Peripheral Blood Microvesicles Secretion Is Influenced by Storage Time, Temperature, and Anticoagulants

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Abstract
Microvesicles (MVs) are small membrane bound vesicles released from various cell types after activation or apoptosis. In the last decades, MVs received an increased interest as biomarkers in inflammation, coagulation and cancer. However, standardized pre-analytical steps are crucial for the minimization of artifacts in the MV analysis. Thus, this study evaluated the MV release in whole blood samples under the influence of different anticoagulants, storage time and various temperature conditions. Samples were collected from healthy probands and processed immediately, after 4, 8, 24 and 48 hours at room temperature (RT) or 4°C. To identify MV subpopulations, platelet free plasma (FFP) was stained with Annexin V, calcine AM, CD15, CD41 and CD235a. Analysis was performed on a CytoFLEX flow cytometer. Procoagulatory function of MVs was measured using a phospholipid dependent activity and a tissue factor (TF) activity assay. Without prior storage, sodium citrate showed the lowest MV count compared to heparin and EDTA. Interestingly, EDTA showed a significant release of myeloid-derived MVs (MMVs) compared to sodium citrate. Sodium citrate showed a stable MV count at RT in the first 8 hours after blood collection. Total MV counts increased after 24 hours in sodium citrated or heparinized blood which was related to all subpopulations. Interestingly, EDTA showed stable platelet-derived MV (PMV) and erythrocyte-derived MV (EryMV) count at RT over a 48 h period. In addition, the procoagulatory potential increased significantly after 8-hour storage. Based on both, this work and literature data, the used anticoagulant, storage time and storage temperature differently influence the analysis of MVs within 8 hours. To date, sodium citrated tubes are recommended for MV enumeration and functional analysis. EDTA tubes might be an option for the clinical routine due to stable PMV and EryMV counts. These new approaches need to be validated in a clinical laboratory setting before being applied to patient studies. ©2016 International Society for Advancement of Cytometry

Key terms
extracellular vesicles; peripheral blood microvesicles; pre-analytics

Microvesicles (MVs) are bioactive submicron (0.1–1 μm) membrane vesicles shed from various cell types during proliferation, activation and apoptosis (1,2). MVs were used in different clinical settings to assess the contribution to various diseases. In the last decades, MVs received an increased interest as biomarkers in inflammation (3–5), coagulation (6,7) and cancer (8,9). Therefore, the accurate measurement of MVs in clinical routine is of major importance.

To date, a standardized protocol for the isolation and analysis of MVs is still missing. However, standardized pre-analytical steps are crucial for the minimization of artifacts in the MV analysis. Different approaches and protocols have been tested to analyze the impact of pre-analytical steps on MV analysis including the used
anticoagulant, phlebotomy technique, transportation conditions, centrifugation steps and freeze-thaw cycles (10–12). So far, only recommendations exist for the pre-analytical handling of blood samples for MV analysis.

In the daily clinical routine, sample processing and/or analysis can be delayed for several hours. Interestingly, most studies only evaluated short time blood storage for up to 4 hours prior to analysis (10,13,14). This time delay may be exceeded in the clinical routine especially in hospitals without in-house laboratory.

Therefore, the objective of the present study was to assess the impact of the used anticoagulant, short- and long-time storage and storage temperature on MV enumeration and MV-dependent procoagulant activity using standardized flow cytometry, a phospholipid-dependent and tissue factor (TF) activity assay.

METHODS

Blood Collection

21 healthy adult volunteers were included in the study. Probands were free of acute or chronic disease and were not taking any medication. The study was approved by the local ethics committee (EK 1266/2014). All donors gave informed consent prior to blood drawing.

Blood samples were drawn directly in the laboratory and immediately processed. Blood was drawn from the antecubital vein with a 21-gauge butterfly needle without tourniquet. The first milliliters were used for routine blood count analysis. Samples were collected into 3.2% sodium citrate, K_{2}EDTA and lithium heparin plastic tubes (Greiner Bio-One, Kremsmünster, AUT). For each evaluated time point a separate tube was used.

Sample Storage and Processing

We investigated the influence of storage temperature [room temperature (RT) or 4°C] and duration on human peripheral blood immediately after blood drawing or 4, 8, 24, and 48 hours thereafter. Therefore, all tubes were stored vertically and motionless in the refrigerator or workbench over the study period. After the indicated time, tubes were once centrifuged at 2,500g for 15 minutes at RT without brake. The resulting platelet-poor plasma (PPP) was gently transferred into a polypyrrole tube and centrifuged at 13,000g for 5 minutes at RT. The platelet-free plasma (PPP) was distributed in aliquots, shock frozen in liquid nitrogen and subsequently stored at −80°C prior to analysis.

