

ORIGINAL ARTICLE

Impact of pre-analytical parameters on the measurement of circulating microparticles: towards standardization of protocol

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Summary. *Background:* Microparticles (MP) are small vesicles of 0.1–1 µm, released in response to activation or apoptosis. Over the past decade, they received an increasing interest both as biomarkers and biovectors in coagulation, inflammation and cancer. Clinical studies were conducted to assess their contribution to the identification of patients at cardiovascular risk. However, among the limitation of such studies, pre-analytical steps remains an important source of variability and artifacts in MP analysis. *Objectives:* Because data from the literature are insufficient to establish recommendations, the objective of the present study was to assess the impact of various pre-analytical parameters on MP measurement. **These parameters included the type of collection tube,** phlebotomy conditions, transportation practices, centrifugation steps and freezing. *Methods:* **MP were assessed by three methods: flow cytometry using a standardized approach, a thrombin generation test (Calibrated Automated Thrombogram®) and a procoagulant phospholipid-dependent clotting time assay (STA®-Procoag-PPL).** *Results:* The main results show that the **three major pre-analytical parameters which impact on MP-related data are the delay before the first centrifugation, agitation of the tubes during transportation and the centrifugation protocol.** *Conclusions:* Based on both this work and literature data, **we propose a new protocol that needs to be validated on a larger scale before being applied for multicenter studies.**

Keywords: microparticles, pre-analytics, standardization.

Introduction

Microparticles (MP) are sub-micron-sized vesicles released from cell membranes in response to activation or apoptosis [1].

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They are generally defined as 0.1–1 µm membrane fragments exposing the anionic phospholipid phosphatidylserine (PS) and membrane antigens representative of their cellular origin. It is now well recognized that MP behave as vectors of bioactive molecules playing a role in blood coagulation, inflammation, cell activation and cancer spreading [2–4]. In clinical practice, circulating MP originating from blood and vascular cells are elevated in a variety of prothrombotic and inflammatory disorders, cardiovascular diseases, autoimmune, infectious diseases and cancer [5–7]. In these clinical settings, MP counts may be useful in identifying patients at risk of vascular disorders and for monitoring the response to treatment [8,9]. The accurate measurement of circulating cell-derived MP is therefore of potentially major importance.

Pre-analytical conditions represent an important source of variability and potential artifacts in the analysis of MP. Unfortunately, data in the literature are sparse [10–14]. **In 2004, a forum described the methodology applied by six teams working in the field whose practices were widely spread out by different reviews [15].** However, the data supporting these pre-analytical choices remain generally unpublished and so far data are insufficient to establish recommendations.

The objective of the present study was therefore to assess the relative impact of various pre-analytical parameters during the sample processing on MP measurements including phlebotomy, transport, plasma preparation and storage. The effects of these pre-analytical parameters were evaluated with blood samples drawn from healthy volunteers, using three methods: standardized flow cytometry, a thrombin generation test and a procoagulant phospholipid-dependent clotting time. Results of this study allow delineating an updated blood processing protocol for MP measurements.

Materials and methods

Donors and blood collection

Blood samples were taken from healthy volunteers which gave informed consent. Donors were between 20 and 60 years old (63% women, 37% men). Donors were free of any overt

disease and were not taking any medication. Their hematological parameters, including hemoglobin, platelet count, white blood cell count and differential, were in the normal range.

Blood samples were drawn directly in the laboratory, at the same time in the morning. Fasting was not mandatory. If not otherwise specified, samples were drawn from the antecubital vein, with the help of a light tourniquet, by a qualified person using a butterfly device with a 21-gauge needle. The first few millilitres were discarded to avoid the potential artifact generated by the contact phase activation. Except specific testing, blood was collected in a 3.2% (0.109 M) citrated plastic tube (Vacuette[®], 3.5 mL; Greiner Bio-one, Frickenhausen, Germany). For each evaluated pre-analytical parameter, one individual tube per condition was collected from the same donor.

Sample processing

All samples were processed by the same experienced operator. After a delay as mentioned, samples were generally centrifuged once at 2500 *g* for 15 min at room temperature (RT) (centrifuge JOUAN CR3i; Thermo Scientific, Waltham, MA, USA) with a light brake only. From each tube, the platelet-poor plasma (PPP) was collected and transferred into a polypropylene hemolysis tube with a micropipette. Aspiration was stopped 1 cm above the buffy coat to avoid disturbing the buffy coat. Plasma (PPP) was centrifuged a second time at 2500 *g* for 15 min at RT. The platelet-free plasma (PFP) was collected into a fresh tube using a micropipette, while leaving about 100 μ L PFP at the bottom of the tube. The collected PFP was homogenized by light vortexing and split into 600- μ L aliquots in 1.5-mL Eppendorf tubes. If needed, samples were stored at -80 °C for various lengths of time as indicated.

MP content was evaluated using three methods: flow cytometry, thrombin generation test and procoagulant phospholipid-dependent clotting time (Data S1).

Anticoagulants and phlebotomy conditions

The effect of the anticoagulant in the collection tube was studied on samples issued from 10 healthy donors using various anticoagulant tubes including citrated tubes (glass tube, 4.5 mL, Vacutainer[®] 0.109 M; BD, Plymouth, UK), CTAD tubes (Citrate-Theophyllin-Adenosine-Dipyridamole, Vacutainer[®], BD), EDTA (Venosafe[®], Terumo, Somerset, NJ, USA) tubes and heparinated tubes (Vacutainer[®]). The citrated tube was used as a reference. The time between blood draw and first centrifugation was set at 1 h at RT with no agitation. MP were analyzed on fresh samples. In additional experiments, the impact of citrate and CTAD over the time before the first centrifugation was studied in an additional 10 blood samples. The respective impact of each of these anticoagulants was evaluated after collection at 1, 2 and 3 h without agitation at RT.

