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Measurement of platelet aggregation, independent of patient platelet count: A flow-cytometric approach

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Essentials

- Platelet function may influence bleeding risk in thrombocytopenia, but useful tests are needed.
- A flow cytometric platelet aggregation test independent of the patient platelet count was made.
- Platelet aggregation was reduced in thrombocytopenic patients with haematological cancer.
- High platelet aggregation ruled out bleeding tendency in thrombocytopenic patients.

Summary

Background: Methods for testing platelet aggregation in thrombocytopenia are missing. **Objective:** To establish a flow cytometric test of *in vitro* platelet aggregation independent of the patient platelet count and examined the association of aggregation with bleeding history in thrombocytopenic patients. **Patients/Methods:** We established a flow cytometric assay of platelet aggregation and measured samples from healthy individuals pre-incubated with antiplatelet drugs and samples from two patients with inherited platelet disorders. Then, we included 19 healthy individuals and 20 patients with platelet counts ≤50 x10^9/L, diagnosed with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). We measured platelet aggregation, and platelet activation by platelet surface expression of activated glycoprotein IIb/IIIa, P-selectin, and CD63 after addition of agonists: Collagen-related peptide, thrombin receptor-activating peptide(TRAP) and adenosine disphosphate(ADP). **Results:** The platelet aggregation assay exhibited a low intra-serial coefficient of variation ≤3%. Similar results were obtained for platelet-rich plasma and isolated platelets at platelet counts >10 x10^9/L, otherwise platelet isolation was required. Platelet aggregation percentage decreased with increasing antiplatelet drug concentration. Platelet aggregation in patients was reduced compared with healthy individuals: 42%(interquartile range (IQR) 27-58) versus 66%(IQR 60-67) for TRAP; 41%(IQR 25-48) versus 70%(IQR 69-72) for collagen-related peptide; and 44%(IQR 30-53) versus 65%(IQR 46-72) for ADP. Platelet activation after stimulation was reduced in patients and correlated with platelet aggregation, e.g.r=0.78-0.81when stimulated with collagen-related peptide). Platelet aggregation had a negative predictive value of 100% for bleeding tendency among patients. **Conclusion:** The established platelet aggregation assay was useful for thrombocytopenic patients, and improved the identification of bleeding risk.

Introduction

Platelets are pivotal in primary hemostasis, and bleeding risk is a concern in thrombocytopenic individuals.[1] Before the availability of platelet transfusions, bleeding was a contributing factor in 67% of deaths among patients with leukemia.[2] Unfortunately, clinically relevant bleeding still occurs on 17-25% of days in hospitalized cancer patients with platelet counts below $80x10^9$/L.[3] Despite equally low platelet count, not all patients with thrombocytopenia experience bleeding and the reason for this remains unknown.

Impaired platelet function may contribute to the increased bleeding risk in thrombocytopenic patients. However, the topic is poorly understood because most platelet function tests exhibit methodological limitations at low platelet counts.[4] For example, results from light transmission aggregometry (LTA), the gold standard for measuring platelet function, is recommended for platelet counts above 150 $x10^9$/L.[5] In contrast, flow cytometry is suited to investigate platelet function in thrombocytopenia because it measures the characteristics of each platelet.[4] Recently, flow cytometry has been applied to quantify platelet aggregation based on principles adapted from assays of leukocyte aggregates. [6] Flow cytometry has been sparsely applied to study platelet function in patients with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS) [7-9], and has never been used to measure platelet aggregation in these patients. We hypothesize that independently of the patient platelet count, platelet aggregation in patients with AML or MDS is reduced compared to healthy individuals. Further, we hypothesize that in patients with AML or MDS, bleeding tendency is associated with reduced platelet aggregation.

In this paper we present a flow-cytometric platelet aggregation assay. The assay was applied to measure platelet aggregation in patients with AML and MDS, and we determined the association with the patients' bleeding tendency.

Methods

Study design and participants

For flow-cytometric platelet aggregation assay, we used blood from healthy blood donors ≥18 years. They were allowed to take over-the-counter drugs up to 24 hours before sampling. Plasma from one male AB RhD^+ donor was used as a matrix in all flow-cytometric platelet aggregation experiments. Patients were ≥18 years, diagnosed with AML or MDS in accordance with current guidelines [10]. Patients had a platelet count of $\leq 50 \times 10^9$/L at the time of sampling. Exclusion criteria were treatment within 14 days with drugs that affect platelet function or platelet transfusion within 7 days, or exposure to procedures or interventions such as central venous catheter insertion or chemotherapy on the day of sampling. Patients were questioned (by PJV) on the occurrence of spontaneous bleeding within the month prior to study inclusion. Bleeding was graded by use of the bleeding assessment tool of the International Society on Thrombosis and Haemostasis[11], clinically relevant bleeding corresponded to a score of ≥1 and was registered as absent or present. Further, we included two patients with known inherited platelet disorders and thrombocytopenia.

The study was approved by the Regional Scientific Ethical Committees of Southern Denmark (s-20150161) and the Danish Data Protection Agency (2008-58-0035). Participants gave written informed consent, and the study was conducted in accordance with the guidelines of the 2013 Declaration of Helsinki.

Blood samples

Blood was drawn from a peripheral vein using a 21-gauge needle into BD Vacutainer tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA) containing either 3.2% (0.109M) trisodium citrate for platelet function measurements or EDTA for haematology parameters on Sysmex XN-9000 (Sysmex, Kobe, Japan).

Flow cytometry platelet aggregation assay

The final protocol

Platelet-rich plasma was obtained by centrifugation of citrate-anticoagulated blood (Fig 1) for 10 minutes at 200 *g,* and transferred into Nunc™ Cryotubes (Thermofischer Scientific, Denmark). To prevent platelet activation during the next centrifugation, we added acid-citrate-dextrose solution (10% volume/volume) and prostaglandin E1 (Alprostadil 0.9 µmol/L, Tocris, Bristol, United Kingdom). Then the sample was centrifuged for 10 minutes at 1000 *g* during which platelets were pelleted. The platelet pellet was washed and resuspended in dilution buffer (NaCl 134 mM, KCL 2.9 mM, $MgCl_2$ 1 mM, glucose 5.6 mM, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 20 mM, pH 7.4). The resting state of platelets was confirmed by flow cytometry following P-selectin antibody labeling (eBioscience, San Diego, CA, USA).

From isolated platelets, two fractions with platelet counts of $144x10^9$/L and $16x10^9$/L were made by adjustment with dilution buffer and labeled with Calcein acetoxymethyl ester (AM) Ultrapuregrade (CAMU) and Calcein AM Violet 450 (CV450), respectively (both from eBioscience, San Diego, CA, USA). CAMU and CV450 stock was prepared according to manufacturer instructions and diluted with PBS to a working stock concentration of 1 µM for CAMU and 21 µM for CV450. From each of these solutions 5 µL was added per 100 µL platelet suspension. Fractions were incubated in the dark for 15 minutes at 37°C.

