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BRIEF REPORT

Platelet function testing at low platelet counts: When can you trust your analysis?

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Abstract

Background: Although flow cytometry is often brought forward as a preferable method in the setting of thrombocytopenia, the relative effects of low sample counts on results from flow cytometry-based platelet function testing (FC-PFT) in comparison with light transmission aggregometry (LTA) and multiple electrode aggregometry (MEA) has not been reported.

Objectives: To compare the effects of different sample platelet counts (10, 50, 100, and 200 × 10^9 L^{-1}) on platelet activation measured with FC-PFT, LTA, and MEA using the same anticoagulant and agonist concentrations as for the commercial MEA test.

Methods: Platelets were stimulated with two commonly used platelet agonists (ADP [6.5 μmol L^{-1}] and PAR1-AP [TRAP, 32 μmol L^{-1}]). The specified sample platelet counts were obtained by combining platelet-rich and platelet poor hirudinized plasma in different proportions with or without red blood cells.

Results: For FC, P-selectin exposure and PAC-1 binding was reduced at 10 × 10^9 L^{-1} after stimulation with PAR1-AP (by approximately 20% and 50%, respectively), but remained relatively unchanged when ADP was used as agonist (n = 9). The platelet count-dependent effects observed with PAR1-AP were eliminated when samples were pre-incubated with apyrase, implying that reduced purinergic signaling was the main underlying factor (n = 5). Both aggregometry-based PFTs showed a 50% reduction at 50 × 10^9 L^{-1} and more than 80% reduction at 10 × 10^9 L^{-1}, irrespective of agonist used (n = 7).

Conclusions: Although FC-PFT is generally preferable to aggregometry-based PFTs in situations with low sample platelet counts, a careful optimization of experimental parameters is still required in order to eliminate platelet count-related effects.

KEYWORDS

platelet activation, platelet aggregation, platelet count, platelet function tests, thrombocytopenia

Essentials

- Data comparing the effect of low sample platelet counts (PC) in different platelet function tests (PFTs) is scarce.
- This study compares the effects of decreasing PCs on results from three common PFTs.
- As expected, results from aggregometry-based PFTs were heavily affected by low PCs.
- For flow cytometry PFTs, smaller effects were observed, affecting parameters dependent on paracrine (cell-to-cell) signaling.

1 | INTRODUCTION

Apart from being indispensable for the diagnosis of platelet function disorders (PFD), platelet function tests (PFTs) also show promise as bleeding risk stratification tools in other situations where a compromised platelet function can be suspected, such as in mild bleeding disorders (MBDs),[1] hematologic malignancies,[2] infectious conditions,[3] or immune thrombocytopenia (ITP).[4] However, as these conditions are often accompanied by various degrees of thrombocytopenia, uncertainties regarding how they perform when sample platelet counts are low[5] remain one factor limiting the clinical utility of PFTs at present.

The empirical basis for concerns regarding the performance of PFTs in the face of low platelet counts mainly consists of laboratory studies involving aggregometry-based PFTs, either light transmission aggregometry (LTA) or multiple electrode aggregometry (MEA). For LTA, the PFT still considered as gold standard for clinical use, there is considerable disagreement in the literature regarding how sample platelet count affect analytical precision. One study report significant effects when platelet counts are decreased only slightly to levels still within the normal range (150-450 × 10^9 L^{-1}),[6] while other studies report a more robust performance of LTA, with significant negative effects only occurring when platelet counts decrease well below the normal range.[7,8] As a result, the current recommendations from the Platelet Physiology Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) for LTA on samples with a low platelet count remain somewhat vague, with a caveat issued for possible detrimental effects on analytical precision when platelet levels fall below 150 × 10^9 L^{-1}.[9] For MEA, which is designed as a point-of-care test with commercially available reagents in standard doses to enable testing also in less-specialized hospitals, results have been shown to be more heavily affected,[7,10-13] with a roughly proportional relationship between sample platelet concentration and the signal-to-noise ratio.[7,13] However, few studies has included an attempt to quantify the effects when sample platelet counts drop below 50 × 10^9 L^{-1}, levels commonly encountered in samples from patients with conditions associated with thrombocytopenia.