To determine the influence of tourniquet and needle type during venepuncture, blood was collected from healthy volunteers using both arms successively. Two different tourniquet

pressure conditions (20 and 80 mmHg) were applied to 10 healthy volunteers. A Butterfly system (SAFETY blood collection set, 21-G, Vacuette[®]; Greiner Bio-one) was compared with a straight needle (BD Eclipse[™] blood collection needle 21-G) for phlebotomy on five healthy donors.

Transportation

To evaluate the impact of agitation on MP numbers and procoagulant function, samples from 10 healthy donors were analyzed after a 2-h delay at RT. During that time, tubes received no agitation, gentle agitation that was a single inversion of the tube just before the first centrifugation or a strong agitation provoked by a continuous rotation (2 h) on a laboratory wheel. The condition without agitation was chosen as a reference. In additional experiments, blood samples from 10 healthy donors were submitted to our usual hospital transportation system, being carried from a clinical department located three floors upstairs to the lab. The transport was performed in a double plastic bag where tubes were unsupported or alternatively in special boxes where tubes were maintained immobile. Boxes (MiniboxXL[®]; CML, Nemours, France) enabled the tubes to be transported vertically and others (Thermabso[®], CML) enables tubes to be transported horizontally. Tubes remaining motionless in the up-right position in our laboratory were used as a reference.

In 20 healthy donors, the influence of delay between blood collection and the first centrifugation was tested. The shortest possible time that we used as a reference was 5 min before the 1st centrifugation, then after 20 min, 1, 2, 3 and 4 h at RT.

The impact of transportation variables (agitation and delay) were also evaluated on different subpopulations of MP (PS positive MP [Total MP = TMP], platelet-derived MP [PMP] and erythrocyte-derived MP [EryMP]) comparing tubes that were maintained motionless to tubes that underwent a gentle agitation with delays ranging from 5 min to 3 h at RT.

Centrifugation steps

In 30 healthy donors, the effect of two centrifugation protocols was compared. Protocol A was two centrifugations at 2500 *g* for 15 min as described above and protocol B was the commonly used protocol for MP analysis [15]. It was used as a reference and consisted of one centrifugation at 1500 *g* for 15 min followed by a second centrifugation at 13 000 *g* for 2 min. In this experiment, the delay in blood processing was set at 2 h and analysis was carried out on both fresh and frozen plasma.

To evaluate the content of residual platelets in PFP, plasma were centrifuged using Cytospin 2 (Shandon Southern Instruments, Inc., Sewickley, PA, USA). In all, 500 μ L of PFP was placed in a disposable chamber (Cytofunnel[®]; Thermo Scientific, Villebon, FR, USA) mounted with a filter card. Ten samples were processed at 350 *g* for 10 min. The supernatant was absorbed by the filter card, while cells were deposited on a 6-mm-diameter circular area on a slide through the hole present

in the filter paper strip. Slides were subsequently stained with Giemsa, visualized under a microscope at $1000\times$ magnification and pictures were taken randomly. A semi-quantitative estimation was performed using a scoring system from 0 to 4 points in which 0 means no platelet and four more than three platelet clumps by field. Dilutions of a platelet-rich plasma show that platelets can be detected with sensitivity below 1 platelet per μL (data not shown).

Storage

In 10 healthy volunteers, the impact of freezing was evaluated comparing PFP frozen samples at -80°C for 1 week and fresh samples. Results were then compared with PFP frozen over a 12-month period. In alternative experiments, samples ($n = 10$) directly frozen at -80°C were compared with samples preliminary snap frozen in nitrogen before being stored at -80°C for a 1 week. Aliquots were thawed quickly (3 min) in a water bath at 37°C and then homogenized before analysis. The influence of the thawing temperature was also tested in 10 additional healthy donors comparing thawing at 37°C (reference) to thawing at RT or slow thawing on ice.

Statistical analysis

For statistical analysis, all data were analyzed with GraphPad Prism software v.4.0. (GraphPad Software, San Diego, CA, USA). For each individual experiment, absolute values were transformed as a percentage of variation compared with one condition chosen as a reference and fixed at 100%. Then, the

mean percentage of variation of all the experiments was calculated and graphically represented. Wilcoxon's matched paired test was used to compare different pre-analytical conditions with a confidence interval (CI) of 95%. A P -value < 0.05 was considered as significant. To determine the correlation between fresh and frozen samples, Spearman's correlation test was used with a CI of 95%.

Results

Blood collection

We first evaluated the impact of the anticoagulant in the collection tube on MP counts and procoagulant activity (Fig. 1A). TMP counts measured by FCM as indicated in the method section, were dramatically increased in EDTA and heparin tubes 15 ± 13 fold ($P = 0.0039$) and 4.3 ± 0.38 fold ($P = 0.0037$), respectively, as compared with the citrated tube. Noteworthy, both PMP and EryMP subpopulations are affected by EDTA (data not shown). In our conditions, we did not find evidence of a significant difference in MP counts or MP-dependent procoagulant activity between Citrate and CTAD tubes. We therefore investigated the effect of time-delay according to the anticoagulant (Fig. 1B). Accordingly, MP numbers, thrombin generation and coagulation time obtained with CTAD tubes did not significantly differ from those obtained with citrated tubes over a 3-h delay between blood sampling and the plasma/blood cells separation. Altogether these results indicate that CTAD did not provide any added value. Thus, the more widely used citrate anticoagulant