Donor plasma was prepared by double centrifugation of citrate-anticoagulated blood at 1000 *g* for 10 minutes with disposal of the sediment after each centrifugation cycle, and stored at -80°C.

For the platelet aggregation test, 70 µL of thawed donor plasma and 35 µL of each of the CV450 or CAMU labeled platelet fractions were mixed in 1.5 mL Safe-Lock tubes (Eppendorf, Hamburg, Germany) (hereon designated the "platelet mix"). Then 3.5 µL of agonist was added and to a final concentrations of 2.2 µg/mL collagen-related peptide (, Dr. Richard W. Farndale, University of Cambridge, United Kingdom), 244 µM adenosine diphosphate (ADP; Sigma-Aldrich, St. Louis, Missouri, USA) or 244 µM thrombin receptor-activating peptide (TRAP; SFLLRN; JPT Peptide Technologies GmbH, Berlin, Germany). An unstimulated platelet mix was analyzed in parallel and included all components except agonist. To induce aggregation, tubes were shaken at 1000 rpm for 5 minutes in an Eppendorf Thermomixer® comfort (Eppendorf, Hamburg, Germany) and thereafter 25 µL was transferred into 225 µL fixation buffer (0.2% formaldehyde in phosphate-buffered saline). Samples were acquired using a FACSCanto II flow cytometer without compensation, and data analyzed with FACS Diva software (Becton Dickinson, Franklin Lakes, New Jersey, USA). Dot plot quadrants (Q1-Q4) were set using the unstimulated platelet mix (Fig 2, left). Platelet aggregation was reported as the percentage of Calcein-AM double-positive events (positive for both of CV450 and CAMU) out of all CV450 positive events. The mean forward scatter of double-positive events was used as approximation of the average platelet aggregate size.

Optimization of the protocol

The effect of platelet count on platelet aggregation was investigated using serial dilutions of donor platelets while keeping a fixed ratio between the two differently labeled platelet fractions. The optimum shaking time was found by transferring the platelet mix into fixation buffer at different time points. The effect of plasma was determined by systematically varying the fraction of plasma in the final mix. Dose-response curves for agonists were obtained. The intermediary precision was determined by analysis of separately prepared samples obtained from two individuals, six samples per individual. As supplement, we tested arachidonic acid (Sigma-Aldrich, St. Louis, Missouri, USA), ristocetin (Roche Diagnostics, Mannheim, Germany) and protease-activated receptor 4 (AYPGKF-NH_2; Bachem, Bubendorph, Schwitzerland) as agonists. We also evaluated the effect of replacing one platelet fraction in the mix with platelets from a healthy donor, n=4. The effect on platelet aggregation of inhibitors in varying concentrations, Abciximab 0.1-40 µg/mL, Ticagrelor 0.006-20 µM, both from Selleckchem (Houston, Texas, USA) or Vorapaxar 3.13-500 nM (SCH530348, Axon Medchem, Groningen, Netherlands) was evaluated. Samples were preincubated with the drug for 0.5 hour (Abxicimab) or 1 hour (Ticagrelor and Vorapaxar) at 37°C. In all experiments, generic reagents were from Sigma-Aldrich. Sizing beads were Megamix from Biocytex (Marseille, France).

Flow cytometric platelet aggregation in whole blood and platelet-rich plasma

After modifying the protocol, we applied it to donor samples of whole blood or platelet-rich plasma, n=6. One undiluted fraction of the sample was labeled with CAMU while another was diluted 1:10 volume/volume with dilution-buffer and labeled with CV450. Seventy µL dilution-buffer instead of donor plasma was added to the mix.

Light transmission aggregometry

LTA using 96-well aggregometry was performed as previously described [12]. Platelet aggregation of Calcein-AM labeled versus unlabeled platelets was compared, n=6 for each agonist.

Platelet reactivity and platelet surface receptor expression

For testing platelet reactivity *in vitro*, 5 µL aliquots of whole blood were incubated for 10 minutes at room temperature in dilution buffer with 1g/L bovine serum albumin and fluorophore-conjugated monoclonal antibodies (mAbs): phycoerythrin (PE)-conjugated GPIb (CD42b, clone HIP1, dilution factor in final sample (DF) 78), allophycocyanin (APC)-conjugated P-selectin (CD62p, clone Psel.KO2.3, DF 325) (both eBioscience, San Diego, CA, USA), and phycoerythrin-cyanine 7 (PE-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 dilution buffer was added. Agonists were ADP 12.8 µM, TRAP 10 µM or collagen-related peptide 1.1 µg/mL to a final sample volume on 65 µL. A negative sample was incubated with mAbs against CD63, CD42b and PAC1, and with an anti-P-selectin matched APC-conjugated isotype control (eBioscience, San Diego, CA, USA). EDTA (10 mM) was added to inhibit specific binding of PAC1 and CD63 mAbs. [13, 14]

In a separate protocol we evaluated expression of platelet surface receptors as described [13]. Briefly, we used mAbs for PE-Cy7-conjugated GPIIb (CD41a; clone HIP8, DF 325), eFluor450-conjugated GPIX (CD42a; clone GR-P, DF 78), PE-conjugated GPIb, APC-conjugated GPIa (CD49b; clone P1H5, DF 39) and FITC-conjugated GPIIIa (CD61; clone VI-PL2, DF 130) or matching isotype controls, all from eBioscience (San Diego, CA, USA).

In both protocols, incubation was stopped by the addition of fixation buffer. Samples were acquired on the FACSCanto II and processed using Kaluza software 1.3 (Beckman Coulter, California, USA). Platelets were gated based on GPIb expression and scatter pattern. The results were expressed as mean fluorescence intensities (MFI) or, in the platelet reactivity assay, as the percentage of platelets positive for P-selectin, CD63 and/or PAC1 when compared to the negative sample.

Statistics

Categorical variables are reported as frequencies and percentages. The distribution of data for continuous variables was evaluated by the Shapiro-Wilk test of normality and/or inspection of histograms. For continuous, normally distributed data, means and standard deviations (SD) are reported; otherwise, medians and interquartile ranges (IQR) are reported. For evaluation of protocol settings and effects of antiplatelet drugs, the paired Student's t-test was used. Group comparisons of non-normally distributed data were carried out with the Mann-Whitney U test (unpaired results) or the Wilcoxon signed-rank test (paired results). Correlations were tested non-parametrically and reported as Spearman's rho. Missing values were omitted (two patients did not have platelet activation markers). Statistical procedures were performed using STATA 14.0 (StatCorp, College Station, Texas, USA) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). P-values were two-sided.

Results

The final protocol

A representative plot of platelet aggregation is shown in Fig 2. CAMU-CV450 double-positive events were present in all stimulated platelet mixes. These events were on average larger (higher forward scatter) and more granular (higher side scatter) than single-positive

events. Platelet surface expression of GPIb and P-selectin confirmed that all double-positive events consisted of activated platelets. Megamix bead comparisons confirmed the size of single-positive events to be within the size range of normal platelets. Thus, double-positive events were taken to represent platelet aggregates. Calcein-AM labeling did not reduce platelet aggregation as measured by LTA; collagen-related peptide-induced platelet aggregation was 83% for CAMU-labeled platelets and 79% for unlabeled platelets, p=0.18, n=6.

