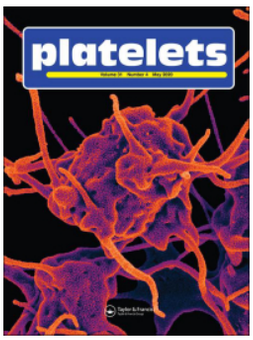

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LETTER

Whole blood platelet aggregation measurement by Multiplate™: potential diagnostic inaccuracy of correcting the results for the sample platelet count

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Dear Editor,

Measurement of platelet aggregation *in vitro* is useful to identify defects of platelet function; in some laboratories, it is also used to monitor antiplatelet therapy [1]. The gold standard technique is light transmission aggregometry (LTA), which measures changes in light transmission through a platelet suspension [2]. Multiplate™ is based on impedance aggregometry, which measures the increase in electrical impedance between two electrodes immersed in platelet suspensions or whole blood, which occurs when stimulated platelets adhere to the electrodes and aggregate [1]. Increased impedance is recorded as tracings of increasing aggregation units and expressed as Area Under the Curve (AUC). Thus, the extent of platelet aggregation measured by Multiplate™ is a function of the volume of the platelet mass accumulated on the electrodes, which depends not only on the ability of platelets to aggregate but also on the sample platelet count. The tight relationship between AUC and the platelet count (even within its physiological range) was demonstrated by experiments in which the platelet count of washed platelet suspensions was decreased to predefined levels by the addition of increasing volumes of buffer [3]. In contrast with Multiplate™, results of LTA are not affected by platelet count within its physiological range [3]. To overcome the confounding effects of platelet count on the results obtained by Multiplate™, it was suggested to express the results as the ratio between AUC and the sample platelet count (AUC/platelet count ratio) [4]. However, platelet count is not the only parameter potentially affecting platelet aggregate size, platelet volume being another plausible candidate. Since the mean platelet volume (MPV) is inversely proportional to the platelet count [5], it would emerge as a variable affecting the Multiplate™ results when they are expressed as AUC/platelet count ratio, which rules off the influence of platelet count.

To test whether MPV affects the values of AUC/platelet count ratio, we retrieved the data obtained in a previously published study in which platelet aggregation was measured, by both LTA (Chrono-log, 560, Mascia Brunelli, Milano, IT) and Multiplate™ (Roche Diagnostics, Milano, IT) in parallel, in patients with Essential Thrombocythemia (ET) on chronic aspirin treatment (100 mg o.d.) and healthy controls who were treated with aspirin (100 mg o.d., Bayer, Milano, IT) for 7 days before blood sampling [6].

We analyzed data from 44 subjects (29 ET; 15 HC; 15 males; median age [IQ range]: 59 [49–70] years) whose platelet counts (391 [271–649] $\times$ 10^9/L; ET: 479 [374-749] *vs* HC: 234 [188–284], $p < .0001$) and MPV values (7.95 [7.3–8.5] fL; ET 7.6 [6.9–8.3] *vs* HC: 8.5 [8.0–9.5)], $p = .0004$) were available. Blood samples had been collected in lepirudin (400 ATU/mL, Verum Diagnostica, Munich, DE); platelet-rich plasma (PRP) was prepared as described [6]. Statistical analysis was performed using GraphPad Prism version 9.0 (GraphPad Software Inc., San Diego, CA, USA). Differences between groups were analyzed using the Mann-Whitney test. Coefficients of correlation were calculated by the nonparametric correlation Spearman's test. All tests were two-tailed and a p-value of <0.05 was chosen as the cutoff level for statistical significance.

To rule out differences in platelet aggregability in the two study groups, which would be caused by abnormalities of platelet secretion in ET [6], we analyzed the results of platelet aggregation induced by ADP (4 µmol/L) (Sigma Aldrich (Milano, IT), which does not cause platelet secretion in aspirin-treated platelets [2]. The

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validity of our approach was confirmed by the finding that there was no difference in the extent of ADP-induced platelet aggregation measured by LTA in ET patients and controls (median values of percent light transmission [IQ ranges]: ET, 68 [61.5–70.5]; controls, 68 [62–73]; p = .8040).