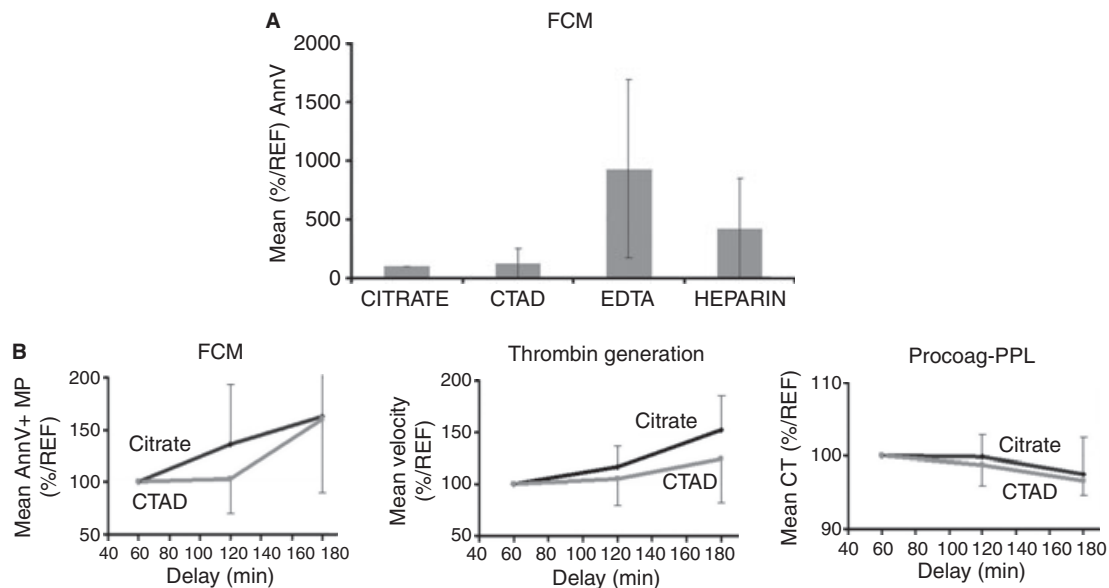


Fig. 1. Impact of anticoagulant on circulating microparticles. All blood samples were drawn from healthy donors ($n = 10$) on various anticoagulants and kept upright at room temperature without agitation. (A) The effect of anticoagulant present in collection tubes was compared amongst citrate (100% = Flow cytometry [FCM] 190MP per $\mu\text{L} \pm 120$), CTAD, EDTA and heparin, after a common delay of 1 h. (B) The effect of a delay before the first centrifugation (1, 2 and 3 h) was compared between Citrate and CTAD. Black line: citrate tubes (absolute values for the 100% control = FCM 220 MP per $\mu\text{L} \pm 100$; thrombin generation [TG] $2.9 \text{ nm min}^{-1} \pm 1.6$; coagulation time [CT] $100.8 \text{ s} \pm 8.3$), gray line: CTAD tubes (100% = FCM 250 MP per $\mu\text{L} \pm 230$; TG $3.8 \text{ nm min}^{-1} \pm 1.4$; CT $107.2 \text{ s} \pm 7.1$), $**P < 0.01$; $***P < 0.001$. Error bars = standard deviation.

remains the most appropriate for MP analysis in these conditions.

To determine the influence of tourniquet and needle type during venepuncture, blood was collected successively on both arms of each healthy volunteer ($n = 10$). Interestingly, in our hands, application of a light (20 mmHg) compared with a strong (80 mmHg) tourniquet did not significantly differ in terms of TMP counts ($6.0 \pm 59\%$, $P = 0.91$), thrombin generation ($-13.2\% \pm 26$, $P = 0.30$) or coagulation time ($0.7 \pm 4.9\%$, $P = 0.73$). Similarly, the use of a butterfly vs. a straight needle for phlebotomy ($n = 12$) did not significantly modify MP-dependent tests (FCM: $36 \pm 40\%$, $P = 0.06$; thrombin generation: $11.2 \pm 32\%$, $P = 0.20$; coagulation time: $-0.8\% \pm 2.9$, $P = 0.28$).

Transportation

Conditions of routine blood tubes transportation may vary greatly in terms of generated agitation. To evaluate the impact of agitation, blood samples ($n = 10$) were submitted to a gentle agitation (+) or a strong agitation (+++) and compared with an absence of agitation (0). As illustrated in Fig. 2A, a gentle agitation does not significantly impact on MP counts or the associated thrombin generation activity, and has only a weak influence on the clotting time. In contrast, a strong agitation induces a critical, although highly variable, increase in MP counts ($+250 \pm 240\%$, $P = 0.006$), in thrombin generation ($+470\% \pm 390\%$, $P = 0.002$) and a significant decrease in clotting time ($-40\% \pm 10\%$, $P = 0.002$). In order to better mimic the real situation in our hospitals, blood tubes

transported either unsupported as usual in double plastic bag or purposely kept immobile using transportation boxes were compared with reference tubes that remained motionless in the lab ($n = 10$) (Fig. 2B). The immobile condition was tested both in horizontal or vertical positions ($n = 10$). As a result, unsupported transport strongly increased MP counts by 180% ($\pm 90\%$, $P = 0.002$), thrombin generation by 140% ($\pm 90\%$, $P = 0.002$) and clotting time decreased by 30% ($\pm 5\%$, $P = 0.002$). Interestingly, the influence of agitation during transportation was almost entirely prevented when tubes were maintained vertically (Fig. 2B) whereas horizontal transportation gave results similar to unsupported transportation.

The effect of a time delay between blood collection and the first centrifugation was evaluated from 5 min to 4 h ($n = 20$) (Fig. S1). As soon as 1 h after blood was drawn, a significant increase in MP amount ($40 \pm 35\%$, $P = 0.02$) was detected. After 4 h, MP counts and thrombin generation values were increased by 80% ($\pm 55\%$, $P = 0.006$) and 180% ($\pm 70\%$, $P = 0.002$) respectively. These results correlate with a significant shortening of the coagulation time after 3 h ($-5\% \pm 4\%$, $P = 0.002$). Altogether, these data indicate that a time delay influences MP counts and procoagulant functions as soon as 1 h after blood is withdrawn.

