

Plasma centrifugation does not influence thrombin-antithrombin and plasmin-antiplasmin levels but determines platelet microparticles count

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Abstract

Introduction: Centrifugation is an essential step for plasma preparation to remove residual elements in plasma, especially platelets and platelet-derived microparticles (PMPs). Our working hypothesis was that centrifugation as a preanalytical step may influence some coagulation parameters.

Materials and methods: Healthy young men were recruited (N = 17). For centrifugation, two protocols were applied: (A) the first centrifugation at 2500 x g for 15 min and (B) at 2500 x g for 20 min at room temperature with a light brake. In protocol (A), the second centrifugation was carried out at 2500 x g for 15 min, whereas in protocol (B), the second centrifugation involved a 10 min spin at 13,000 x g. Thrombin-antithrombin (TAT) and plasmin-antiplasmin (PAP) complexes concentrations were determined by enzyme-linked immunosorbent assays. PMPs were stained with CD41 antibody and annexin V, and analyzed by flow cytometry method. Procoagulant activity was assayed by the Calibrated Automated Thrombogram method as a slope of thrombin formation (CAT velocity).

Results: Median TAT and PAP concentrations did not differ between the centrifugation protocols. The high speed centrifugation reduced the median (IQR) PMP count in plasma from 1291 (841-1975) to 573 (391-1010) PMP/ μ L (P = 0.001), and CAT velocity from 2.01 (1.31-2.88) to 0.97 (0.82-1.73) nM/min (P = 0.049). Spearman's rank correlation analysis showed correlation between TAT and PMPs in the protocol A plasma which was (ρ = 0.52, P < 0.050) and between PMPs and CAT for protocol A (ρ = 0.74, P < 0.050) and protocol B (ρ = 0.78, P < 0.050).

Conclusion: Centrifugation protocols do not influence the markers of plasminogen (PAP) and thrombin (TAT) generation but they do affect the PMP count and procoagulant activity.

Key words: cell-derived microparticles; blood coagulation tests; centrifugation; preanalytical phase

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Introduction

Preanalytical phase conditions, like procedures for sample collection, test tubes or conditions of sample storage were subjected to previous studies. However, there is scarce evidence of the influence of sample processing procedures on fibrinolysis parameters or thrombin generation (1). Centrifugation is an essential step for plasma preparation to remove residual blood cell elements in plasma, especially platelets. For routine coagulation tests, single centrifugation at 1500 x g for no less than 15 min at room temperature is a standard, whereas specialized coagulation tests (i.e. lupus anticoagulant or protein C activity) require double centrifugation (2). Using relative centrifugal forces over

1500 x g is not recommended as they are consistently associated with analytical and biological interferences, i.e. hemolysis or platelet activation (3). On the other hand, a short centrifugation time influences results of conventional coagulation testing (APTT - activated partial thromboplastin time and fibrinogen concentrations) and the role of residual blood elements (microplatelets, microparticles - MPs) is proposed (4). The other preanalytical factor, which may significantly influence coagulation testing, is inappropriate sampling by an untrained phlebotomist, which may cause a significant increase in thrombin generation parameters (5).

Our working hypothesis was that centrifugation as a preanalytical step may influence some coagulation parameters. As the information on the role of platelet-derived microparticles (PMPs) in plasmin and thrombin activation *in vitro* is scarce, this study was designed to investigate the presence of platelet-derived microparticles in generation of thrombin-antithrombin (TAT) and plasmin-antiplasmin (PAP) complexes with regard to centrifugation.

Materials and methods

Study design

The study was designed according to the protocol provided by the Vascular Biology Scientific and Standardization Committee International Society on Thrombosis and Hemostasis (VB SSC ISTH) Collaborative Workshop - 2 Preanalytical Phase group (6). The study was approved by the Ethical Committee of the Jagiellonian University Medical College (KBET/257/B/2011).