We found a statistically significant correlation between platelet count in whole blood and the extent of platelet aggregation measured by Multiplate™ (r = 0.78, $p < .0001$) [3] (Figure 1). There was no correlation between MPV and AUC (r = −0.22, $p = .16$); however, there was a statistically significant correlation between MPV and AUC/platelet count ratio (r = 0.41, $p = .006$) (Figure 1). There was no statistically significant correlation between the extent of ADP-induced platelet aggregation measured by LTA and the platelet count in PRP (r = −0.18, $p = .26$), MPV (r = 0.11, $p = .51$), or the light transmission/platelet count ratio (r = 0.27; $p = .095$) (Figure 1). We repeated the analysis on data from 28 subjects with normal platelet count (150–450×10^9/L), obtaining similar results with both Multiplate and LTA (not shown).

The results of our analysis confirm our hypothesis that, in addition to platelet count, MPV also contributes to the extent of platelet aggregation measured by Multiplate™. This association was observed only when we analyzed the AUC/platelet count ratio, which rules off the overwhelming contribution by the platelet count. This observation highlights the potential pitfalls in expressing platelet aggregation results obtained by Multiplate™ as the AUC/platelet count ratio, which would provide inaccurate results particularly in samples from thrombocytopenic patients. Indeed, it is likely that the relative contribution of MPV increases with the decrease in platelet count, which, as already mentioned, is the predominant contributor to the results obtained by Multiplate™. Moreover, most congenital and acquired conditions associated with thrombocytopenia are characterized by the presence of very large platelets [7–10], which would substantially affect the results. Therefore, the calculation of the AUC/platelet count ratio, which extrapolates the observed extent of platelet aggregation in thrombocytopenic samples to the expected extent in the same samples at normal platelet count (Figure 2), is prone to overestimate platelet aggregability in patients with thrombocytopenia and high MPV (Figure 2). Obviously, underestimation of platelet aggregability would result when the AUC/platelet count ratio is calculated for samples with the less common inherited microthrombocytopenias [8].

As an example of potential overestimation of AUC in macrothrombocytopenias, it has recently been shown that, compared to healthy controls and patients with chronic hepatitis, patients with cirrhosis (many of whom with thrombocytopenia) displayed increased AUC/platelet count ratio when ADP-induced whole blood platelet aggregation was measured by Multiplate™, leading the authors to conclude that cirrhotic patients have increased platelet aggregation [11]. This conclusion contrasts with the results of most published studies, which reported defects of platelet aggregation, generally measured by LTA, in cirrhotic patients [12], and is likely explained by the fact that platelets from most cirrhotic patients with moderate/severe thrombocytopenia are large or even "giant" (Figure 2) [9,10].

It is plausible that the influence of MPV on platelet aggregation measured by Multiplate™ is due not only to the size of platelet aggregates contributing to changes in electrical impedance between the two electrodes but also to the increased aggregability of platelets, which has been detected in previous reports in which platelet aggregation was studied by LTA [5]. Our results obtained with LTA are compatible with this possibility, because we did find some correlation between MPV and the light transmission/platelet count ratio,