Then the influence of combined agitation and time delay was tested on different MP subsets. As shown in Fig. S2, a time-dependent increase in PMP counts was observed after 3 h. As expected, the increase was much higher under agitation ($+170\%$, $P = 0.0078$) than without agitation ($+120\%$, $P = 0.012$). In contrast, we did not observe any influence of time delay on EryMP in this time frame. Thus these results

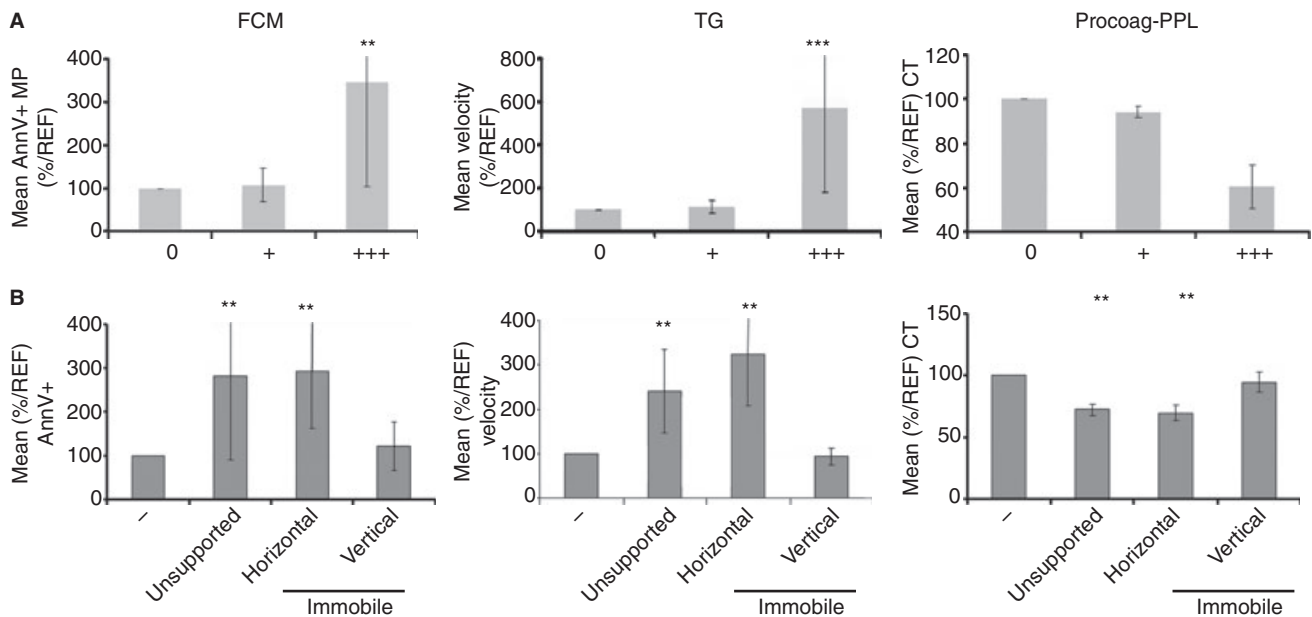


Fig. 2. Impact of agitation and transportation on circulating microparticles. All blood samples were drawn from healthy donors ($n = 10$) and centrifuged after a 2-h delay at room temperature (RT). (A) Impact of agitation 0: unsupported; +: gentle agitation; +++: strong agitation. 100% = flow cytometry (FCM) 280 MP per $\mu\text{L} \pm 180$; thrombin generation (TG) $3.4 \text{ nm min}^{-1} \pm 1.4$; coagulation time (CT) $93.9 \text{ s} \pm 3.9$. (B) Impact of transportation: 0: static; mobile: in plastic bags; immobile: in special boxes. 100% = FCM 200 MP per $\mu\text{L} \pm 80$; TG $2.1 \text{ nm min}^{-1} \pm 0.9$; CT $93.9 \text{ s} \pm 4.5$. * $P = 0.05$; ** $P < 0.01$; *** $P < 0.001$. Error bars = standard deviation.

demonstrated that both time delay and agitation affect PMP but not EryMP counts, at least in the described conditions.

Centrifugation

The effect of two different centrifugation protocols both intended to entirely remove platelets was, therefore, compared on 30 healthy donors. Protocol B (1500 *g* 15 min and 13 000 *g* 2 min) was compared with protocol A (two centrifugations of 2500 *g* 15 min). When fresh samples were analyzed, no significant difference was observed between both protocols (Fig. 3A). In contrast, after a freeze–thaw cycle, data showed a significant increase in MP counts (+170 ± 120%, $P = 0.0002$) and velocity (+70% ± 80%, $P = 0.0084$) and a decrease in clotting time (−154% ± 8%, $P < 0.0001$) with protocol B as compared with protocol A. PMP are the main contributor of a AnnV+MP increase with protocol B (data not shown). Interestingly, a correlation of results obtained with fresh and frozen samples with protocol A ($r^2 = 0.95$,

$P < 0.0001$) was much better than obtained with protocol B ($r^2 = 0.23$, $P = 0.045$) suggesting that an interfering artifact may impact on this last protocol (Fig. 3B). Accordingly when the level of residual platelets in these PFP preparations was evaluated on cytospin pellets (Fig. 3C), these were found to be significantly increased in protocol B compared with protocol A, respectively 1.45 points ± 1.5 vs. 0.25 points ± 0.4 ($P = 0.0156$, Fig. 3D). Altogether, these results indicate that, although both protocols create PFP with very low levels of contaminating platelets, protocol A generates less artifactual PMP than protocol B after freezing as a result of a lower level of residual platelets.