Subjects

Seventeen healthy young male volunteers aged between 20 to 40 years (inclusion criteria) were recruited among laboratory personnel between December 2010 and January 2011 (John Paul II Hospital, Krakow, Poland). The exclusion criteria were biochemically confirmed diabetes, renal or liver failure, any chronic disease and active clinical condition confirmed biochemically (C-reactive protein above 3 mg/L), as well as any medication including anti-inflammatory drug treatment, non-steroidal anti-inflammatory drugs, especially acetylsalicylic acid, used within one week prior to the study. All subjects signed their informed consent in accordance with the requirements of the Helsinki Declaration on Scientific Research with Humans, which states that the welfare of the research subject takes precedence over the interests of science and society.

Blood sampling

Samples were collected in the morning (8-11 am) after overnight fasting by one experienced phle-

botomist according to a standardized protocol using S-Monovette individual blood collection system (Sarstedt AG & Co., Nümbrecht, Germany). Venipuncture of the antecubital vein was performed with a 21-gauge needle following the application of a light tourniquet. The first serum tube was used for biochemical analysis (2.7 mL, cat No 04.1943.001), whereas the second and third ones additionally contained citrate 0.106 M anticoagulant (8.2 mL, cat No 01.1606.001). The fourth one was used for hematology analyses (2.7 mL, cat No 05.1167). Anticoagulated tubes were gently mixed by inversion (5 times) and immediately transferred to a nearby laboratory for processing. After phlebotomy, samples were subjected to centrifugation by a SIGMA 3-15 centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). In order to compare the centrifugation methods, two protocols were applied: (A) the first centrifugation at 2500 x *g* for 15 min at room temperature with a light brake and (B) at 2500 x *g* for 20 min at room temperature with a light brake (deceleration program 9.4 s) (Figure 1). Then, platelet poor plasma (PPP) was transferred with a Pasteur pipette into a 15 mL Falcon tube, stopping 1 cm above the buffy layer while avoiding disturbing the buffy layer. In protocol (A), the second centrifugation was performed at 2500 x *g* for 15 min at room temperature, whereas in protocol (B) the second centrifugation involved a 10 min spin at 13,000 x *g*. Platelet free plasma (PFP) was transferred into another Falcon plastic tube leaving about 100 µl of plasma at the bottom, gently homogenized and distributed into 1.5 mL Eppendorf tubes in aliquots of at least 500 µl. The aliquots were immediately frozen at -80 °C and stored for further investigations. For TAT, PAP, Calibrated Automated Thrombogram (CAT) and PMPs analysis, samples were randomly selected. In order to avoid cryoprecipitation, citrated plasma were thawed in a water bath at 37°C for no longer than 15 min.

Methods

For all subjects, biochemical parameters were performed by means of the Dimension Xpand Plus integrated chemistry system (Siemens AG, Erlangen, Germany). Complete blood count was performed

using a Sysmex K-4500 (Sysmex Europe GmbH - Europe - Norderstedt, Germany) automated hematology analyzer, while routine coagulation tests (prothrombin time - PT, APTT, fibrinogen) were performed on a BCS analyzer (Dade Behring, Marburg, Germany). Additionally, solid-phase methods were used for determination of PAP (PAP micro ELISA, DRG Diagnostics International Inc., Marburg, Germany) and TAT (Enzygnost TAT micro, Siemens Healthcare, Marburg, Germany) concentrations. Platelet microparticles (PMPs) were stained with CD41-PE antibody (BioCytex, Marseille, France) and analyzed using AnnexinV-7AAD kits (Gallios, Beckman Coulter, Villepinte, France) in a protocol standardized with Megamix+ beads (BioCytex, Marseille, France) during the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. Details regarding methods can be found in supporting information to the paper by Lacroix *et al.* (6). In the same workshop, procoagulant activity was assayed by means of the thrombin generation method, i.e. CAT (Thrombinoscope BV, Maastricht, The Netherlands). This method employs a fluorogenic thrombin substrate added to PPP that enables the kinetic analysis of thrombin generation induced by tissue factor (TF) in a plasma sample. The parameters include the lag time, time to peak, area under the curve (also known as the endogenous thrombin potential - ETP) and slope of thrombin formation (also known as the CAT velocity index) (7).