Staining Protocol

PPP was thawed in a waterbath at 37°C and immediately processed for immunolabeling. 10 μL of PPP was stained with Pacific Blue-labeled annexin V (ebioScience, San Diego, CA), the intracellular fluorescent dye calcein AM (Life Technologies, Carlsbad, CA), anti-CD15-KromeOrange mAb (clone 80H5), anti-CD41a APC mAb (clone P2) and anti-CD235a-APC-AlexaFluor750 mAb (clone KC16, all from Beckman Coulter, Krefeld, Germany) for 25 minutes at 37°C. True MV events were defined as double positive for annexin V and calcein AM. Calcein AM is a non-fluorescent acetomethoxy derivative from calcein which is transported through the cellular membrane into intact/live particles. In the inner part of the particle structure, it converts Calcein AM to calcein by removing acetomethoxyl, which in turn makes calcein a strong green fluorophore. EVs are further characterized by surface staining for following subpopulations: CD41<sup>+</sup> MVs = platelet-derived MVs (PMVs), CD235a<sup>+</sup> MVs = erythrocyte derived MVs (EryMVs) and CD15<sup>+</sup> MVs = myeloid-derived MVs (MMVs). Prior to the staining, the antibody mixture was centrifuged at 20,000g for 30 minutes to remove fluorescent particles as described elsewhere (15). Stained PPP was diluted 1:100 with annexin binding buffer (ebioScience) containing 2 ATA/mL recombinant Hirudin (Sigma Aldrich, St. Louis, MO) to prevent clot formation. Diluted binding buffer was sterile filtered through a 0.2 μm mesh to reduce background noise. For standardization of the enumeration of MVs in PPP we performed comparison experiments using TruCount bead tubes (BD Biosciences, San José, CA) alone or combined with plasma samples diluted 1:100. These beads have an approximate size of 4.2 μm and are designed for absolute enumeration of events via flow cytometry. The number of the enumerated MVs/μL was calculated as (($\text{# of events in region containing MVs}/\text{# of events in absolute count bead region}) \times (\text{# of beads per test/test volume})$. 4.2 μm TruCount beads were out of the dynamic range of the particle gating as described later in Figure 1A and could not be measured in the same plot. We therefore used an additional dotplot using FSC and SSC of the 488 nm laser in which TruCount beads were counted (Supporting Information Fig. 1). Additionally we compared the amount of measured TruCount beads with the volume measurement of the CytoFLEX (events/μL) and could not detect significant differences.

Flow Cytometric Analysis of Microvesicles

Flow cytometry was performed using a CytoFLEX flow cytometer (Beckman Coulter). The flow cytometer was equipped with 405 nm, 488 nm and 638 nm lasers to detect up to 13 fluorescence parameters. The CytoFLEX is equipped with a more sensitive SSC resulting in higher particle resolution compared to the FSC.

For daily routine, the CytoFLEX was turned on accordingly the manufacturers’ recommendations. Afterwards the machine was cleaned with filtered double distilled water from a freshly opened bottle for 30 minutes. As sheath fluid reagent double distilled water was used.

The CytoFLEX used during this study had a three laser pathway: red laser (638 nm) – blue laser (488 nm) – violet laser (405 nm). This means that normally the blue laser serves as the triggering laser, the red laser gets a positive time delay and the violet laser a negative time delay. The time delays are controlled daily during the standardized QC startup procedure.

To standardize the instrument for daily measurement and to detect MVs standard filter configuration was changed so that the SSC from the 405 nm violet laser (VSSC) was used as trigger signal to discriminate the noise instead of the
Figure 1. Flow cytometric analysis of microvesicles (MVs). (A) Fluorescence-gated polystyrene beads of different sizes were used to determine the MV gate (dotted line) between the 0.1 µm and 0.9 µm bead peaks. (B) Using the defined MV gate, all events positive for annexin V and calcein AM were defined as “true” MV events and were further analyzed for surface marker staining. (C) 0.1 µm fluorescence beads were detectable using the 405 nm side scatter (VSSC). (D) Two gating strategies based on the lower detection limit (0.1 µm or 0.2 µm beads) were used to evaluate the influence of the flow cytometer detection limit and the annexin V+calcine+ MV counts. Gating down to 0.1 µm (solid line, white bar) beads resulted in a significantly higher MV count compared to 0.2 µm (dotted line, gray bar) gating in all three tested anticoagulants. (E) To check the background noise of the used cytometer, filtered distilled water was measured and events were counted for 2 minutes resulting in a maximal background of 36 events/sec. (F) Serial PFP dilutions were used to determine the influence on the concentration of detected MVs to exclude the possibility of swarm effects. The solid line indicates the calculated regression line with a $R^2 = 0.99$. * $P < 0.05$; MV = microvesicles; VSSC = violet side scatter. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

