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Title: Measurement of platelet count with different anticoagulants in thrombocytopenic patients and healthy subjects: accuracy and stability over time

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The accuracy of platelet count measurement is important for the diagnosis and management of thrombocytopenia, which is defined as platelet count <150x10^9/L(1), although new age- and sex-specific reference intervals should be adopted(2). An exception is immune thrombocytopenia (ITP), which is diagnosed when the platelet count is lower than 100x10^9/L (3-5). Although the relationship between platelet count and the bleeding risk has never been validated in the clinical setting, platelet count guides treatment choice for thrombocytopenic patients who have to undergo surgical interventions, invasive procedures or delivery, or for prophylaxis with platelet transfusions of patients undergoing myeloablative chemotherapy (4-6).

Pseudothrombocytopenia, a spuriously low platelet count caused by antibody-induced *in vitro* platelet agglutination, is observed in 0,07%-0,27% of tripotassium-ethylenediaminetetraacetic acid (K3EDTA)-anticoagulated blood samples (7, 8). Failure to identify pseudothrombocytopenia may impose the physician to request further, unnecessary and expensive diagnostic tests and expose patients to unnecessary, expensive and potentially dangerous treatments. We showed that some degree of time-dependent platelet agglutination in K3EDTA-blood also occurs in some patients with thrombocytopenia, leading to underestimation of their platelet count. Collection of blood samples in citrate-tris-pyridoxalphosphate (CPT) instead of K3EDTA attenuated the severity of this artefact, but did not completely prevent it (9). More recently, it was shown that the frequency of psudothrombocytopenia was lowest in blood samples anticoagulated with magnesium sulphate ($MgSO_4$) (10, 11).

Aims of our study were to extend our previous observations (9) by: 1) comparing platelet counts by hematology analyzer in blood samples collected in four different anticoagulants, including $MgSO_4$, with those obtained by flow cytometry, which is the gold standard method for platelet counting (12); 2) evaluating the effect of time elapsed since blood sampling on platelet counts; 3) comparing the effects of different anticoagulants on the accuracy and stability over time of platelet count measurement in healthy subjects and thrombocytopenic patients.

One-hundred patients with thrombocytopenia were enrolled in the study (M/F, 49/51, median age 67 years, IQR=46-76): 3 had inherited thrombocytopenia and 97 had the following

acquired thrombocytopenias: ITP (n=42), hypersplenism-associated thrombocytopenia (n=31), multifactorial thrombocytopenia (n=11), gestational thrombocytopenia (n=6), clonal hematopoietic disease (n=4), other/unknown forms (3 patients were lost at follow-up when the diagnostic work-up was still ongoing) (n=6). One-hundred healthy subjects (M/F, 49/51, median age 36 years, IQR=25-45) were recruited among blood donors of our Hospital.

Venous blood samples were drawn using a 21-gauge butterfly needle. Non-anticoagulated blood diluted 1:40 with PBS was tested for platelet count by flow cytometry (BD FACS Canto, Becton Dickinson) immediately after sampling, in the presence of an anti-CD41a monoclonal antibody (Becton Dickinson). The following anticoagulants were used to collect blood samples for platelet counting by COULTER® LH 750 Hematology Analyzer (Beckman Coulter,): K3EDTA (Sarstedt AG&Co.), Citrate-Theophylline-Adenosine-Dipyridamole (CTAD) (Becton Dickinson), home-made Citrate-Tris-Pyridossalphosphate (CPT, 17 mmol/L tri-sodium citrate+11.3 mmol/L pyridoxal 50-phosphate+24.76 mmol/L Tris) (9) and $MgSO_4$ (Sarstedt AG&Co.). Cell counts were performed in each anticoagulated sample immediately after sampling (t0) and after 20 (t20), 40 (t40), 60 (t60), 120 (t120), 180 (t180) minutes storage at room temperature. The coefficients of variation (CV) were 2.67% for flow cytometry and 2.52% for the hematology analyzer.

Four categories of clinically relevant platelet counts were predefined: 100-149x10^9/L (very mild thrombocytopenia of any type except ITP, with very low bleeding risk); 50-99x10^9/L (any type of thrombocytopenia with low bleeding risk); 20-49x10^9/L (any type of thrombocytopenia with intermediate bleeding risk; need for treatment in selected patients with concomitant conditions increasing the bleeding risk); <20x10^9/L (any type of thrombocytopenia with high bleeding risk; need for treatment in most instances). The study was approved by the Ethics Committee of Ospedale San Paolo, Milano,.

Data are shown as medians and ranges. Differences were analysed using Friedman's analysis of variance (ANOVA) followed by Dunn's *post hoc* test. Differences between frequencies were analysed using the Mc Nemar’s test. P values <0.05 were considered statistically significant.