Statistical analysis

It was established whether the continuous data followed the normal distribution by the Kolmogorov-Smirnov test. Continuous variables are presented as the mean value \pm standard deviation (SD) or the median and the interquartile range (IQR). Differences between mean values were verified using the Student's *t* test when the distribution of variables was normal. In other cases, the Mann-Whitney U test was applied. Correlation of normally distributed variables was examined by Pearson's correlation test and the (*r*) parameter was used. The Spearman's rank correlation test (ρ) was used if variables were not normally distributed. In addition, power analysis was done for

each parameter. P value below 0.05 was set as the level of significance. Analyses were performed with Statistica Version 10 (StatSoft, Inc., Tulsa, USA) software.

Results

Hematology and coagulation parameters were in the laboratory reference range (Table 1). Most of hematology and coagulation (PT, APTT) parameters, body mass index (BMI), glucose and PAP were normally distributed. The other parameters including TAT concentrations and other biochemical parameters were skewed. With regard to demographic parameters, the subjects were representative of Polish male population at their age with respect to BMI and smoking habits. In addition, a detailed analysis of biochemical and hematology parameters was performed in order to look for any possible relationships between those parameters and PMPs or coagulation tests. No such correlations were found in our study group.

Analysis of TAT concentration in plasmas separated according to protocol A utilizing two subsequent centrifugations at 2500 $\times g$ for 15 min revealed that one subject demonstrated an extremely high TAT concentration (5.74 $\mu\text{g/L}$) (Figure 2A, asterisk). This high TAT concentration corresponded with an increased but not extreme PAP level (414 $\mu\text{g/L}$). In the sample separated using protocol B (first centrifugation at 2500 $\times g$, the second one: 10 min spin at 13,000 $\times g$), we did not observe any increase in TAT (1.90 $\mu\text{g/L}$), and PAP was 402 $\mu\text{g/L}$. Median concentrations of TAT did not differ between samples separated by protocol A and protocol B: 1.38 (1.21-1.76) vs. 1.36 (1.10-1.54), $P = 0.480$. Similarly, mean concentrations of PAP in samples separated by the different protocols were not statistically different: 329 ± 68.5 vs. 331 ± 70.2 , $P = 0.920$ (Figure 2B).

We observed a correlation between TAT measured in the protocol A and protocol B samples: $\rho = 0.59$, $P < 0.05$. Likewise, PAP concentrations revealed a correlation between these two protocols with $r = 0.94$, $P < 0.001$. These correlations are presented on graphs (Figure 3, A and B).

TABLE 1. Demographic parameters and routine hematology, coagulation and biochemistry tests results in study subjects (N = 17).

Parameter	N = 17	Reference value
Demographic parameters		
Age	34 (min 22 - max 39)	
BMI (kg/m ²)	23.4 (20.8-26.0)	
smoking (N)	5/17	
Hematology parameters		
White blood cell count (10 ⁹ /L)	7.05 (5.71-7.70)	3.80-10.00
Neutrophil count (10 ⁹ /L)	3.67 (2.56-4.20)	2.00-7.80
Lymphocyte count (10 ⁹ /L)	2.28 (2.04-3.00)	0.60-4.10
Monocyte count (10 ⁹ /L)	0.60 (0.55-0.74)	0.20-1.00
Eosinophil count (10 ⁹ /L)	0.22 (0.12-0.29)	0.00-0.70
Basophil count (10 ⁹ /L)	0.04 (0.02-0.05)	0.00-0.20
Red blood cell count (10 ¹² /L)	5.15 (4.88-5.44)	4.20-6.00
Hemoglobin concentration (g/L)	153 (144-157)	140-180
Hematocrit (L/L)	0.445 (0.43-0.46)	0.40-0.54
Mean corpuscular volume (fL)	87.4 (84.3-88.3)	80.0-99.0
Mean corpuscular hemoglobin (pg)	29.2 (28.5-30.1)	27.0-35.0
Mean corpuscular hemoglobin concentration (g/L)	337 (329-344)	320-370
Red blood cell distribution weight (%)	12.9 (12.3-13.3)	11.5-16.0
Platelet count (10 ⁹ /L)	238 (209-263)	140-440
Mean platelet volume (fL)	10.8 (10.0-11.0)	7.20-12.50
Coagulation parameters		
Prothrombin time (% of normal ratio)	105 (95-112)	70.0-130.0
Activated partial thromboplastin time (s)	29.3 (27.5-30.1)	25.0-36.0
Fibrinogen (g/L)	2.43 (2.23-2.64)	1.80-3.50
Biochemistry		
Creatinine (μmol/L)	83.0 (81.0-86.0)	62.0-106.0
C-reactive protein (mg/L)	0.80 (0.55-1.64)	<3.00
Total cholesterol (mmol/L)	4.63 (4.11-5.45)	<5.00
High-density lipoprotein cholesterol (mmol/L)	1.30 (1.12-1.77)	>1.00
Low-density lipoprotein cholesterol (mmol/L)	1.06 (2.27-3.36)	<3.00
Triglycerides (mmol/L)	1.06 (0.78-1.42)	<1.00
Glucose (mmol/L)	5.34 (5.00-5.80)	3.30-5.60