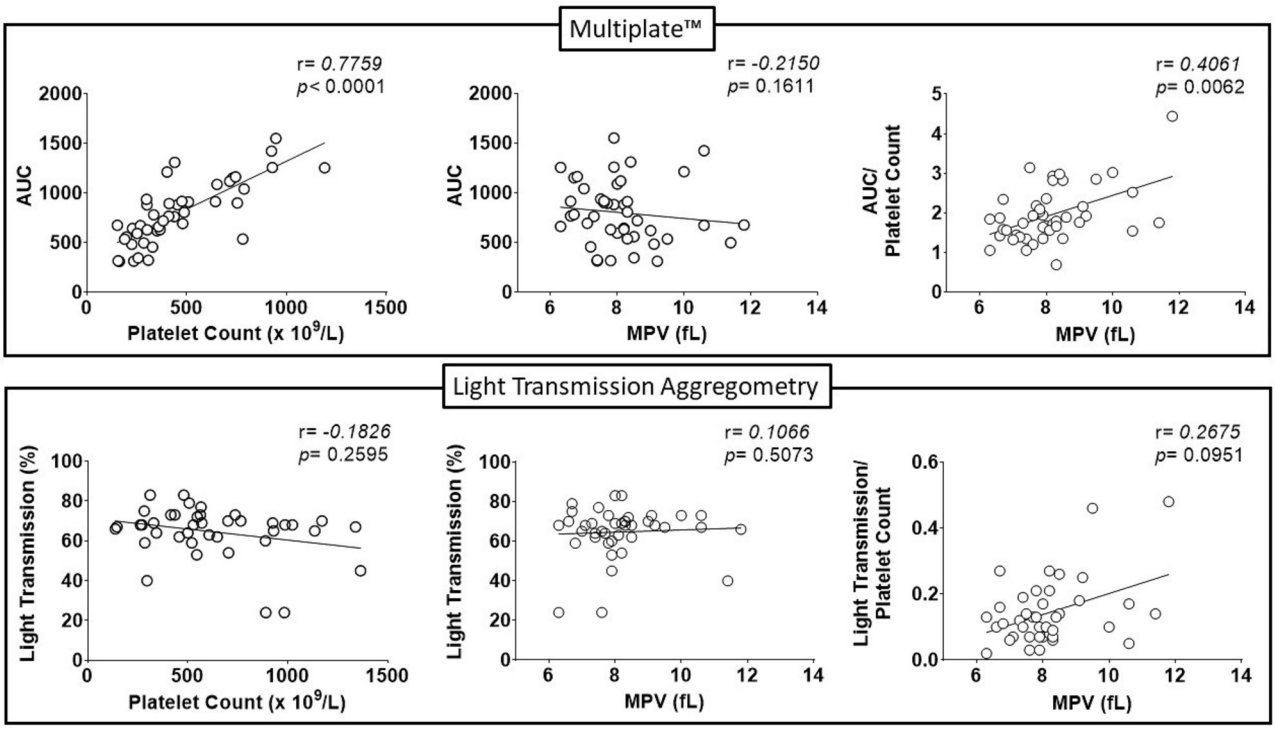

Figure 1. Correlations between platelet count or mean platelet volume (MPV) with platelet aggregation or platelet aggregation/platelet count ratio. Upper panel: platelet aggregation was measured in lepirudin-anticoagulated whole blood by Multiplate™. Lower panel: platelet aggregation was measured in lepirudin-anticoagulated platelet-rich plasma by Light Transmission Aggregometry. N = 44 (Essential Thrombocythemia patients = 29; healthy controls = 15). Correlation coefficients were calculated on data obtained in a previously published study in which platelet aggregation was measured in ET patients and healthy subjects by both LTA and Multiplate™ in parallel [6] (see text for details). AUC: Area Under the Curve of the aggregation tracings obtained by Multiplate™.