In thrombocytopenic patients, the median platelet count measured at t0 in non-anticoagulated blood by flow cytometry was not significantly different from those measured by hematology analyzer in K3EDTA, CPT- and $MgSO_4$-anticoagulated samples, but significantly higher than in CTAD-samples (Table1): this difference (5%) was higher than the CV of the method (2.7%). The agreement (Cohen's kappa, *k*) between flow cytometry and hematology analyzer in categorizing patients to the four prespecified bleeding risk categories at t0 was optimal (k=80-100%) for all anticoagulants: k=0.88 for K3EDTA-; k=0.86 for CTAD; k=0.90 for CPT; k=0.93 for $MgSO_4$. There were no statistically significant differences among median platelet counts at t0 in healthy subjects (Table 1).

The median platelet counts of thrombocytopenic patients were significantly lower at t180 than at t0 with all tested anticoagulants (Table 1). The time-dependent decrease in platelet count was faster with K3EDTA than with the other anticoagulants: compared with t0, a significantly lower platelet count was observed at t40 with K3EDTA and at t120 with the other anticoagulants (Table 1). The mean percent reductions of median platelet counts observed ranged between 4% and 17%, which were higher than the CV of the method (2,5%).

Due to the observed time-dependent *in vitro* decrease in their platelet counts, some patients would have been assigned to higher bleeding risk categories at t180, compared to t0: 16 patients (16%) considering blood samples in K3EDTA, 9 (9%) in CPT, 8 (8%) in CTAD and 5 (5%) in $MgSO_4$ (Table 2): the differences between K3EDTA and CTAD ($p=0.034$) or $MgSO_4$ ($p=0.0045$) were statistically significant. In contrast, only 2 patients (2%) in K3EDTA, 1 (1%) in CTAD, 4 (4%) in CPT and 2 (2%) in $MgSO_4$ shifted from higher-risk to lower risk category, with no statistically significant differences among anticoagulants (Table 2). Compared with the CV of the method (2.5%), shifts from lower to higher risk categories were higher, while those from higher to lower-risk categories were of a similar magnitude.

In healthy subjects, the platelet count was more stable over time: statistically significant decreases in platelet counts from t0 were observed at t60 and t180 in K3EDTA, at t120 and t180 in CTAD (table 1). The degree of reduction was always <2%.

This is the first study comparing platelet counting by hematology analyzer in whole blood samples collected in four different anticoagulants to the results obtained by flow cytometry in non-anticoagulated samples, which is the “gold standard” for platelet count determination (12). Our study shows that the baseline platelet counts measured by hematology analyzer agree with those measured by flow cytometry, both in thrombocytopenic patients and healthy controls, independently of the anticogulant used, with the exception of CTAD. In addition, the agreement with flow cytometry in assigning thrombocytopenic patients to four pre-defined bleeding risk categories, based on the baseline platelet count, was optimal for all tested anticoagulants.

Our study also shows that the platelet count in anticoagulated blood samples from patients with different types of thrombocytopenia decreases *in vitro* over time when blood samples are stored at room temperature before testing. The decrease was observed with any anticoagulant, although it was more remarkable with K3EDTA. The same pattern of *in vitro* decrease in the platelet count was not observed in blood samples from healthy controls. The mechanism responsible for this artefact is likely the same that is responsible for pseudothrombocytopenia, which is caused by platelet agglutination mediated by antibodies against the platelet GPIIb-IIIa complex. In accordance with the results of a previous study in subjects with pseudothrombocytopenia (10), the time-dependent *in vitro* decrease in platelet count was minimal in blood samples anticoagulated with $MgSO_4$. The observation that no changes in platelet count occurred *in vitro* over time in samples from healthy subjects suggests that antibodies that cause thrombocytopenia may also be responsible for the observed anticoagulant-dependent *in vitro* decrease in platelet count.

The results of our study have relevant clinical implications, because therapeutic decisions for thrombocytopenic patients are largely based on their platelet counts. In our cohort of thrombocytopenic patients, the platelet count in K3EDTA, CTAD, $MgSO_4$ and CPT samples significantly decreased during 180 min storage of blood samples at room temperature. Based on their platelet counts at t180 some patients would have been assigned to a higher bleeding risk category, possibly inducing physicians to start unnecessary, expensive and potentially toxic treatments. Although this misclassification of patients was observed with all tested anticoagulants,

its magnitude was significantly lower with CTAD and $MgSO_4$ than with K3EDTA. Considering that the time lapse occurring in everyday clinical practice between blood sampling and laboratory measurement of blood cell count in large hospitals could be even longer than 180 minutes, our findings are relevant to common clinical practice.

Based on the results of our study, $MgSO_4$ should be recommended for collecting blood samples for platelet count measurement, because it was associated with the lowest *in vitro* decrease in platelet count, in agreement with the demonstration of a very low prevalence of pseudothrombocytopenia in $MgSO_4$-anticoagulated samples (10, 11, 13). However, $MgSO_4$ does not completely prevent the artefact in all samples and is more expensive than K3EDTA. An easier and more practical suggestion would be to repeat platelet count measurements in K3EDTA-samples immediately after blood withdrawal, before taking any therapeutic decision in patients with critical thrombocytopenia.