Values are presented as median with interquartile interval (Q1-Q3).
 BMI – body mass index.

Generally, we observed that both the number of platelet-derived microparticles (annexin V and CD41 positive PMPs) and procoagulant activity of plasma (thrombin generation in the CAT method) were significantly influenced by different plasma centrifugation protocols (Figure 2, C and D). The

second, high speed centrifugation (protocol B) reduced the median PMP count in plasma from 1291 (841-1972) PMP/μl in protocol A to 573 (391-1010) PMP/μl in protocol B (P = 0.010). Similarly, the velocity of thrombin formation was significantly influenced by centrifugation procedure: 2.01 (1.31-

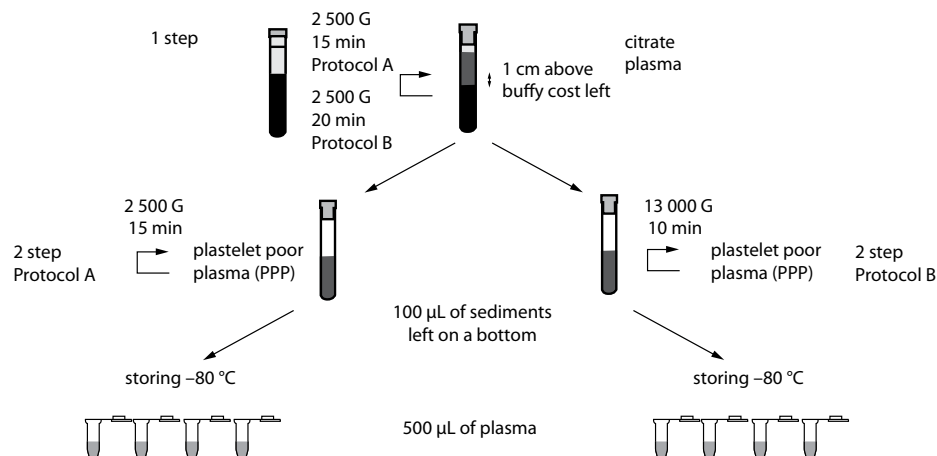


FIGURE 1. Schematic representation of centrifugation protocols and plasma collection.