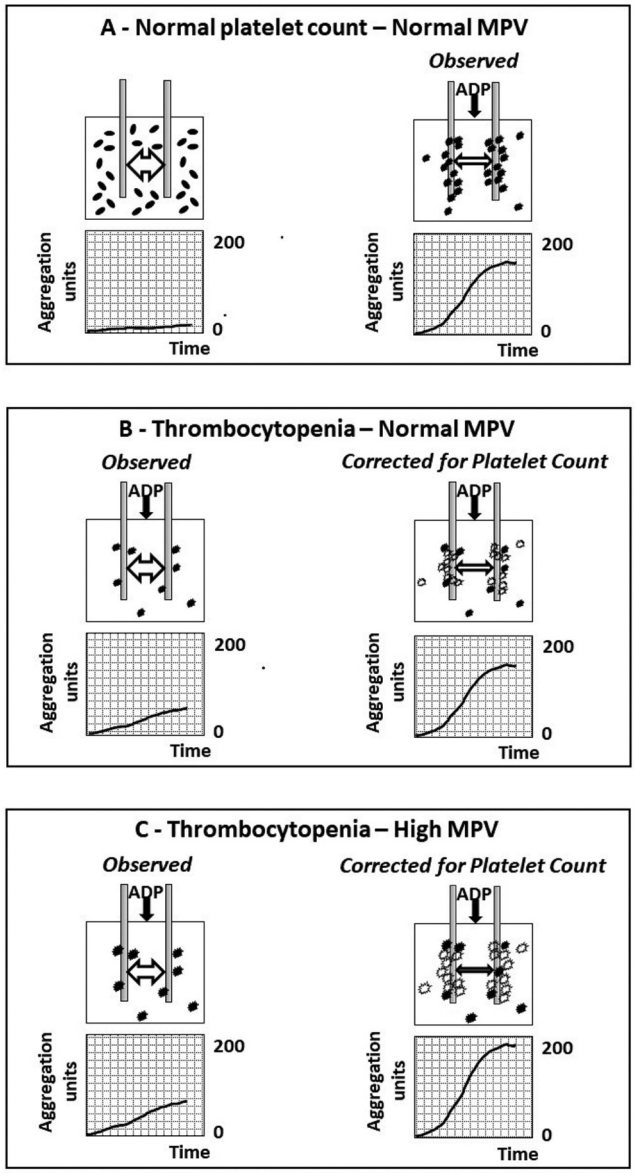

Figure 2. Effects of platelet count and mean platelet volume (MPV) on platelet aggregation measured by Multiplate™. (a): Electric conductance (left-right double arrow) is high between two electrodes immersed in a sample of resting platelets (discs) [left]. Upon ADP stimulation, platelets are activated (spiny spheres) and adhere to electrodes and to each other (aggregation), increasing the impedance, which is recorded as tracings of increasing aggregation units, and expressed as area under the curve (AUC) [right]. (b) and (c) [left]: ADP-induced platelet aggregation in samples with thrombocytopenia and normal (b) or high MPV (c); (b) and (c) [right]: ADP-induced platelet aggregation in the same thrombocytopenic samples as in [left], after correction for platelet count by expressing results as AUC/platelet count ratio: this procedure is equivalent to extrapolating the results obtained in the thrombocytopenic samples to those that would have been obtained in the same samples by addition of "virtual platelets" (white spiny spheres) with the same MPV, to reach normal platelet count [right]. Aggregation tracings in the figure are not based on real data and should be considered illustrative.

although it was not statistically significant and was lower than that with AUC/platelet count. Independently of the mechanism(s) responsible for the correlation between MPV and AUC/platelet count, our data show that its use for expressing the results of whole blood platelet aggregation measured by Multiplate™ may solve the problem of the confounding effect of platelet count but, at the same time, amplifies the additional confounding effect of MPV, especially in patients with macrothrombocytopenia. Overall, these pitfalls of the Multiplate™ question the accuracy of the instrument for the study of platelet aggregability and perhaps account for the insufficient diagnostic accuracy of this method to detect defects of platelet function [1,13] and its poor sensitivity to inhibition of platelet aggregation by antiplatelet agents compared to other tests [14]. We believe that, especially in patients with mild thrombocytopenia, platelet aggregation studies should be performed by LTA, because its results are not affected by a broad range of platelet count. For patients with more severe thrombocytopenia, which would also affect the results obtained by LTA [3], alternative, surrogate methods should be used, such as, for instance, flow cytometry [1].

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Author contributions

MS acquired, analyzed and interpreted the data, revised and approved the final manuscript; GMP, AT interpreted the data, revised and approved the final manuscript; MC developed the concept, designed the study, interpreted the data, wrote the draft of the manuscript and approved the final manuscript.

Data sharing statement

All data will be available upon request to the corresponding author.

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