Storage

The impact of storage was evaluated on PFP samples from 10 healthy donors. First, the effect of one single cycle of freezing at −80 °C followed by thawing 1 week later was evaluated (Fig. 4A). Only a limited increase in MP counts (+15%

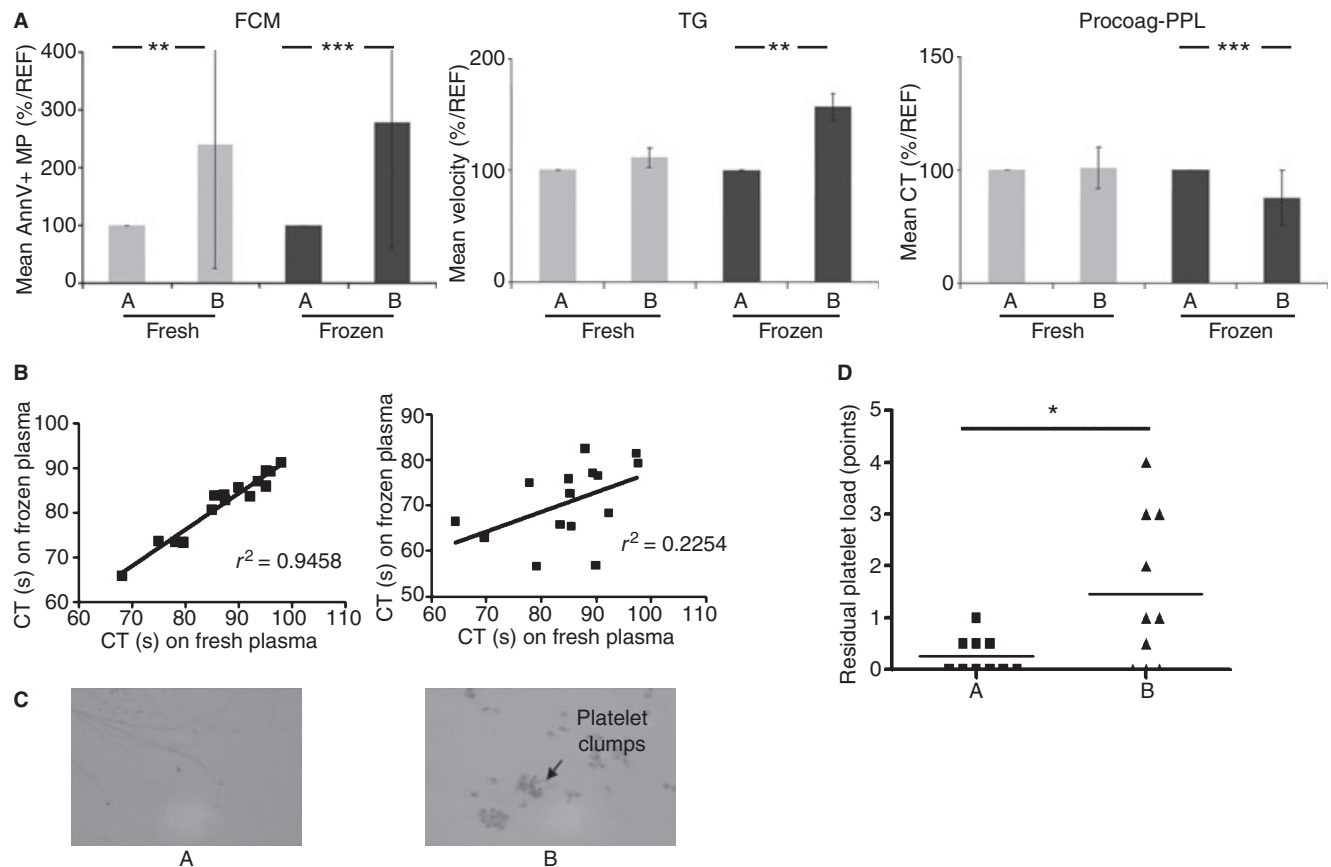


Fig. 3. Impact of centrifugation conditions on circulating microparticles. Two centrifugation protocols (A: 2 × 2500 *g* 15 min, B: 1500 *g* 15 min per 13 000 *g* 2 min) were compared on citrated blood samples from healthy donors ($n = 30$). (A) The effect of freezing after protocol A and B. Gray box: fresh plasma, 100% = flow cytometry (FCM) 630 MP per $\mu\text{L} \pm 910$; thrombin generation (TG) 4.84 $\text{nm}/\text{min}^{-1} \pm 4.91$; coagulation time (CT) 88.3 s ± 8 . Black box: frozen plasma, 100% = FCM 860 MP per $\mu\text{L} \pm 950$; TG 6.53 $\text{nm}/\text{min}^{-1} \pm 4.60$; CT 82.7 s ± 8 . (B) Comparison of coagulation times between fresh and frozen plasma samples ($n = 15$), using protocol A (left-hand graph) and protocol B (right-hand graph). (C) Representative pictures of cell pellets from platelet-free plasma (PFP) subjected to cytospin (2000 *g* 10 min) showing the presence of residual platelets either alone or in clumps (arrow). (D) Semi-quantitative estimation of the residual platelet load in PFP obtained after a dual centrifugation with either the A or B protocol. The scoring system spans from 0 to 4 points in which 0 means no platelet, 1 means one clump per microscope field, etc. and four greater than three platelet clumps per field. * $P = 0.05$; ** $P < 0.01$; *** $P < 0.001$. Error bars = standard deviation.

$\pm 11\%$, $P = 0.16$) and procoagulant activity ($+32\% \pm 23\%$, $P = 0.0039$ for thrombin generation and $-5\% \pm 3.5\%$, $P = 0.049$ for Procoag-PPL) was observed. Second, the impact of storage delay was evaluated between 1 week and 1 year (Fig. 4B). In these conditions, no major change in MP counts, thrombin generation or clotting time was observed after 12 months at -80°C . Third, snap-freezing in liquid nitrogen before storage at -80°C was compared with a direct -80°C freezing procedure. In our conditions, no significant change was observed (Fig. 4C). Finally, the impact of thawing conditions was evaluated comparing thawing at 37°C in a water bath, at RT and on ice (Fig. 4D). Thawing samples at RT resulted in a significant increase in thrombin generation ($+15\% \pm 15\%$, $P = 0.0020$) and a decrease in coagulation time ($-3.5\% \pm 2.2\%$, $P = 0.021$). Thawing on ice increased thrombin generation activity ($+20\% \pm 13\%$, $P = 0.0020$), but did not induce any statistically significant modification on either FCM-based MP counts or CT (Fig. 4D). Altogether these results suggest that deep-freeze storage conditions do not strongly influence MP analysis when performed adequately.