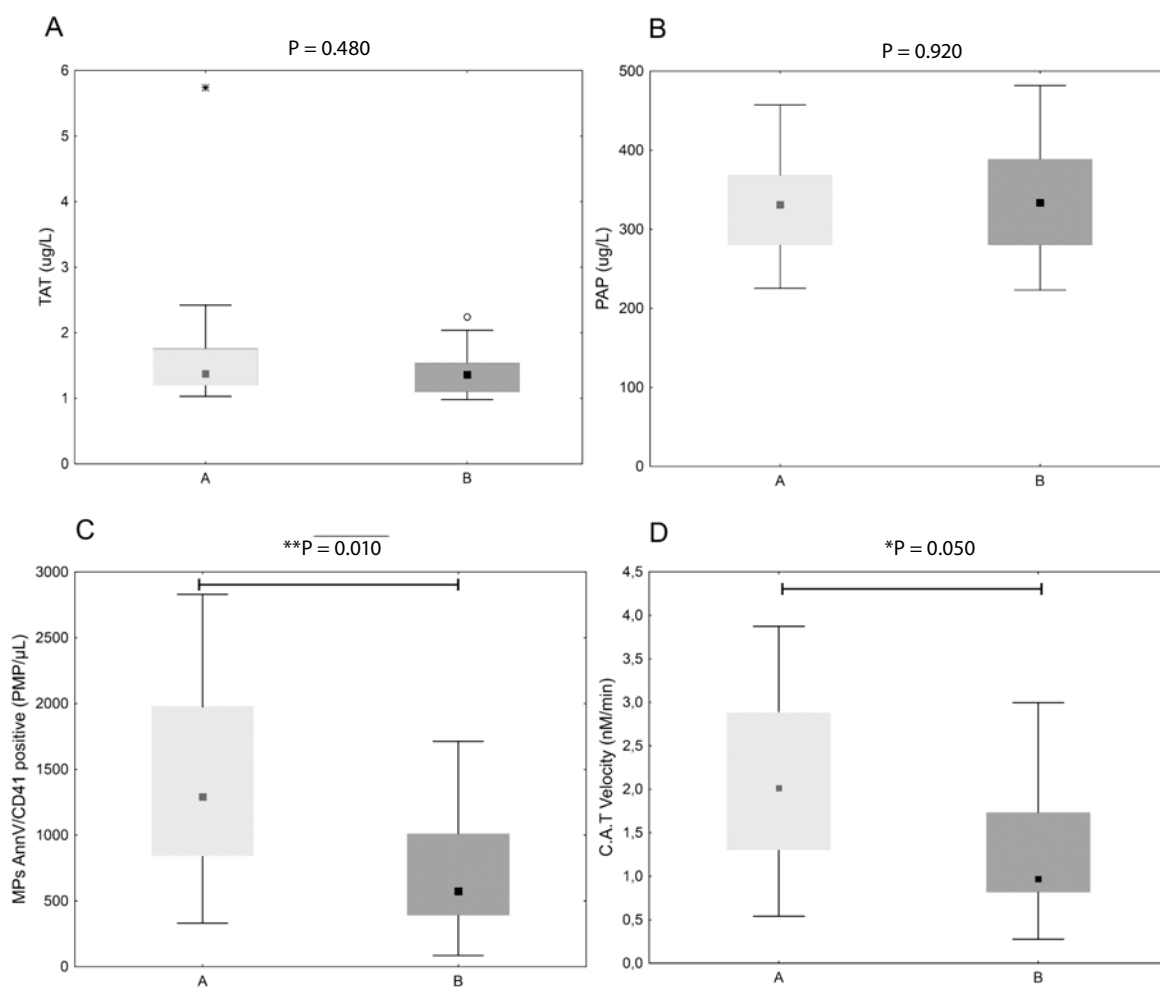


FIGURE 2. Comparison of TAT (thrombin-antithrombin complexes) (A), PAP (plasmin-antiplasmin complexes) (B), platelet-derived microparticles number (C) and thrombin generation (CAT) (D) in human plasma obtained by 2 different centrifugation methods: (A) the first centrifugation at 2500 x g for 15 min and the second one at 2500 x g for 15 min at room temperature, (B) the first centrifugation at 2500 x g for 20 min and the second one for 10 min at 13,000 x g at room temperature with a light brake.