Optimization of the protocol

Flow cytometric platelet aggregation declined at platelet counts $<15x10^9$/L and was significantly reduced at platelet counts $\leq 5x10^9$/L (Fig 3 A). Platelet aggregation results depended on the shaking time (Fig 3 B), the plasma fraction (Fig 3 C) and agonist concentration (Fig 3 D), n=3. In the final protocol, we used a plasma fraction of 0.50, 5 minutes of shaking and a platelet count of $40x10^9$/L in the final mix (equivalent to 5.6 million platelets per test). It corresponded to approximately one blood tube (4.5 mL) per test at platelet counts on $5x10^9$/L. In the final assay, we used the lowest agonist concentration that resulted in maximal platelet aggregation. However, to ensure that the maximum aggregation for ADP was reached, we chose a higher agonist concentration because the variation in platelet aggregation results among healthy donors was higher than observed for the other agonists. The CV% for repeated preparations from blood drawn concomitantly from the same individual was 1% for TRAP, 2% for ADP and 3% for collagen-related peptide. For platelet mixes, the mean relative change in platelet aggregation over 90 minutes was 0-2%. The agonists arachidonic acid, ristocetin and AYPGKF-NH_2 were suitable for the assay with aggregation responses above 50%.

Mixing patient platelets (CAV450 labeled, 1 part) with donor platelets (CAMU labeled, 9 parts) yielded an average absolute increase in aggregation on -7 - 12% for ADP, 10-20% for collagen-related peptide and 3-12% for TRAP compared to platelet mix with patient platelets only.

The platelet aggregation assay is sensitive to the effects of antiplatelet drugs

Abciximab and Ticagrelor inhibited TRAP- and ADP-induced platelet aggregation. Abciximiab also inhibited collagen-related peptide-induced platelet aggregation (Fig 4). At low Abciximab concentrations, ADP-induced platelet aggregation appeared slightly increased with increasing Abciximab concentration. Vorapaxar selectively inhibited TRAP-induced platelet aggregation. Platelet aggregate size decreased progressively with increasing antagonist concentrations (Fig 4).

Flow-cytometric platelet aggregation in patients with inherited platelet disorders

We included a male patient with genetically confirmed Wiscott-Aldrich syndrome, no bleeding tendency and a platelet count of $45x10^9$/L and a female patient with suspected MYH-9 related thrombocytopathy (dominant inheritance of macrothrombocytopenia, Döhle leukocyte inclusions), no bleeding tendency and platelet count of $54x10^9$/L. Both displayed normal flow-cytometric platelet aggregation as compared to healthy individuals (data not shown).

Flow cytometric platelet aggregation in whole blood and platelet-rich plasma

Calcein-AM labeled platelets in whole blood and platelet-rich plasma. In whole blood samples, platelets were undetectable after stimulation. The platelet aggregation in platelet-rich plasma based samples was comparable to results from mixes with isolated platelet. The

median difference in platelet aggregation percentage between platelet mix and platelet rich was -4.8 % (full range -8.5- 2.0) for TRAP; 0.8 % (full range -3.4-0.8) for ADP, and 11,0% (full range 8.0-11.6) for collagen-related peptide.

Characteristics of patients with AML or MDS and healthy individuals

Nineteen blood donors and 20 patients with AML (n=18) or MDS (n=2) were included. Among the patients with AML, 12 were newly diagnosed and eight had relapsed disease. None had acute promyelocytic leukemia. The median platelet count was 24 $x10^9$/L (IQR 14-32) for patients versus 243$x10^9$/L (IQR 206-337) for healthy individuals. There was no difference in mean platelet volume: 11.1±1.1 fL for patients versus 10.8±1.1 fL for healthy individuals, p=0.58.

Flow-cytometric platelet aggregation in patients with AML and MDS

Patients with AML or MDS, overall, exhibited reduced platelet aggregation in comparison with healthy individuals (Fig 5 A). Spontaneous platelet aggregation in unstimulated samples was low (≤4%) in the two groups. Average aggregate size after stimulation with collagen-related peptide was higher for patients than for healthy individuals: mean FSC±SD, 20,008±3499 MFI and 16,932±2746, p=0.01, respectively. No significant differences in mean platelet aggregate size were observed for the other agonists: mean±SD, TRAP 16,643±803 MFI versus 15,766±412 MFI, p=0.35; ADP 20,320±1389 MFI versus 20,934±1139 MFI, p=0.74 for patients versus healthy individuals.

Flow-cytometric platelet aggregation was associated with bleeding history

Six patients (all AML) had a history of bleeding within the month prior to inclusion: 3 had epistaxis (recurrent and lasting >10 minutes or requiring medical attention), one had

abnormal vaginal bleeding (lasting more than 7 days), one had overt gastrointestinal bleeding, and one had a large haematoma requirering medical contact. Overall, flow-cytometric platelet aggregation was lower in patients with bleeding tendency compared to patients without (Fig 5 A) and in fact, results were able to identify all patients without bleeding tendency (Fig 5 B). Platelet aggregate size was not related to bleeding tendency (data not shown).

Platelet reactivity and surface receptor expression

With regard to unstimulated samples, the median percentage of P-selectin-positive platelets was significantly higher in patients compared to healthy individuals (Table 1). Upon platelet stimulation, platelets became activated in all samples. That is the percentage of activated GPIIb/IIIa, CD63 and P-selectin increased relative to unstimulated samples (all $p<0.05$). However, the median percentages of platelets positive for activated GPIIb/IIIa, CD63 and P-selectin after stimulation were significantly lower in patients. Also, MFI-values after platelet stimulation were lower in patients than healthy individuals, except for CD63 and P-selectin expression in ADP-activated samples (Supplemental Table 1), There was no difference in platelet activation in terms of percentage of platelets positive for the marker (Fig 6) or MFI-values between patients with and without bleeding tendency(Supplemental Table 2), $p>0.05$ for all).,. The expression of GPIX (CD42a) was higher in patients than in healthy individuals, while no differences were observed in the other platelet surface receptors. For patients, there was a correlation between platelet aggregation and platelet reactivity when using the same (Fig 6).

Discussion

We established flow-cytometric assay for evaluating *in vitro* platelet aggregation in patients with thrombocytopenia. The assay demonstrated that patients with AML or MDS had decreased platelet aggregation, and platelet aggregation results identified patients with bleeding tendency.