Relative impact of pre-analytical parameters on circulating MP analysis

The various studied pre-analytical parameters were classified according to their relative impact on MP numeration, a MP-dependent thrombin generation test and MP-dependent coagulation time. As displayed in Fig. 5, agitation, time delay, transportation and the centrifugation protocol were clearly identified by the three analytical methods as the three major parameters that impact on MP analysis.

Discussion

To our knowledge, this is the first study to analyze the relative impact of different pre-analytical parameters on circulating MP determined simultaneously by standardized FCM-based enumeration and using two pro-coagulant function tests. During the pre-analytical processing of the samples from healthy donors, agitation during transportation, time delay, and centrifugation conditions were identified as the three major parameters that have the strongest effects on MP measurement.

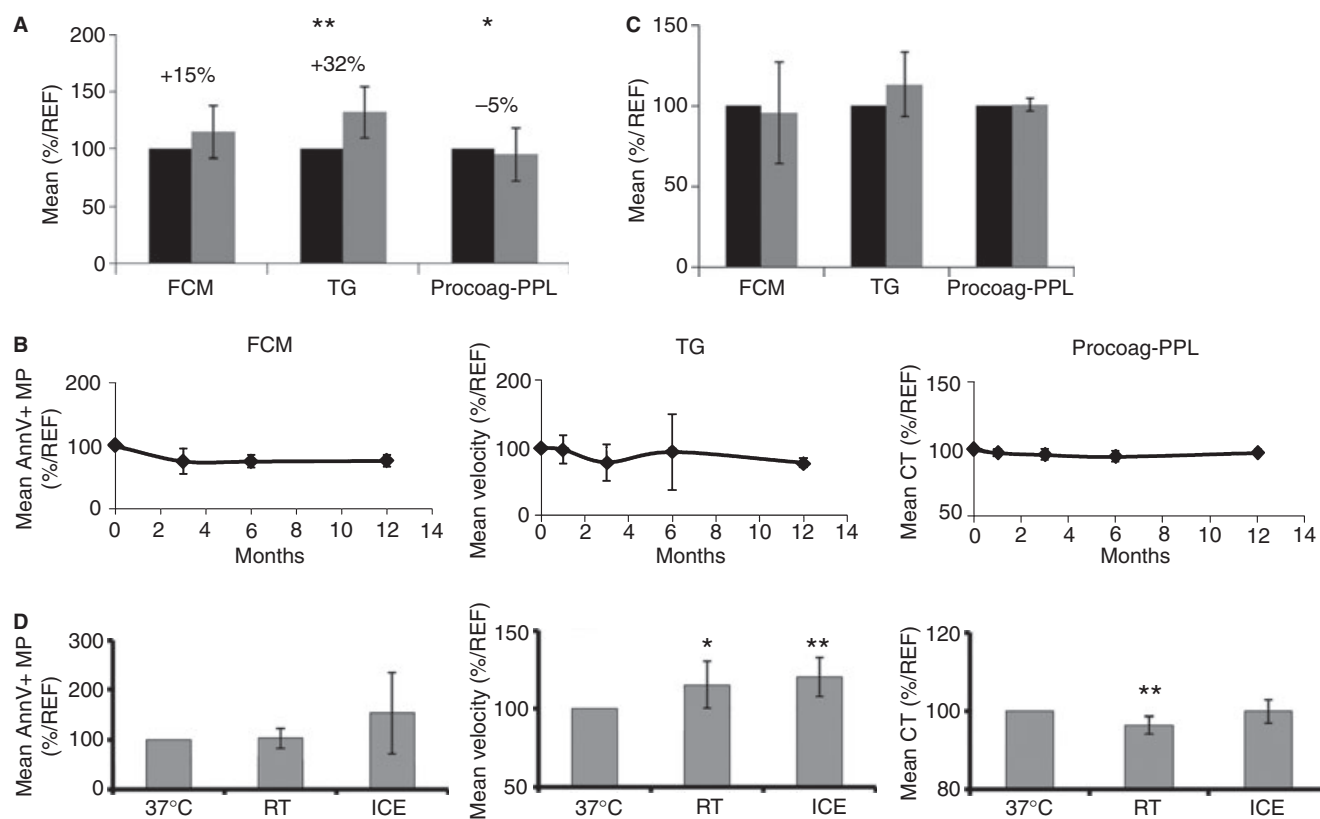


Fig. 4. Impact of plasma storage conditions on circulating microparticles. All tests reported here were operated on platelet-free plasma (PFP) prepared from healthy volunteers ($n = 10$) using centrifugation protocol A. (A) The effect of freezing was evaluated comparing PFP samples frozen at -80°C for 1 week (gray bars) with fresh samples (black bars). 100% = flow cytometry (FCM) 630 MP per $\mu\text{L} \pm 910$; thrombin Generation (TG) $4.84 \text{ nm min}^{-1} \pm 4.91$; coagulation Time (CT) $88.3 \text{ s} \pm 8$. (B) The effect of storage at -80°C was monitored over 12 months. Reference (100%) values were taken after 1 week of storage. 100% = FCM 800 MP per $\mu\text{L} \pm 500$; TG $4.72 \text{ nm min}^{-1} \pm 1.16$; CT $84.2 \text{ s} \pm 8.6$. (C) The effect of deep temperature freezing conditions was studied comparing direct storage at -80°C used as reference (black bars) with the addition of a previous snap-freezing step in nitrogen (gray bars). 100% = FCM 250 MP per $\mu\text{L} \pm 160$; TG $3.96 \text{ nm min}^{-1} \pm 1.8$; CT $91.2 \text{ s} \pm 4$. (D) The effect of thawing temperature was studied comparing 37°C used as a reference, room temperature (RT) and thawing on ice (ICE). 100% = FCM 470 MP per $\mu\text{L} \pm 220$; TG $3.0 \text{ nm min}^{-1} \pm 1.4$; CT $88.4 \text{ s} \pm 10.2$. * $P = 0.05$; ** $P < 0.01$; *** $P < 0.001$. Error bars = standard deviation.