normally used 488 nm FSC. When the VSSC was used as a trigger signal the noise was significant lower in comparison to the 488 nm SSC when beads were used as standardization reference as described below (16). The standard filter configuration for the 488 nm laser was 525/40BP, 582/42BP, 610/20BP, 690/50BP, 780/60BP for the 638 nm laser 660/20BP, 712/25BP, 780/60BP and for the 405 nm laser 450/45 nm BP, 525/40 nm BP, 610/20 BP, 660/20 BP, 780/60 BP and at a free position a 405/10 BP filter was placed. The changes in filter configuration to detect MVs were the following: the 405 nm laser filter
Figure 2. To demonstrate that the cytometer is clean and that there is no increasing noise when measuring the Gigamix solution, beads were measured at flow rates from 10 µL/min to 180 µL/min. For this purpose Gigamix beads were diluted 1:5 in distilled water. As can be seen in Figure 2A the number of the 100 nm beads (middle gray peak in the light gray area at the lower left end of the logarithmic scale) is very small and within the range of noise (light gray area). Increasing the flow rate makes the 100 nm beads more “visible” which clearly indicates that only the beads signal was increased but not the background noise. Also all other peaks are more prominent so that it is obvious that by increasing the flow rate the background noise is not increased and remains stable. With this assay we could also show that up to 120 µL/min flow rate a linear increase of measured particles was detectable (Fig. 2B). The upper range of measured particles was about 2,800 events/sec before they left the linear range and showed coincidence/swarming effects with increasing flow rates.

Position 450/45 nm was changed to 405/10 nm, the 525/40 nm was changed to 450/45 nm and the 610/20 nm was changed to 525/40 nm. 660/20 BP and 780/60 BP regarded to the 405 nm laser as well as the filters from the 488 nm and the 638 nm lasers were left unchanged.

For daily calibration of the flow cytometer fluorescent polystyrene beads (Megamix FSC & SSC Plus, BioCyte, Marseille, Fra) were used in sizes of 0.1, 0.16, 0.2, 0.24, 0.3, 0.5, and 0.9 µm. VSSC and FL1 channel gain were set so that the beads were visible as depicted in Figure 1A/C. The VSSC threshold was set as the trigger channel below the 0.1 µm bead population which gave us an acceptable noise of about 30–200 events/sec. For better discrimination of the beads, Megamix bead solution (Fig. 1A) was gated excluding the background noise. Background noise in Figure 1C was due to the Megamix bead solution. As can be seen in Figure 1E, measurement of filtered double distilled water generates a minimal background of approximately 36 events/sec. A rectangular gate was set between the 0.1 µm and 0.9 µm bead populations and defined as MV gate (Fig. 1A–1C).

To demonstrate that the machine is clean and that there is no increasing noise when measuring the Megamix solution, beads were measured at the flow rates from 10 µL/min = 180 µL/min. For this purpose Megamix beads were diluted 1:5 in distilled water. The triggering signal was set to the SSC of the 405 nm laser and was not combined with a fluorescence trigger. As can be seen in Figure 2A the population of the 100 nm beads is very small and within the range of noise. Increasing the flow rate makes the 100 nm beads more “visible” which clearly indicates that only the beads signal was increased but not the background noise (light grey). Also all other peaks are more prominent so that it is obvious that by increasing the flow rate the background noise is not increased and remains stable. With this assay we could also show that up to 120 µL/min a linear increase of measured particles was detectable. The upper range of measured particles was about 2,800 events/sec before they left the linear range (Fig. 2B).

Coincidence and/or swarm effects might be a serious problem when MVs are measured by flow cytometry (17). To avoid swarm effects dilution assays were additionally performed with platelet-free plasma (PPF) before MV analysis. For this purpose PPF was serially diluted from 1:2 to 1:1,000 and measured with a flow rate of 10 µL/min. From this and from the experiments with beads as described above we performed our assay with a maximum event rate up to 2,000 events/sec at the lowest flow rate (10 µL/min) for each anticoagulant.