The use of flow cytometry for measuring platelet aggregation has been proposed previously.[6, 15] The flow-cytometric assay presented here is optimized for testing in patients with thrombocytopenia. In contrast to most other available platelet aggregation methods [4], this assay exhibits a low CV% (≤3%) with repeated measurements of stimulated platelet mixes and a low inter-individual variation among healthy individuals.
For the final protocol, we mixed platelets with CAMU-labeled platelets in excess. It increased the likelihood that CV450-labeled platelets would aggregate primarily with CAMU-labeled platelets and create double-positive events. The used 1:10 (platelet count/platelet count) ratio, yielded the highest platelet aggregation percentages. As results were affected by the platelet count ratio, it needed to remain constant to ensure comparability of samples. By chance, a fraction of CV450 labeled platelets expectedly will aggregate with platelets of the same dye. The fact that the highest platelet aggregation we observed was 78%, indicates the level of (undetectable) CV450 single-positive platelet aggregates. In agreement, CV450 single-positive platelets were activated by platelet stimulation as judged by P-selectin expression.

Platelet aggregation was reduced for platelet counts <10-15x10^9/L in the final platelet mix. This finding corresponds to the clinical observation that the risk of spontaneous, clinically significant bleeding increases considerably at platelet counts ≤10 x10^9/L.[3, 16] Thus, the effect of platelet transfusions might partly be the increased platelet count facilitating platelet

aggregate formation by optimizing rheological properties independently of the function of transfused platelets. This also points to the limits in the use of whole blood or platelet-rich plasma in the flow-cytometric assay when testing samples from severely thrombocytopenic patients since platelet counts cannot be increased by concentration. Apart from this, the assay worked with platelet-rich plasma from donors and yielded results comparable to those obtained using isolated platelets. The use of platelet-rich plasma is a simpler approach and is feasible for patients with mildly reduced platelet counts. It could be clinically useful since most platelet aggregation methods are not valid in thrombocytopenic samples [4]. Other assay modifications could be of interest; patient platelet mix combined with donor plasma could be compared to a platelet mix with autologous plasma and thereby enable evaluation of the hemostatic properties of a patient's plasma. In previously published studies platelet mixes were based on a combination of patient and donor platelets [6, 17] . We found this approach to yield higher platelet aggregation percentages compared to approaches using patient platelets only. Our concern is therefore, that mild platelet function defects could be missed by this patient-donor platelet mix approach. On the other hand, it resembles the possible mechanism of transfusing donor platelets to a patient and might be an appropriate *ex vivo* model for measuring the effects of platelet transfusion.

The *in vitro* effects of platelet inhibitors and the dose-response curves of the flow-cytometric assay were similar to what have been observed using LTA. Thus, Ticagrelor inhibited ADP-induced platelet aggregation and to a lesser extent TRAP-induced platelet aggregation. This effect could be mediated through the P_2Y_{12} receptor.[18, 19] Vorapaxar selectively inhibited TRAP-induced platelet aggregation,[20] and Abciximab significantly reduced platelet aggregation by all agonists. However, at low concentrations (<0.3 mg/L), Abciximab seemed to paradoxically activate ADP-induced platelet aggregation. This finding might be due to an

intrinsic property of Abciximab for activating GP IIb/IIIa and cause fibrinogen binding. At low Abciximab concentrations, results in platelet aggregation due to access to ligand-induced binding sites on the receptor.[21, 22] This result emphasizes the high sensitivity of the present assay. Collagen-related peptide induced platelet aggregation was not fully inhibited with Abciximab. It has been shown that some agonists recruit unblocked internal GPIIb/IIIa receptors and thereby induce platelet aggregation despite presence of an inhibitor [23, 24]. The two patients with inherited platelet disorder (Wiscott-Aldrich Syndrome or MYH-9 related thrombocytopenia) and thrombocytopenia were shown to have normal platelet aggregation. This is in agreement with none of the two having a bleeding tendency and further documents the application of the flow-cytometric platelet aggregation assay in patients with thrombocytopenia.

Patients with AML or MDS had reduced platelet aggregation and lower platelet activation after stimulation. The cause is likely multifactorial and not explained by reduced expression of platelet surface receptors. Previous studies have investigated the association between platelet activation and bleeding in thrombocytopenic patients with AML/MDS [8, 9] or ITP [9, 25]. Leinoe *et al*. found lower platelet activation in AML patients than healthy individuals [8]. AML patients with bleeding the same day had lower P-selectin, CD63 and GPIb, but only P-selectin expression predicted new bleeds within 28 days [8]. Psaila *et al*. found a higher expression of GPIIb/IIIa and P-selectin on stimulated platelets in AML/MDS patients with bleeding versus no bleeding [9]. In ITP, high P-selectin and high GPIIb/IIIa expression in stimulated samples were associated with low bleeding score [9, 25]. Although results are conflicting, current evidence is in favor of the hypothesis that impaired platelet function is associated with bleeding in patients with thrombocytopenia. In addition, we showed that reduced platelet aggregation was associated with bleeding tendency, while in our study platelet activation markers were not. Thus, flow cytometric platelet aggregation could be a

stronger indicator of bleeding risk than platelet activation markers. In agreement, our study shows that for some agonists, a large change in platelet aggregation was associated with only a small change in the expression of platelet activation markers in stimulated samples. Platelet aggregation depends on multiple factors including platelet activation, membrane composition and granule contents. Therefore, it is plausible that a direct measurement of platelet aggregation reflects of platelets' haemostatic capacity. Further, platelet aggregation was superior to the platelet count for identifying a positive bleeding history and particularly useful for ruling out bleeding tendency with 100% certainty. It must be noted, however, that we only had retrospective data on bleeding, and the ability of flow-cytometric platelet aggregation to predict bleeding must be confirmed in a prospective study.

Some other limitations exist. Compared to LTA, higher agonist concentrations were needed for maximum platelet aggregation. However, the required agonist concentration was equally high for isolated platelets and platelet-rich plasma and was thus not related to the handling during platelet isolation procedure. Further, the labeling itself did not affect platelet aggregation as tested with LTA. Thus the high agonist concentration required in this assay might be related to the way aggregates are detected. The platelet aggregates detected in the flow cytometric assay might be too small to change absorbance readings in LTA and therefore would remain undetected in LTA. The results from this platelet aggregation assay might not be representative for platelet function in whole blood as large platelets are removed during centrifugation [26]. Also, results are based on a fraction of platelets in the mix. Finally, donors were allowed to take over-the-counter medicine up to 24 hours before sampling. For some donors this may have reduced platelet aggregation. If so, the presented differences between platelet aggregation in AML patients and donors are conservative estimates.

The flow cytometric platelet aggregation assay has several potential applications. It might be used for bleeding risk stratification and evaluation of efficacy of platelet transfusion. Further, it provides the possibility of identifying patients eligible for antiplatelet therapy despite being thrombocytopenic and for the evaluation of the efficacy of antiplatelet therapy. This research area is lacking evidence because thrombocytopenic patients were generally excluded from studies on antiplatelet treatment.[27, 28] Finally, the assay could facilitate the diagnostic work-up of inherited platelet disorders.

In conclusion, we established a flow-cytometric platelet aggregation assay, found that patients with AML and MDS had reduced platelet aggregation and that low platelet aggregation identified bleeding tendency.