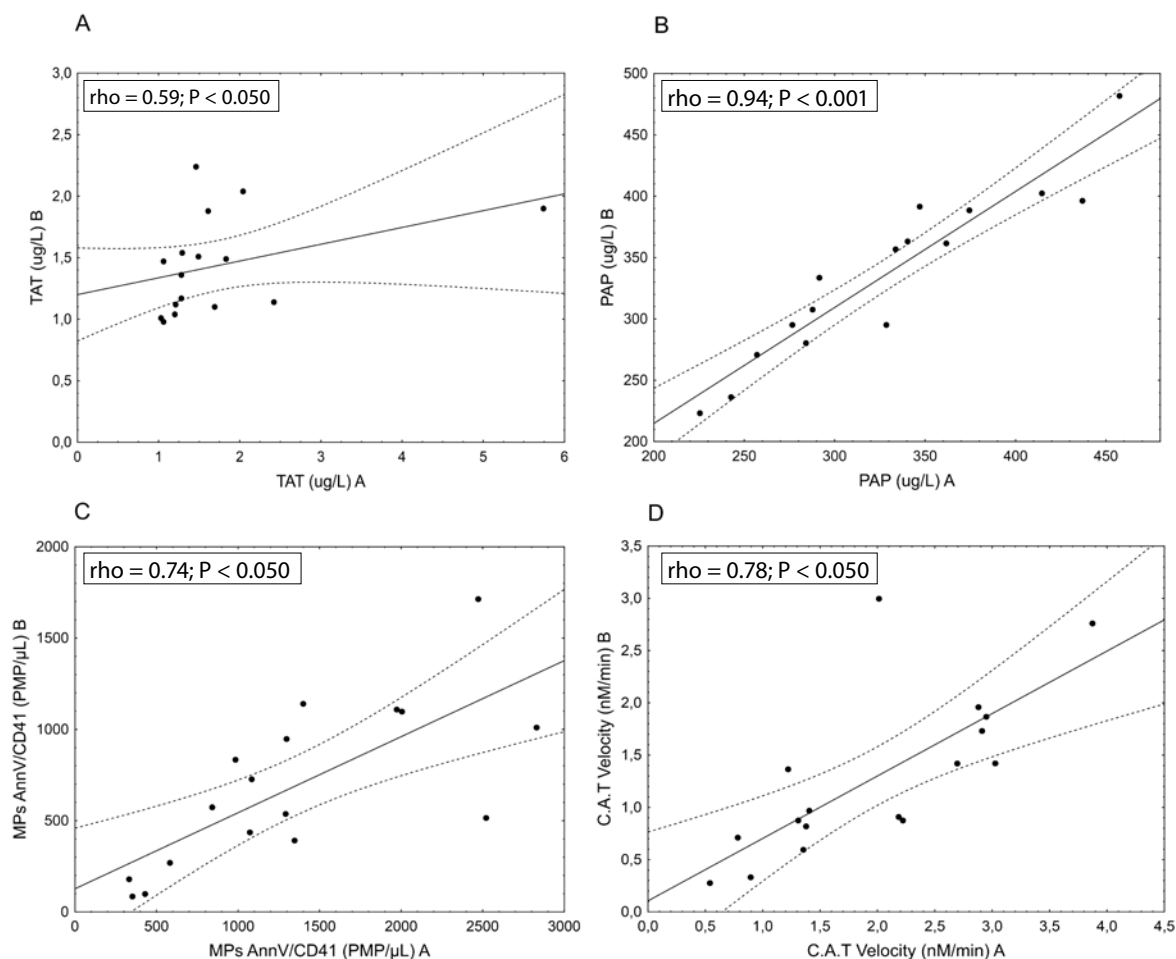


FIGURE 3. Graphical presentation of correlations between two centrifugation protocols (Protocol A and Protocol B) for TAT (thrombin-antithrombin complexes) (A), PAP (plasmin-antiplasmin complexes) (B), platelet-derived microparticles number (C) and thrombin generation (CAT) (D) parameters. The data are presented as Spearman’s (A,C,D) and Person’s (B) statistics.

2.88) nM/min for protocol A vs. 0.97 (0.82-1.73) nM/min for protocol B (P = 0.049). Despite this significant effect of the separation methods on the PMPs count and CAT results, we observed significant correlations between PMPs and CAT obtained for these two protocols: rho = 0.74 (P < 0.050) and rho = 0.78 (P < 0.050), respectively (Figure 3, C and D).

Additionally, we analyzed the correlation between TAT and PAP concentrations with respect to the PMP count and CAT velocity. The only correlation found between TAT and PMPs in the protocol A was (rho = 0.52, P < 0.050).