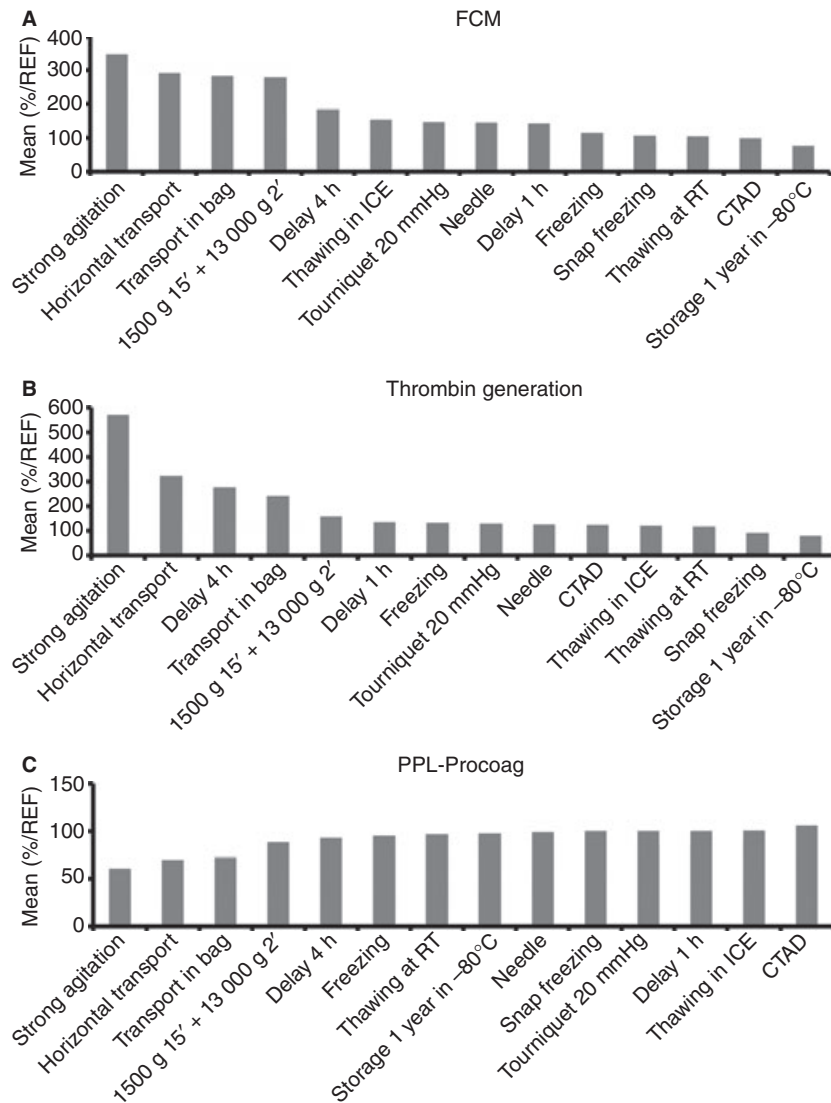


Fig. 5. Relative impact of the different pre-analytic parameters on the measurement of circulating microparticles. Regarding strong agitation, horizontal transport and transport in a bag, the reference was a motionless tube; for centrifugation at 1500 g 15' + 13 000 g 2', the reference was 2 × 2500 g 15'; regarding the delay of 4 h, the reference was the delay 5'; regarding thawing on ice, the reference was thawing at 37 °C in a water bath; regarding tourniquet 20 mmHg, the reference was tourniquet 80 mmHg; regarding the needle, the reference was a butterfly; for the delay 1 h, the reference was delay 5'; for freezing, the reference was fresh samples; regarding snap freezing, the reference was freezing directly at -80 °C; regarding thawing at room temperature (RT), the reference was thawing at 37 °C in water bath; for CTAD, the reference was Citrate; regarding storage 1 year at -80°C, the reference was storage 1 week at -80 °C.

Provided that cells can be easily activated during sample processing and storage, the optimal measurement of circulating MP necessitates cautions to avoid *ex vivo* MP generation and to obtain complete removal of platelets to enable storage.

Because of the major interference of cell activation, we followed practices commonly used in hemostasis laboratories to limit this kind of artifact. Blood collection was performed with a large size, 21-gauge needle, a diameter large enough to avoid *in vitro* hemolysis, EryMP generation and platelet activation [12,15–17]. The first 2 to 3 mL of blood was discarded as already recommended to avoid the effects of the vascular damage caused by venepuncture [18,19]. Only a light tourniquet was allowed during phlebotomy although we did

not observe a significant difference between light or strong tourniquet in our conditions. Moreover, in accordance with Lippi *et al.* [20] evaluating pre-analytical conditions for routine hemostasis analysis, we did not observe a significant difference between a straight needle or butterfly device for blood collection.