Authorship and Conflict-of-interest statement

P. J. Vinholt, C. Nielsen, H. Frederiksen, A. M. Hvas and U. Sprogøe designed the study. P. J. Vinholt, C. Nielsen and H. Frederiksen recruited patients and conducted the experiments. P. J. Vinholt and C. Nielsen performed data management. All authors contributed to data interpretation, critical writing and final approval of the manuscript.

Disclosure

A. M. Hvas has received speaker fees from CSL Behring, Leo Pharma, Bayer Health care, Bristol-Myers Squibb and Boehringer-Ingelheim and research support from CSL Behring, Octapharma and Leo Pharma. The other authors have no conflicts of interest.

Figure Legends

Figure 1: Workflow for the flow cytometric test of platelet aggregation

Figure 2: Representative flow-cytometric platelet aggregation of stimulated and unstimulated samples from a healthy individual

The left plot represent is an unstimulated sample used for setting quadrants (Q1-Q4). Boxed plots represent results after gating on calcein-AM violet 450 positive events. The plots in the top box represent unstimulated samples while the plots in the bottom box are results from a collagen-related peptide stimulated sample. Within each box the left plots show mean fluorescence intensity of calcein Violet 450 AM- (y-axis) and calcein-AM Ultrapure grade-labeled cells (x-axis) (A & C). The right plots (B & D) are forward (FCS) and side scatter properties (SSC) of platelets. Blue events represent platelet aggregates defined as calcein-AM double positive events.

Figure 3: Effects of platelet count, shaking time and plasma concentration on platelet aggregation

The figures present results from healthy individuals, n=3 per experiment; # indicates the composition of the final platelet aggregation assay. Results are mean and standard deviations.

3a) The effect of platelet count on platelet aggregation in the sample test tube. Samples were prepared by serial dilutions of platelets in dilution buffer without altering other aspects of the protocol. * $p < 0.05$ compared results obtained using settings for the final assay.

3b) Platelet aggregation and the relationship with shaking duration at 37°C and 1000 rpm after stimulation with agonists in samples from three individuals.

3c) Relationship between the fraction of the sample comprising plasma from a healthy AB RhD+ blood donor and the aggregation response of platelets from healthy donors.

3d) relationship between agonist concentration and platelet aggregation on samples from three individuals.

ADP, adenosine diphosphate; TRAP, thrombin receptor activating peptide.

Figure 4: Effect of antiplatelet drugs on platelet aggregation in healthy individuals

ADP, adenosine diphosphate; TRAP, thrombin receptor activating peptide.

Samples from healthy individuals were pre-incubated with antiplatelet drugs (n=3). Curves and columns show mean platelet aggregation and the relationship between platelet aggregation and drug concentration. Bars represent standard deviations. * $p < 0.05$ compared with platelet aggregation with the same agonist but no drugs added, paired Student's t-test. Drugs tested were Abciximab (A), Ticagrelor (B), and Vorapaxar (C). Figure D presents platelet aggregation in ADP stimulated samples preincubated with different concentrations of Abciximab.

Figure 5: Platelet aggregation in patients with AML or MDS compared with healthy individuals

ADP, adenosine diphosphate; TRAP, thrombin receptor activating peptide; PPV, positive predictive value, NPV, negative predictive value. Triangles represent patients with a positive bleeding history within the past month.

A: Platelet aggregation in patients with AML or MDS and a platelet count $<50 \times 10^9$/L (full circles) compared with healthy individuals (open circles). Horizontal lines indicate the median platelet aggregation in each group. Groups were compared with the Mann-Whitney U-test. Patients with a history of bleeding had lower platelet aggregation than patients without bleeding; $p<0.05$, Wilcoxon signed-rank test.

B: The relationship between TRAP-induced platelet aggregation and platelet count among patients with AML or MDS. Negative predictive value and positive predictive value were calculated at cut-points of 38% for TRAP induced platelet aggregation and 10 $x10^9$/L for platelet count (dottet lines).

For other agonists, the positive predictive value for bleeding was 79% and negative predictive value 100% at a cutpoint of 40% platelet aggregation.

Figure 6: Correlation between platelet aggregation and platelet reactivity measurements in patients with AML or MDS

ADP, adenosine diphosphate; TRAP, thrombin receptor-activating peptide.

Plot show correlation between platelet aggregation results and platelet reactivity markers among patients with AML or MDS (n=18). Results of platelet reactivity is given as percentage of platelets positive for CD63, P-selectin or activated GPIIb/IIIa after agonist stimulation. Agonists used were collagen-related peptide (first row), TRAP (second row) and ADP (third row). Correlations are reported as Spearman's rho. Triangles represent patients with a positive bleeding history within the past month.

Table 1. Platelet reactivity in patients with AML or MDS versus healthy individuals

ADP, adenosine diphosphate; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; MFI, mean fluorescence intensity; TRAP, thrombin receptor activating peptide.

The results are presented as median and interquartile ranges and p-values based on the Wilcoxon rank-sum test.