As a result for optimal particle measurement in each used anticoagulants a dilution of 1:400 with a maximum event rate of 2,000 events per second was performed (Fig. 1F). It should be noted that the counting limit of the CytoFLEX (CytExpert Software Version 1.2) is up to 30,000 events/sec. However, to avoid swarming/coincidence effects, in our assay we used a maximum event rate of 2,000 events/sec as described above. To avoid carry-over effects between each sample measurement we performed a washing step with filtered double distilled water for 2 minutes at an increased flow rate of 60 µL/min.
Figure 3. Impact of used anticoagulant on microvesicle (MV) counts. Blood samples (n = 7) were drawn from healthy adult volunteers and immediately analyzed via flow cytometry. (A) Two different staining protocols were used to identify MVs: a single staining protocol using annexin V (SP-MV; dark grey bar) and a double staining protocol using annexin V and the intracellular dye calcein AM (DP-MV; white bars). Calcein AM is an intracellular dye which is activated by esterases in intact cells or vesicles. Therefore, we considered double positive events as “true” MV events to exclude unspecific staining of annexin V. (B/C) Specific surface staining of DP-MVs revealed the distribution of MV subpopulations (PMV = platelet derived microvesicles; EryMv = erythrocyte derived microvesicles; MMV = myeloid derived microvesicles) in the differently used anticoagulants underlining the divergent effects of different anticoagulants on peripheral blood cells and their MV release. * P < 0.05; error bars = standard deviation; N.S. = non significant

Functional Analysis

The procoagulatory function of MVs was assessed by a phospholipid dependent procoagulatory assay (ZYMUPHEN MP-activity assay, Aniara diagnostic, West Chester, OH). Briefly, citrated PFP was incubated on annexin V coated microwells, washed and a Factor Xa-Va mixture containing calcium and prothrombin was added. Dependent on the MV count, and therefore the phospholipid concentration, the thrombin generation was measured on an absorbance plate reader at 405 nm (Varioskan, Thermo Fisher Scientific, Waltham, MA).

For the MV-associated TF activity measurement, MVs were isolated from platelet free plasma (PFP) by centrifugation at 18,000 g for 20 min at 4°C, washed twice with Hank’s balanced salt solution and resuspended in 100 μL of HBSA. Samples were incubated with either mouse anti-human TF antibody or a control antibody for 15 min at RT, and then 50 μL aliquots were added to duplicate wells of a 96-well plate. Next, 50 μL of HBSA containing 10 nM activated factor VII (FVIIa), 300 nM factor X (FX) and 10 mM CaCl₂ was added to each sample and the mixture was incubated for 2 h at 37°C. Activated FX (FXa) generation was stopped by the addition of 25 μL of 25 mM EDTA HBSA buffer. 25 μL of the chromogenic substrate was then added and incubated at 37°C for 15 min. Finally, absorbance was measured at 405 nm. The TF-dependent FXa generation was determined by subtracting the amount of FXa generated in the presence of TF antibody from the amount of FXa generated in the presence of the control antibody.

Statistical Analysis

SPSS Statistics 22.0 (IBM, Armonk, NY) was used for statistical analysis. Data visualization was realized using SPSS and Adobe Illustrator CS6 (Adobe Systems, San José, CA). Flow Cytometric data were analyzed using FlowJo vX (FlowJo L.C, Ashland, OR). Statistical analysis was performed using multi-way analysis of variance (ANOVA) for repeated measurement. Further statistical calculation was performed using...
Table 1. MV counts after immediate blood drawing from healthy adult volunteers

<table>
<thead>
<tr>
<th>ANTICOAGULANT</th>
<th>SP-MV</th>
<th>DP-MV</th>
<th>PMV</th>
<th>ERMV</th>
<th>MMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>61277 ± 31307</td>
<td>30078 ± 20272</td>
<td>1991 ± 1872</td>
<td>1885 ± 1714</td>
<td>26310 ± 18406</td>
</tr>
<tr>
<td>Heparin</td>
<td>69762 ± 25501</td>
<td>5311 ± 4027</td>
<td>4519 ± 3904</td>
<td>804 ± 486</td>
<td>1121 ± 536</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>16696 ± 13518</td>
<td>2072 ± 1838</td>
<td>1751 ± 1733</td>
<td>1080 ± 934</td>
<td>175 ± 135</td>
</tr>
</tbody>
</table>

Peripheral blood MV counts of healthy adult probands (n = 7) after immediate blood drawing displaying higher variability within the probands. SP-MV = single positive microvesicles; DP-MV = double positive microvesicles; PMV = platelet derived microvesicles; EryMV = erythrocyte derived microvesicles; MMV = myeloid-derived microvesicles; Data are presented as mean ± standard deviation.