Sodium citrate is the most commonly used anticoagulant in hemostasis laboratories. It acts by chelation of free calcium ions, thus making the *ex vivo* calcium unavailable to the coagulation system [21,22] and prevents leukocytes and platelets from degranulation [23]. Because calcium is a key player in membrane phospholipid remodeling, citrate is believed to prevent, at least partially, the vesiculation process [24]. Even if

platelet reactivity may vary among different tubes brands [25], our data confirm that citrated anticoagulant generated less artifactual MP as compared with heparin or EDTA. EDTA is known to induce a P-selectin-dependent platelet activation process [11] that may result in pseudo-thrombopenia and platelet aggregates on blood smears. Moreover, EDTA tubes contain an extremely high concentration of potassium [26], whose consequence on vesiculation is unknown. The platelet activation inhibitors contained in CTAD tubes may be more effective in inhibiting *ex vivo* platelet microparticle formation [27–29]. However, in our conditions, we did not evidence a significant difference in MP counts or MP-dependent procoagulant activity between Citrate and CTAD tubes. As a whole, although the present study needs to be performed on the other 109-mm citrate tubes, this comparison of the most commonly used anticoagulants identifies citrate as the most appropriate for MP analysis.

Because samples from healthy volunteers were collected directly in the lab, it was possible to study the impact of delay between blood sampling and the first centrifugation with a short delay of 5 min serving as reference. In such conditions, our results show that a time delay before the first centrifugation influences TMP and PMP counts as well as procoagulant activity as soon as 1 h, consistent with a PMP increase already described in other studies [27,29,30]. However, the increase during the first 2 h remains moderate in comparison to other factors suggesting that this delay, which remains compatible with current practices in hospital labs, may be acceptable. Noteworthy, we did not evidence a significant change in EryMP in such a short time delay, contrary to what may happen during storage of blood units for several days [31].

No study has yet evaluated the impact of transportation on MP analysis. We did not show a significant impact of moderate agitation on EryMP in the present study in contrast to a previous study on red blood cell units [31]. We observed that the impact of agitation induced by a common hospital transportation system was similar to the effect of strong agitation. Interestingly, transporting blood tubes in a vertical rather than a horizontal position, via the use of special transportation boxes, limited the extent of *in vitro* MP generation. The data identify agitation as a crucial step and suggest that such transportation boxes should be used for MP studies when patients' samples are not directly collected in the lab.

The centrifugation conditions, speed and time vary widely among studies [12,32]. The initial centrifugation intended to generate cell-free plasma is 1500–2500 $\times g$ for 15–20 min in most studies. However, platelets persist after a single centrifugation step [11]. An additional centrifugation step of 13 000 $\times g$ for 2 min ensures the generation of PFP [33,34]. After this 2nd centrifugation, the plasma should be carefully aspirated leaving the bottom 1 cm undisturbed [35]. Because high speed centrifugation is not always readily available, we compared the currently recommended protocol of 1500 g 15 min + 13 000 g 2 min [36,37] to a more routine lab-adapted protocol for example 2 \times 2500 g 15 min [38]. The present study demonstrated the best efficiency of the second protocol in terms of

platelet removal efficiency and thus stability after deep-freeze storage. The more complex tube handling involved in the high-speed centrifugation may partly explain such a difference.

Because, in most cases, samples must be stored before analysis, several teams have already studied the impact of a freeze–thaw cycle on MP measurements. Studies where platelets were insufficiently depleted resulted in obvious differences in MP counts after thawing [39]. In proper conditions, however, although repeated freeze–thaw cycles are known to significantly alter the number of PMP [30,40,41], no major impact of deep freezing was observed as shown in studies on PMP [40] and EryMP [31]. The influence of freezing on endothelial-derived MP (EMP) was inconclusive in the present study because of their small amount in healthy subjects. The literature remains contradictory on this topic. Simak *et al.* [42] did not observe any variation in EMP counts after freezing; in contrast, important changes were observed by van Ierssel *et al.* [19]. In our conditions, MP-dependent procoagulant activity as well as both TMP and PMP counts remained constant up to 12 months after storage at -80°C .

Some teams advocate snap-freezing of PFP in liquid nitrogen before storage at -80°C [43] to reduce ice crystal formation. Based on MP counts by FCM, thrombin generation and phospholipid-dependent coagulation time, we did not find any significant difference comparing a previous snap-freezing step with direct storage at -80°C . However, more subtle structural changes or modifications in the intracytoplasmic MP content cannot be excluded.

Different methods regarding thawing have been described for thawing MP samples including thawing slowly on ice [35,44]. Thawing for several minutes at 37°C may prevent the formation of ice crystals [27,45,46]. In our hands, thawing on ice resulted in a significant artifactual generation of procoagulant activity.

Collectively, the results of the present study and data from the literature allow us to propose the following protocol for MP analysis (Table 1) involving: (i) the use of plastic-citrated tubes; (ii) a blood collection device with large needle size and discarding the first milliliters of blood; (iii) a time delay of a maximum 2 h before the first centrifugation;

Table 1 Proposed pre-analytical protocol for the analysis of circulating microparticles

Pre-analytical parameters	Recommendations
Nature of tube	Citrated tubes
Blood collection	Large needle size (21-G minimum) and discarding the first milliliters of blood
Time delay before the first centrifugation	< or = 2 h
Tubes transportation	Appropriate boxes that maintain tubes vertically
Centrifugation protocol	Two successive centrifugations: 2500 g 15 min at RT
Storage of PFP	-80°C and a quick thawing in water bath

RT, room temperature; PFP, platelet-free plasma.

(iv) transportation in appropriate boxes that maintain tubes vertically; (v) a centrifugation protocol consisting of two successive centrifugations of 2500 g for 15 min at RT; and (vi) storage of PFP at -80°C with a quick thawing in water bath at 37°C .

In conclusion, the present study shows that the three major pre-analytical points that impact on MP measurement are: (i) the delay before the first centrifugation, (ii) agitation of the tubes during transportation and (iii) the protocol of centrifugation. Based on both the present study and data from the literature, we propose a new pre-analytical protocol for MPs analysis. This protocol needs to be validated by other teams before being applied to multicenter studies.

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Disclosure of Conflict of Interest

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Impact of delay before first centrifugation on circulating microparticles.

Figure S2. Impact of delay and agitation on different subpopulations of microparticles.

Data S1. Materials and methods.

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