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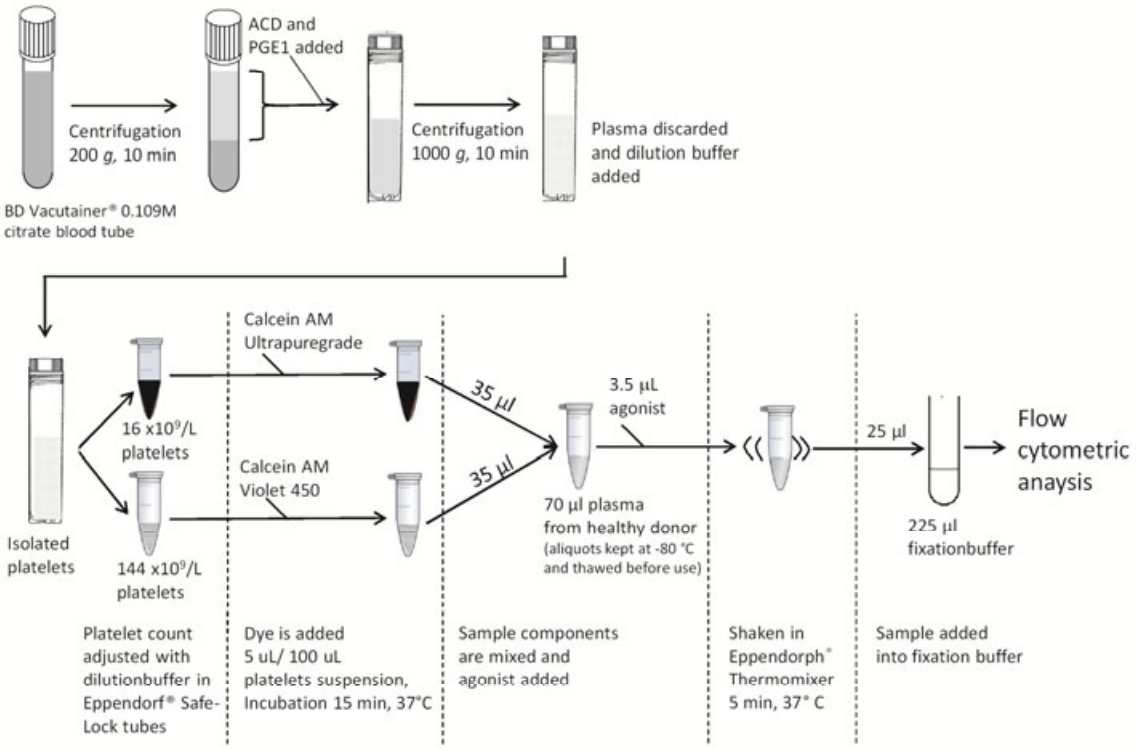
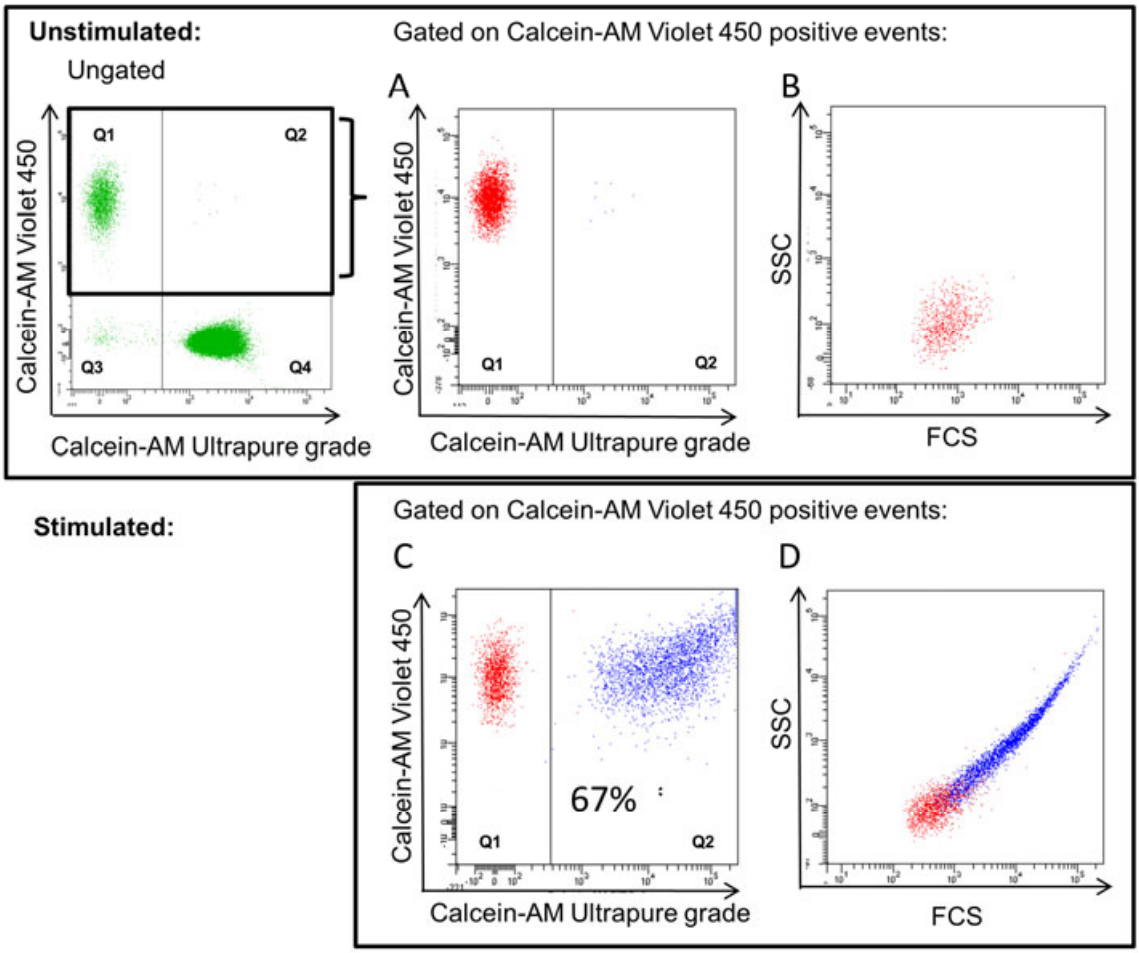