Flow cytometry (FC) has been brought forward as a preferable alternative to aggregometry-based PFTs in the setting of thrombocytopenia.[5,14] Theoretically, FC-PFT certainly represents a major improvement in comparison to LTA and MEA in this context, as the method involves measuring the activation response of individual platelets instead of measuring processes that are dependent on physical contacts between platelets. However, it is nevertheless likely that results from FC-PFT are affected by variations in the sample platelet count to some extent, albeit indirectly. Important platelet activation pathways involving receptors such as the protease activated receptors 1 and 4 (PAR1 and PAR4) and the collagen receptors are partially dependent on autocrine and paracrine stimulation with autacoids, of which the most potent are ADP and thromboxane A_2. As the concentrations of these substances can be expected to vary to an extent proportional to the platelet count after platelet stimulation, the degree of platelet activation observed after platelet stimulation with these agonists using FC-PFT could be anticipated to be reduced when sample platelet counts are low, giving an impression of impaired platelet function, even though the reactivity of the individual platelet might be normal. As it is currently not reported to what extent these factors influence FC-PFT, and how results obtained by the three techniques compare to each other at different platelet counts, this experimental study was performed. For total comparability, all tests were performed in parallel using the standard reagents and conditions for the commercially available MEA test. Hirudin was used as anticoagulant, as platelet responses involving ADP have been shown to be artificially enhanced in the low calcium environment in citrated blood.[15]

2 | MATERIALS AND METHODS

Venous blood was collected from healthy volunteers not taking any drugs that interfere with platelet function for the last 10 days. Blood was collected in hirudin tubes (Roche Diagnostics GmbH, Mannheim, Germany). The procedure for blood collection was approved by the regional ethics review board in Linköping, Sweden.

To obtain platelet-rich plasma (PRP), whole blood was centrifuged at 150 *g* for 15 min at room temperature. After harvesting PRP and discarding of the remaining buffy coat, these tubes were further centrifuged at 2500 *g* for 15 min to obtain red blood cells. Separate tubes were immediately centrifuged at 2500 *g* for 15 min to obtain platelet-poor plasma (PPP). Cell counting of the content of the different fractions was performed using a Swelab Alfa (Boule Diagnostics AB, Spånga Sweden). Blood and PRP with platelet counts of 200, 100, 50, and 10 × 10^9 L^{-1} were created by combining the components in different proportions, calculated from the platelet counts for the different components. For whole blood samples, the hematocrit was adjusted to 40%.

LTA was performed in reconstituted PRP using a Chronolog instrument (Model 560, Chrono-Log, Haverston, PA) and MEA in reconstituted whole blood using the Multiplate instrument (Roche Diagnostics GmbH) according to manufacturer instructions. For consistency, the Multiplate TRAPtest and ADPtest reagents were

used for platelet activation in all experiments in the same final concentrations as in the commercial Multiplate test (final concentration 32 μmol L^{-1} TRAP (PAR1-activating peptide), 6.5 μmol L^{-1} ADP). The reagents were dissolved and stored according to the manufacturer's instructions.

For flow cytometry, 3 μL of blood or PRP was added to 33 μL HEPES buffer (137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 1 mmol L^{-1} $MgCl_2$, 5.6 mmol L^{-1} glucose, 1 g L^{-1} bovine serum albumin, 20 mmol L^{-1} HEPES, pH 7.4, chemicals from Sigma-Aldrich) containing antibodies (0.69 μg mL^{-1} ECD-anti-human-CD41 [Beckman Coulter, Brea, CA], 0.17 μg mL^{-1} PE-anti-human-CD62P [P-selectin], and 0.56 μg mL^{-1} FITC-PAC-1 [Becton Dickinson, Franklin Lakes, NJ, all final concentrations]) and platelet agonists (TRAPtest or ADPtest). For investigation of the role of endogenous ADP for the platelet activation response, apyrase was added to some samples (Apyrase ADP-PREMIUM, Agro-Bio, La Ferté Saint-Aubin, France, final concentration 0.2 U mL^{-1}). The samples were incubated for 10 minutes at room temperature before dilution in 600 μL of HEPES buffer and analysis using a Gallios flow cytometer (Beckman Coulter, Brea, CA). Platelet identification and gating for negative controls were performed as previously described.[16] Platelet activation in resting samples was 2.4 ± 1.4% PAC-1 positive and 10.4 ± 4.9% P-selectin positive platelets (mean ± SD for 72 samples from the nine donors at the different platelet counts and in PRP and reconstituted blood).