Discussion

Several efforts have been made to identify the most suitable procedures for collecting, handling

and preparation of plasma for coagulation analysis (2,3,5). However, preanalytical determinants of plasma microparticles are still not fully revealed.

The study proved that different conditions of plasma centrifugation do not affect TAT and PAP concentrations in healthy donors but do influence the PMP count and plasma CAT activity. To our knowledge, there has been no similar study showing this effect before.

MPs are membrane vesicles of 0.1-1 μm in diameter, shed from normal or pathological vascular endothelial and blood cells (lymphocytes T and B, macrophages) or platelets in response to cell activation and stress. In plasma, activated platelets are the main source of microparticles and platelet-derived MPs account for about 80% of their total number (8).

It has recently been discovered that circulating MPs might be indicators of the fibrinolytic response to an inflammatory or prothrombotic process, however, platelet-derived MPs (which are mainly represented in healthy subjects) do not contribute to plasmin generation (9). Our observation that PAP levels do not correlate with the PMP count supports those data. The PAP complexes test is a useful biomarker of the plasminogen activation endpoint. However, it does not represent plasma fibrinolytic activity. In our study, we may assume that plasminogen activation measurements are not influenced by plasma centrifugation method and that PAP concentration genuinely represents *in vivo* plasminogen activation. Additionally, we observed that the one outlying subject, who showed extremely high TAT results, did not show any significant increase in PAP concentration in the same sample. Therefore, there is no correlation between these two markers and the TAT elevations did not disturb the overall results.

The role of MPs in procoagulant activity and thrombin activation is unquestionable (10), and PMPs are suspected to be the most significant trigger of thrombin generation due to their relative high quantity and tissue factor (TF) activity. Especially for patients with acute myocardial infarction, the relative median number of TF-bearing PMPs is 72.8 (6.2) %. For patients with stable angina, this number is also elevated when compared to control subjects: 56.2 (6.40) % vs. 7.4 (1.6) %, $P = 0.001$, respectively (8). In our study, we observed the correlation between the PMP count and TAT concentration (in protocol A) despite low plasma coagulation activity in healthy subjects *per se*. The lack of correlation between TAT complexes and the PMP count in protocol B suggests that the number of PMPs may influence to some extent the prothrombotic activity *in vitro*. However, this relationship is not clear. The most important observation is that the PMP count and thrombin generation (CAT) were strongly influenced by plasma centrifugation method. It is not surprising since centrifugation (especially ultracentrifugation) is a method of choice for MP separation. In our study, we found the strong correlation between the PMP count and CAT velocity in both plasma centrifugation

protocols. This means that “thrombogenic activity” of plasma is related to the number of MPs.

The main limitation of the study is that the authors did not measure the residual platelet count after centrifugation, which might be the potential source of PMPs. However, higher centrifugation force in protocol B should remove residual platelets before freezing. For some particularly sensitive tests, it is important to ensure that platelets have been properly eliminated to their count below 10×10^9 platelets/L (2). Another limitation is the number of subjects included in the study. Our study was designed to analyze whether different conditions of plasma preparation influence the microparticle count and whether such variance has an effect on thrombin and plasmin activity. For such purpose, our study population was sufficient in quantity ($N = 17$ for each group). Power analysis showed that for PMP analysis performed by the 2-sample independent sample t-test, very high power (1.00) was achieved. In the case of TAT and PAP analysis, the minimal number of samples is 23 to achieve the quantifiable standardized effect (E_s) with a power equal to 0.8. For the CAT analysis, the minimal number of samples is 21. Other limitations like sampling bias and data collection were avoided. In our study, we paid attention on fasting sampling, always between 8.30 and 9.30 PM, from non-related volunteers, by one experienced phlebotomist.

Conclusion

Our study has proved that the centrifugation method does not influence the commonly used markers of plasminogen and thrombin generation (TAT and PAP). We have also found a significant relationship between the PMP count and CAT velocity despite different centrifugation protocols.

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Potential conflict of interest

None declared.

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