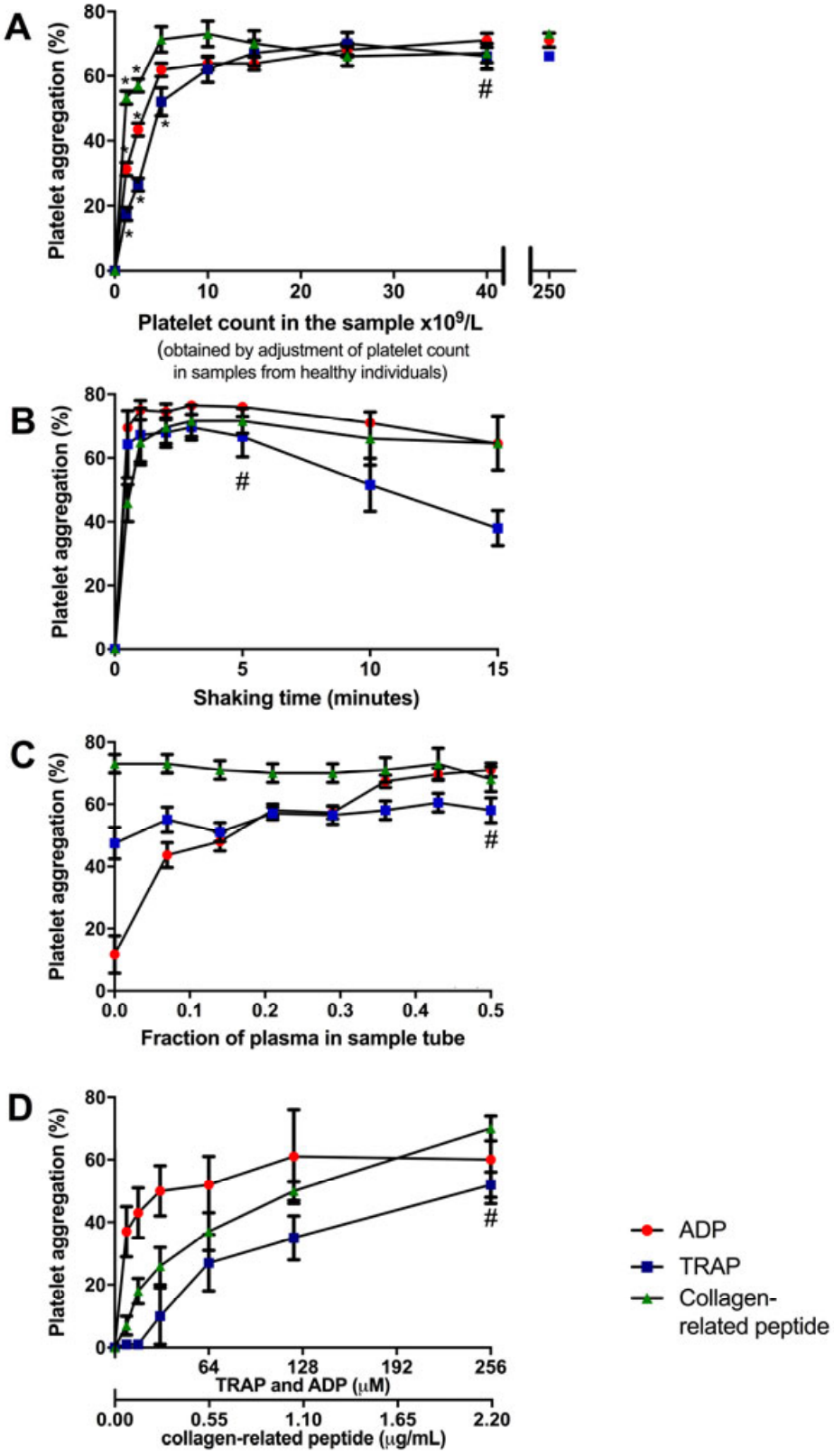
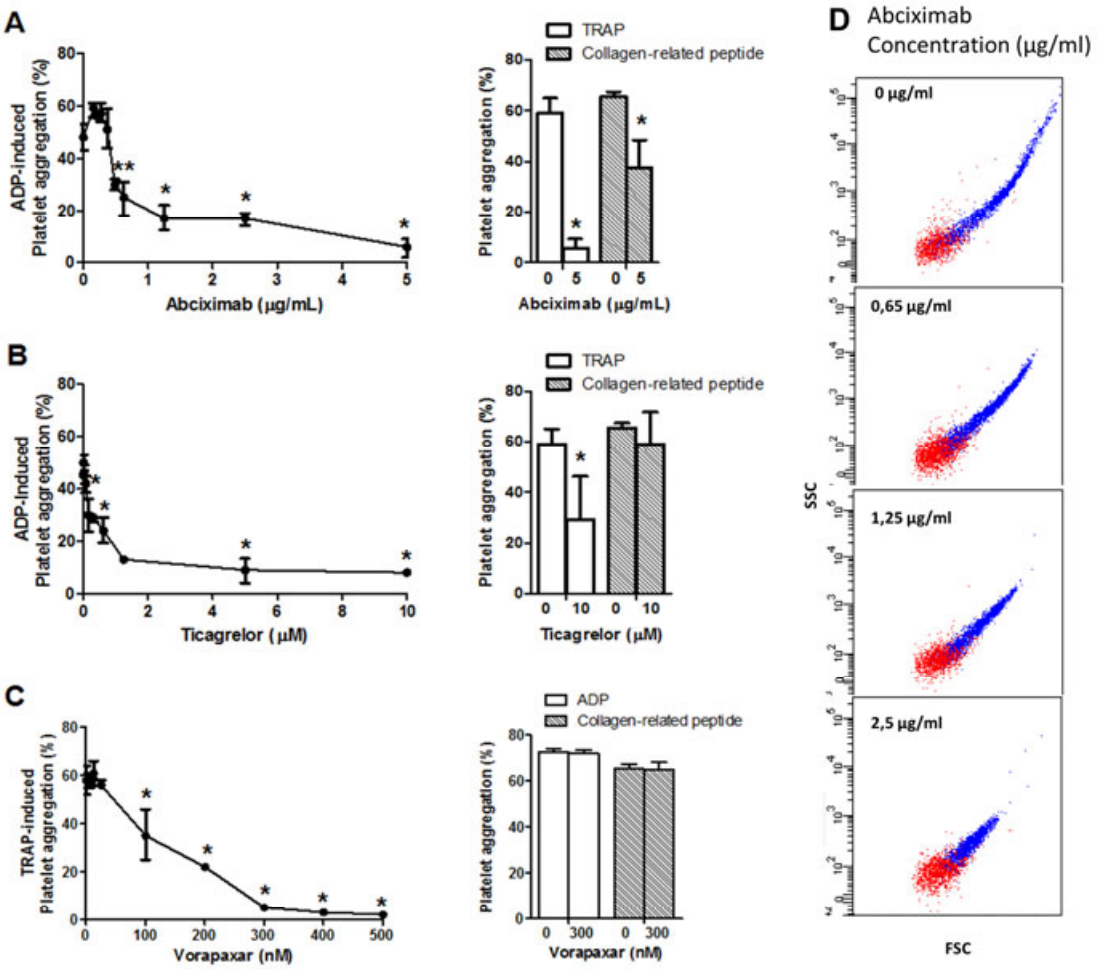
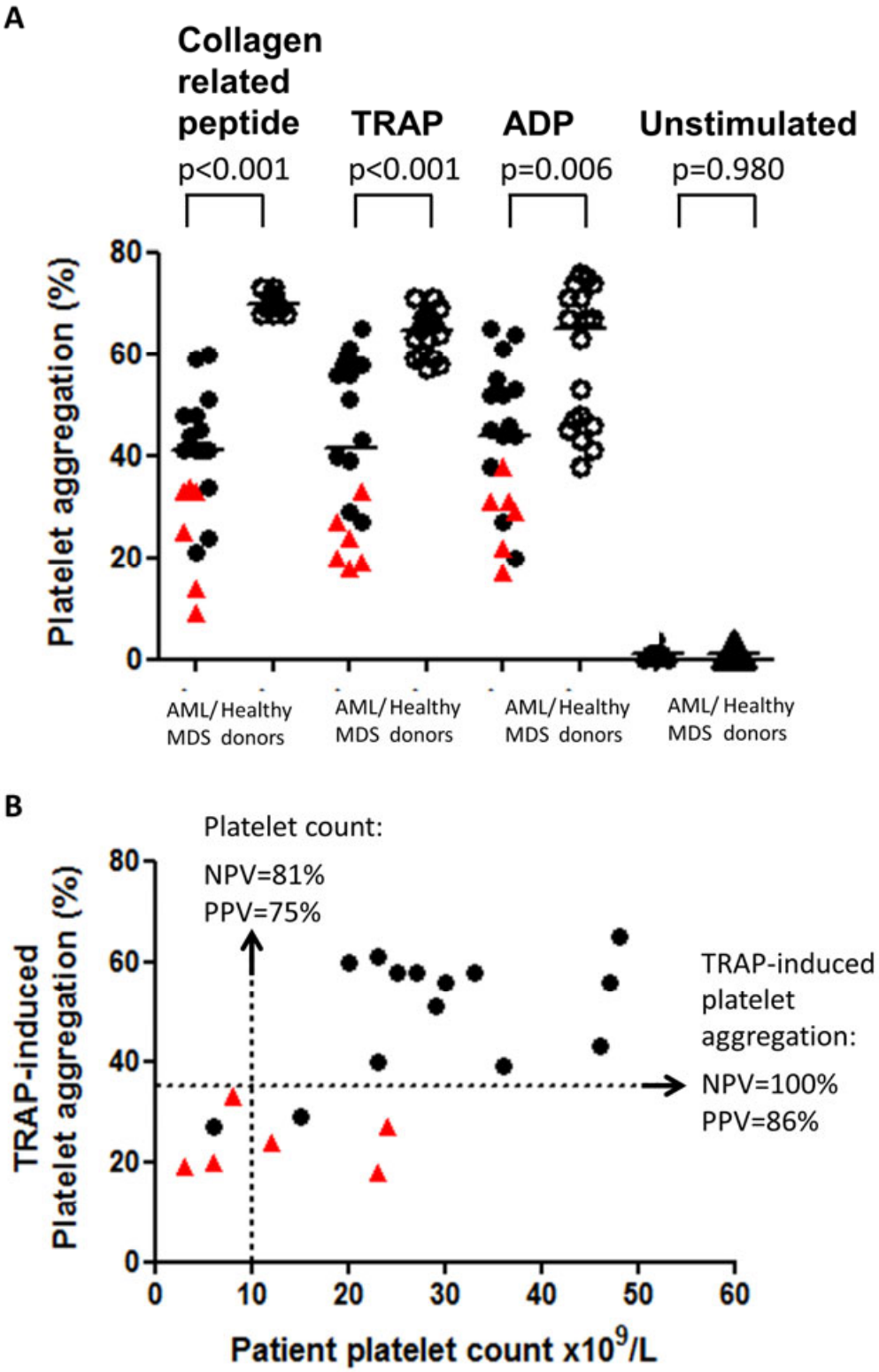