2.1 | Statistics

Statistical analysis was performed using GraphPad Prism v.5.04 (GraphPad Software, La Jolla, CA). Repeated measures analysis of variance (ANOVA), followed by Bonferroni's post-hoc test was performed to compare results at a platelet count of 200 with results for the other platelet counts. As only complete data sets are accepted in ANOVA, the analysis for LTA data is based on samples from 5 donors, otherwise the number of donors are as stated in the figures.

3 | RESULTS AND DISCUSSION

For FC-PFT, decreasing sample platelet counts were associated with statistically significant but slight increases in the binding of activation markers after stimulation with ADP (Figure 1A,B). In contrast, decreasing sample platelet counts were associated with significantly decreased PAC-1 binding after activation with PAR1-AP (Figure 1C,D). These differences were significant at all of the tested platelet counts. The percentage of PAC-1-positive platelets at 10 × 10^9 L^{-1} was 65%-70% of the value at 200 × 10^9 L^{-1} (Figure 2C), while the MFI decreased to approximately 50% of the value at 200 × 10^9 L^{-1} (Figure 2D). Decreasing sample platelet counts were also associated with a slight decrease in P-selectin exposure, but this effect was much less pronounced and significant only at a platelet count of 10 × 10^9 L^{-1} (approximately 80%-85% of the value at 200 × 10^9 L^{-1}, Figure 2D). Interestingly, the association observed between sample platelet count and FC-PFT after stimulation with PAR1-AP was almost completely abolished when apyrase was added to the samples before stimulation (Figure 1E,F). This indicates that decreased paracrine stimulation from ADP is the dominant underlying cause of the reduced PAC-1 binding observed with decreasing platelet counts in samples stimulated with PAR1-AP. It is likely that similar results would be observed also for other activation pathways dependent on secondary stimulation via autologous secreted ADP.

To allow for a comparative analysis of the effects of sample platelet count on the most commonly used PFTs, parallel experiments were also conducted with LTA and MEA. Our results demonstrate dramatic and highly significant effects of sample platelet count on aggregation responses in the tested platelet count interval (Figures 1G,H and 2G,H). For LTA, ADP- and PAR1-AP-induced increases in light transmittance were relatively similar at platelet counts of 200 and 100 × 10^9 L^{-1}, but markedly reduced at 50 × 10^9 L^{-1}. At 10 × 10^9 L^{-1}, no evaluable curves were possible to obtain, and thus 0% aggregation was recorded as result. For MEA, there was a linear decrease in the aggregatory response with lower platelet counts, resulting in significant differences in AUC for all three comparisons (100, 50, and 10 × 10^9 L^{-1}), with no differences between the two tested agonists. These results are consistent with several previous studies.[6,7,10–12]

Our study demonstrates a significant positive association between sample platelet counts and the binding of platelet activation markers when PAR1-AP was used as an agonist. However, this effect was not observed with ADP. Also, the impact of sample platelet count could be eliminated by the addition of apyrase before stimulation with PAR1-AP, to subtract the effects of decreased ADP signaling in samples with a low platelet count, which potentially also explain the similar results reported by Psalia et al[17]

It is possible that the pre-analytical procedures used in this study to achieve different platelet counts have had some influence on the results, as it has previously been demonstrated that addition of autologous PPP to PRP can cause decreased platelet reactivity.[8] However, this effect was only significant when using weak stimuli and could not be demonstrated for stronger stimuli comparable to the ones used in this manuscript. Moreover, the inhibitory effect of autologous PPP reported by Cattaneo et al was very prominent upon treatment with ADP and only marginally affected by treatment with apyrase, whereas the opposite relation was true for the effect of decreasing platelet counts observed in this study for FC-PFT. In fact, we even observed a slight increase in response to ADP at lower platelet counts (Figure 2A,B). This strongly suggests that the PPP we used did not have any detrimental effects on platelet reactivity.