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AUTHORSHIP CONTRIBUTIONS

GMP and MC conceived and designed the study; MP, PS, MS and EV provided study materials and assembled data; GC and GMP analyzed data; GMP, MP and MC wrote the manuscript. All authors revised and gave final approval of the manuscript.

CONFLICT OF INTEREST DISCLOSURES

Gian Marco Podda, Mariateresa Pugliano, Giovanni Casazza, Pietro Soru, Mariangela Scavone, Giuseppina Vismara, Marco Cattaneo declare no conflicts of interests.

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Table 1 – Platelet counts and their variations over time since blood sampling in healthy subjects and thrombocytopenic patients: comparison of different techniques and anticoagulants for blood samples

Time since blood sampling	*Flow citometry*	***COULTER® LH 750 Hematology Analyzer***			
	No anticoagulant	*K3EDTA anticoagulant*	*CTAD anticoagulant*	*CPT anticoagulant*	*$MgSO_4$ anticoagulant*
		A – HEALTHY SUBJECTS (plateletsx10^9/L)			
t0	240 (203-292)	239 (208-288)	238 (211-292)	244 (209-292)	239 (208-290)
t20		237 (206-291)	241 (209-290)	239 (211-294)	238 (207-290)
t40		237 (266-285)	240 (206-288)	239 (211-293)	237 (207-285)
t60		235 (207-286)*	242 (208-300)	237 (211-288)	238 (207-289)
t120		237 (208-282)	237 (207-288)*	241 (210-290)	239 (208-284)
t180		238 (208-289)*	234 (202-287)*	240 (215-284)	236 (208-289)
		B – THROMBOCYTOPENIC PATIENTS (plateletsx10^9/L)			
t0	80 (47-102)	76 (45-99)	76 (46-95)°	83 (46-100)	79 (45-97)
t20		74 (44-96)	74 (43-96)	80 (46-97)	79 (45-95)
t40		72 (43-95)*	75 (45-96)	74 (47-96)	76 (44-95)
t60		68 (43-93)*	77 (47-93)	72 (46-99)	77 (42-94)
t120		68 (43-94)*	69 (47-93)*	74 (41-95)*	76 (44-96)*
t 180		66 (42-92)*	66 (42-91)*	69 (39-97)*	76 (41-95)*

K3EDTA=tripotassium-ethylenediaminetetraacetic acid; CTAD=Citrate-Theophylline-Adenosine-Dipyridamole; CPT=Citrate-Tris-Pyridossalphosphate; $MgSO_4$=magnesium sulfate.

Median values (IQR). Friedman's analysis of variance (ANOVA) followed by Dunn's post hoc test. ANOVA test from t0 to t180 was statistically significant for all anticoagulants. °p<0.05 vs flow cytometry a t0. *p<0.05 vs t0

Table 2 – Shifts in bleeding risk categories from t0 to t180 in 100 thrombocytopenic patients, based on *in vitro* changes in platelet counts from t0 to t180, as a function of the anticoagulant for blood samples

	Shifts to categories at higher bleeding risk *number of shifted patients/total patients at lower risk category at t0 (%)*			
Lower-risk category⇒ higher-risk category	*K3EDTA anticoagulant*	*CTAD anticoagulant*	*CPT anticoagulant*	*$MgSO_4$ anticoagulant*
100-149⇒50-99 (plt x 109/L)	9/24 (38%)	5/21 (24%)	4/25 (16%)	3/23 (13%)
50-99⇒20-49 (plt x 109/L)	5/48 (10%)	2/50 (4%)	5/47 (11%)	1/48 (2%)
20-49⇒0-19 (plt x 109/L)	2/22 (9%)	1/23 (4%)	0/21 (0%)	1/23 (4%)
Total shifts from lower to higher bleeding risk category at t180	**16/100 (16%)**	**8/100 (8%)***	**9/100 (9%)**	**5/100 (5%)***
	Shifts to categories at lower bleeding risk *number of shifted patients/total patients at higher risk category at t0 (%)*			
Higher-risk category⇒ lower-risk category	*K3EDTA anticoagulant*	*CTAD anticoagulant*	*CPT anticoagulant*	*$MgSO_4$ anticoagulant*
50-99 ⇒100-149 (plt x 109/L)	0/48 (0%)	0/50 (0%)	1/47 (2%)	2/48 (4%)
20-49⇒50-99 (plt x 109/L)	1/22 (5%)	1/23 (4%)	3/21 (14%)	0/23 (0%)
0-19⇒20-49 (plt x 109/L)	1/6 (17%)	0/6 (0%)	0/7 (0%)	0/6 (0%)
Total shifts from higher to lower bleeding risk category at t180	**2/100 (2%)**	**1/100 (1%)**	**4/100 (4%)**	**2/100 (2%)**

K3EDTA=tripotassium-ethylenediaminetetraacetic acid; CTAD=Citrate-Theophylline-Adenosine-Dipyridamole; CPT=Citrate-Tris-Pyridossalphosphate; $MgSO_4$=magnesium sulfate

Data were analyzed using the Mc Nemar's test. *p<0.05 vs K3EDTA