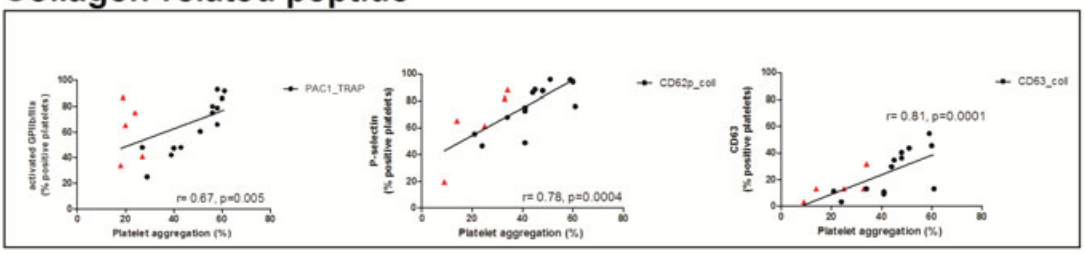
$$\text{* Platelet aggregation (\%)} = 100 \times \left[\frac{\text{Calcein Violet 450 AM double-positive events (Q2)}}{\text{All Calcein Violet 450 AM-positive events (Q1 + Q2)}} \right].$$



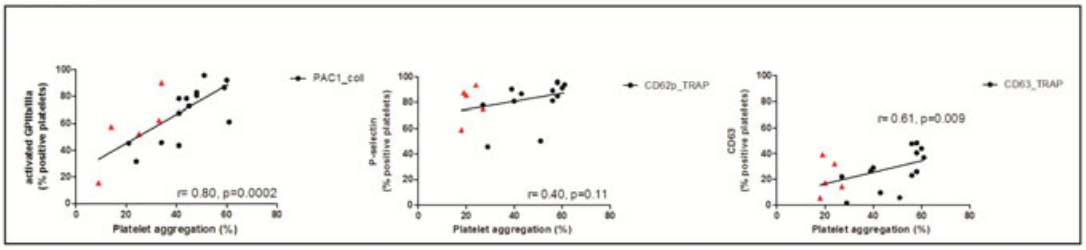




Collagen-related peptide



TRAP



ADP

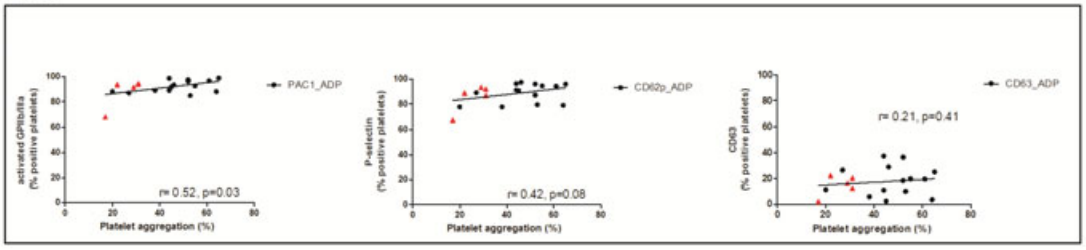


Table 1

		AML/MDS Patients (n=18)	Healthy individuals (n=19)	*p*-value
Platelet reactivity				
No agonist	Activated GPIIb/IIIa, %	4.6 (2.8-9.3)	3.2 (1.1-6.2)	0.07
	CD63, %	0.9 (0.6-1.8)	0.6 (0.3-1.0)	0.13
	CD62p, %	14.4 (8.3-17.3)	7.7 (5.2-11.0)	0.002
ADP 12.8 µM	Activated GPIIb/IIIa, %	91.8 (88.1-94.8)	99.0 (98.0-99.4)	<0.001
	CD63, %	17.3 (9.9-25.1)	36.8 (23.0-42.0)	0.002
	CD62p, %	91.1 (79.5-94.7)	95.5 (93.7-97.2)	0.01
TRAP 10 µM	Activated GPIIb/IIIa, %	62.9 (44.8-76.9)	98.8 (96.2-99.2)	<0.001
	CD63, %	26.0 (14.5-37.0)	80.6 (70.8-82.5)	<0.001
	CD62p, %	85.7 (77.8-91.2)	99.2 (97.1-99.5)	<0.001
Collagen Related Peptide	Activated GPIIb/IIIa, %	70.2 (49.0-81.7)	99.0 (97.9-99.4)	<0.001
2.2 µg/L	CD63, %	13.1 (11.1-36.3)	74.9 (68.9-81.8)	<0.001
	CD62p, %	76.0 (65.2-87.7)	98.6 (96.6-99.0)	<0.001
Platelet surface receptor expression				
	GPIX (CD42a), MFI	12.5 (9.7-13.3)	10.5 (9.2-10.7)	0.04
	GPIb (CD42b), MFI	15.9 (14.4-18.1)	16.6 (15.2-18.0)	0.64
	GPIIb (CD41a), MFI	75.8 (67.7-87.2)	66.5 (53.3-87.6)	0.47
	GPIIIa (CD61), MFI	19.1 (17.8-23.0)	16.4 (14.0-24.4)	0.44
	GPIa (CD49b), MFI	2.9 (2.3-3.4)	3.3 (2.6-4.8)	0.09