Our finding that PAC-1 binding was more affected by decreasing platelet counts than P-selectin exposure is consistent with the notion that decreased ADP signaling represents the major underlying mechanism, as alpha granule release (resulting in P-selectin exposure) is a very early event in platelet activation, whereas stable

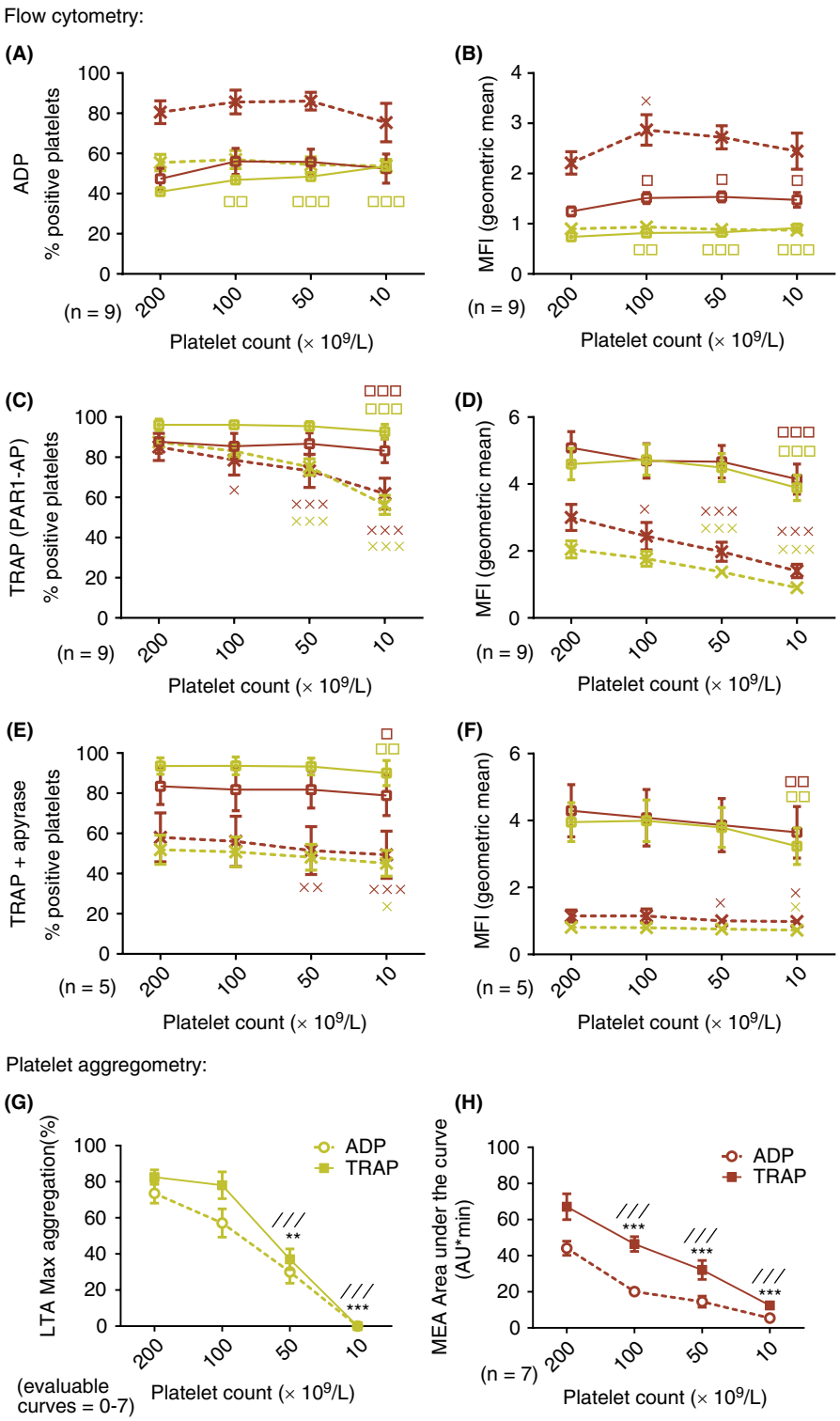

FIGURE 1 (A-F) Platelet activation measured by flow cytometry in response to ADP (A, B, final concentration 6.5 μmol L^{-1}), PAR1-activating peptide (C, D, TRAP, final concentration 32 μmol L^{-1}) or PAR1-activating peptide (32 μmol L^{-1}) in the presence of apyrase (final concentration 0.2 U mL^{-1}, E, F) in samples with platelet counts of 200, 100, 50, or 10 × 10^9 L^{-1}. (A, C, E) Percentage of platelets positive for PAC-1 (activated fibrinogen receptor, x symbols, dashed line) or P-selectin (alpha granule release, square symbols, solid line) in platelet-rich plasma (PRP) (yellow symbols) or reconstituted whole blood (red symbols). (B, D, F) Mean fluorescence intensity (MFI) for the same samples. Symbols (x for PAC-1, □ for P-selectin, in yellow for PRP, in red for whole blood) denote significant differences as compared to the response at 200 × 10^9 L^{-1}. (G) Maximal platelet aggregation in PRP measured with light transmission aggregometry. (H) Area under the curve in reconstituted whole blood, measured with multiple electrode aggregometry (MEA). Symbols (* for ADP, / for TRAP) denote significant differences as compared to the response at 200 × 10^9 L^{-1}. All graphs show mean and standard error of the mean (SEM). */**/*** = $P < 0.05/0.01/0.001$

glycoprotein activation is associated with more sustained platelet activation, dependent on autacoid co-stimulation.[18]

In conclusion, our study implies that FC-PFT is generally preferable to aggregometry-based PFTs in situations with low platelet counts and also provides guidance regarding the magnitude of changes to be expected at different platelet counts. We conclude that in order to minimize the effect of low sample platelet counts on results from FC-PFTs, tests should be performed using ADP as agonist or, when using agonists such as PAR1-AP which are partially dependent on paracrine stimulation, in the presence of apyrase to eliminate the contribution of ADP signaling. We also show that P-selectin exposure is a more robust activation marker than PAC-1 binding in situations with low platelet counts. One caveat in this regard, is that we only tested one agonist concentration for each of the agonists used. Thus, it would be desirable to confirm our findings in future studies including a broader range of agonist concentrations.

