.
<table>
<thead>
<tr>
<th>Marker</th>
<th>Weeks with bleeding episodes (n=52)</th>
<th>Weeks without bleeding episodes (n=62)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FSC (MFI)</strong></td>
<td>2.8 (2.5-3.1)</td>
<td>2.7 (2.5-3.0)</td>
<td>0.52</td>
</tr>
<tr>
<td><strong>No agonist</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activated GPIIb-IIIa positive platelets (%)</td>
<td>2.8 (2.0-6.1)</td>
<td>3.6 (2.2-7.3)</td>
<td>0.25</td>
</tr>
<tr>
<td>CD63 positive platelets (%)</td>
<td>1.9 (1.1-2.6)</td>
<td>1.6 (1.1-2.2)</td>
<td>0.20</td>
</tr>
<tr>
<td>P-selectin positive platelets (%)</td>
<td>22.5 (16.1-29.3)</td>
<td>22.1 (15.2-31.2)</td>
<td>0.85</td>
</tr>
<tr>
<td><strong>Thrombin receptor-activating peptide 10 µM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activated GPIIb-IIIa positive platelets (%)</td>
<td>51.8 (30.3-66.5)</td>
<td>60.3 (47.0-76.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD63 positive platelets (%)</td>
<td>18.5 (10.5-26.7)</td>
<td>20.8 (12.7-28.8)</td>
<td>0.30</td>
</tr>
<tr>
<td>P-selectin positive platelets (%)</td>
<td>83.8 (73.8-88.9)</td>
<td>89.2 (79.7-93.1)</td>
<td>0.004</td>
</tr>
<tr>
<td><strong>Collagen-related peptide, 2.2 µg/L</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activated GPIIb-IIIa positive platelets (%)</td>
<td>48.4 (31.2-60.9)</td>
<td>53.5 (35.4-70.6)</td>
<td>0.14</td>
</tr>
<tr>
<td>CD63 positive platelets (%)</td>
<td>13.9 (6.4-25.4)</td>
<td>15.6 (8.1-23.0)</td>
<td>0.85</td>
</tr>
<tr>
<td>P-selectin positive platelets (%)</td>
<td>70.0 (58.7-80.6)</td>
<td>74.2 (59.9-81.7)</td>
<td>0.43</td>
</tr>
</tbody>
</table>

GP, glycoprotein; FSC, forward light scatter. The results are percent platelets positive for the markers. The results are presented as medians and interquartile ranges, and P-values are based on the Wilcoxon rank-sum test. P<0.005 was considered significant. Data represent differences in platelet activation measured in blood samples undertaken before follow-up and divided in two groups based on whether bleeding occurred within the seven days follow-up period. Data show all seven days follow-up periods.
Figure 1

A

- Blood tube
- Isolated platelets
- Agonist
- Shaken 1000 rpm
- 37°C
- Final sample

B

- Unstimulated
- Patient with AML and bleeding
- Patient with AML and no bleeding

- Platelet aggregation (%): 
  \[ 100 \times \frac{Q2}{Q1+Q2} = 0\% \]

- TRAP-induced platelet aggregation = 10%

- TRAP-induced platelet aggregation = 62%

C

- TRAP
- Collagen-related peptide
- Unstimulated

- Platelet aggregation (%)
- Weeks with: Bleeding n=52, No bleeding n=62
- Bleeding n=52, No bleeding n=62
- Bleeding n=52, No bleeding n=62
- Bleeding n=52, No bleeding n=62

- p=0.005
- p=0.005
- p=0.48
Figure 2

A
CRP-XL-induced platelet aggregation <40%
TRAP-induced platelet aggregation <38%
Activated GPIb/IIa, % positive platelets, TRAP *
P-selectin, % positive platelets, TRAP *
Platelet count, per 10x10^9/L increase
Positive bleeding history
Age, per 1 year increase
Male sex
* per 10% increase

B
CRP-XL-induced platelet aggregation < 40%
TRAP-induced platelet aggregation with <38%
Activated GPIb/IIa, % positive platelets, TRAP *
P-selectin, % positive platelets, TRAP *
Platelet count, per 10x10^9/L increase
* per 10% increase

Odds ratio for bleeding (univariate analysis)

Odds ratio for bleeding (multivariate analysis)
Figure 4

A

Collagen-related peptide-induced platelet aggregation (%)

Visit number

Day 0  Day 7  Day 14

B

Change in collagen-related peptide-induced platelet aggregation (%)

Change in platelet count (day 7 - day 0)

-40  -20  0  20  40  60  80

-40  -20  0  20  40  60

Change in TRAP-induced platelet aggregation (%)

Change in platelet count (day 7 - day 0)

-40  -20  0  20  40  60

-40  -20  0  20  40  60

Platelet transfusion given
No platelet transfusion given

Spontaneous platelet aggregation (%)

Visit number

Day 0  Day 7  Day 14

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