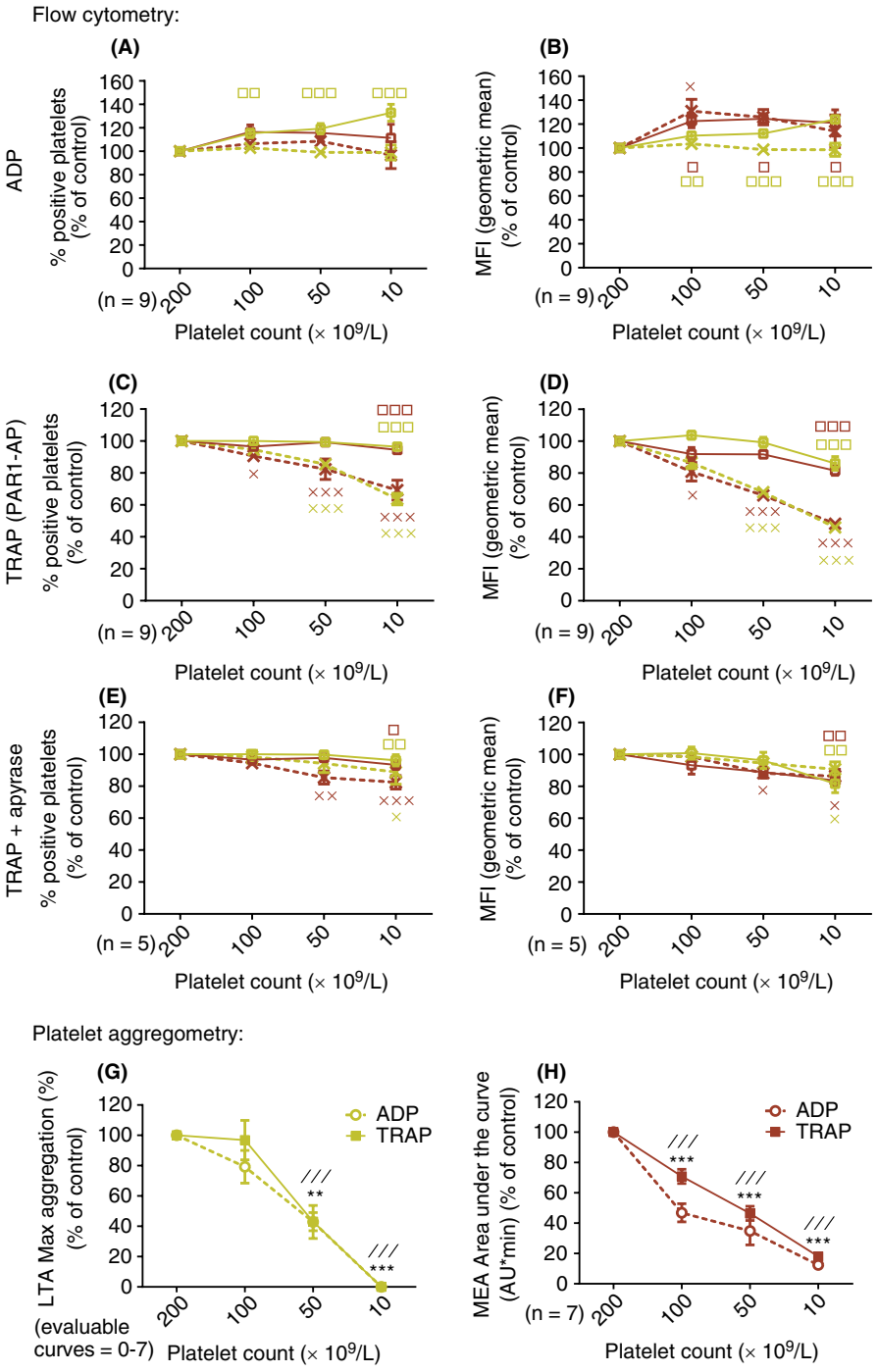

FIGURE 2 Normalized data from Figure 1, with the response of each donor at 200 × 10^9 L^{-1} set to 100%. Symbols (* for ADP, / for TRAP) denote significant differences as compared to the response at 200 × 10^9 L^{-1}. All graphs show mean and standard error of the mean (SEM). */**/*** = $P < 0.05/0.01/0.001$

Future studies are needed in order to establish to what extent FC-PFT can be used to guide clinical decision making in situations with an increased bleeding risk and low platelet counts. This issue is particularly important in view of clinical evidence indicating that the risk of spontaneous bleeding events in severely thrombocytopenic patients is not directly related to the platelet count,[19,20] and that the risk of surgical or obstetric bleeding is higher in patients with inherited disorders of platelet function than in inherited disorders of platelet numbers.[21,22] Also, in another recent study, it was found that although there was an increased incidence of bleeding events during delivery in women with inherited thrombocytopenia and a platelet count of <50 × 10^9 L^{-1}, the increased incidence did not seem to be linearly correlated with platelet counts, as the odds ratio (OR) for bleeding events showed a nonsignificant trend towards lower incidences of bleeding in the tertile of platelets with a platelet count of 49-80 × 10^9 L^{-1} in comparison with patients with a platelet count of > 80 × 10^9 L^{-1}.[23] These observations indicate that clinical decision making needs to be based on knowledge regarding both quantitative and qualitative defects in primary hemostasis.

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AUTHOR CONTRIBUTIONS

NB and SR conceived and designed the study, SR and AM performed the experiments. SR and AS analyzed the data and prepared the figures. NB, SR, AM, and AS wrote the manuscript.

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