Results

The Impact of Used Anticoagulant on MV Count Immediately After Blood Collection

We first evaluated the direct effect of the used anticoagulant in the blood collection tube without any time delay. Further, we compared a double staining protocol with annexin V and calcein AM (DP-MVs) with an often used single annexin V staining (SP-MVs) to identify intact MVs. Both, SP-MVs and DP-MVs counts were significantly increased in EDTA and heparin tubes compared to the sodium citrate reference. Focusing on the staining protocol, SP-MV counts were significantly higher in EDTA (61,277 ± 31,307 vs 30,078 ± 20,272 MVs/μL; P = 0.007), heparin (69,762 ± 25,501 vs 5,311 ± 4,027 MVs/μL; P = 0.001) and sodium citrate (16,696 ± 13,518 vs 2,072 ± 1,838 MVs/μL; P = 0.038) tubes compared to DP-MVs (Fig. 3A). We were also interested in how different anticoagulants affected MV subpopulation counts. Noteworthy, both EDTA (26,310 ± 18,406 MVs/μL; P = 0.0006) and heparin (1,121 ± 536 MVs/μL; P = 0.002) displayed a higher CD15+ MV count compared to sodium citrate (175 ± 135 MVs/μL). Blood collection with heparin resulted in higher, but not significant (4,519 ± 3,904 vs 1,753 ± 1,733; P = 0.142), PMV count compared to sodium citrate. EDTA and sodium citrate displayed comparable PMV counts in MVs; (1,291 ± 1,375 vs 1,211 ± 1,35 MVs/μL; P = 0.985). The EryMV subpopulation was unaffected by the used anticoagulant. Only EDTA showed a slightly higher, but not significant (1,885 ± 1,714 vs 1,080 ± 934 EryMV/μL; P = 0.381), EryMV count compared to the sodium citrate reference (Fig. 3B/C). All data are summarized in Table 1.

Storage Time and Temperature Influence MV Count

As indicated above, we used a double staining protocol to identify intact MVs. Therefore, only DP-MVs were further investigated for the expression of specific surface markers. Sodium citrate tubes stored at RT or 4°C showed stable DP-MV, PMV, EryMV and MMV counts for 8 hours after blood collection. Longer storage of >24 hours resulted in a significant increase of DP-MV, PMV, EryMV and MMV counts compared to the 0 hours timepoint. Interestingly, sodium citrate tubes stored at 4°C resulted in a higher, but not significant, release of PMVs after 8 hours. Furthermore, chilled storage for longer than 24 hours showed stable MMV counts compared to RT storage (Fig. 4A).

Stored heparin tubes showed comparable MV count courses as sodium citrate. Storage at RT or 4°C up to 8 hours resulted in increased, but not significant, DP-MV, PMV, EryMV and MMV counts. Interestingly, cooled storage over 24 hours significantly increased the MV counts in all investigated subpopulation, except MMVs. Heparin tubes stored for 24 or 48 hours at RT resulted in higher, but not significant, counts of total MVs, PMVs and EryMVs (Fig. 4B).

As described above, EDTA exhibited a significantly higher total MV count due to the release of MMVs after blood drawing. However, motionless storage at RT or 4°C decreased the MMV count overtime and therefore the total MV count. Interestingly, EDTA tubes stored at RT displayed stable PMV and EryMV counts for 48 hours. Chilled storage showed slightly higher PMV and EryMV counts overtime (Fig. 4C).

We further analyzed calcein AM+/annexin V- and calcein AM-/annexin V+ effects (Supporting Information Fig. 2). AnnV exhibits positive signals for CD41/CD295/CD15 expression which might be unspecific binding to plasma contents caused by, e.g., protein and/or free floating membrane particles resulting in high counts of only single positive AnnV events. As can be seen in Supporting Information Figure 3 showing fresh drawn PPP of healthy adults (in our study time-point 0 hours), we found no significant expression of CD41, CD293 or CD15 on single positive calcein events. Comparing starting time point 0 with end point 48 hours, an increase of calcein+CD41+/ particles was detected. However there was no significant detection of CD293+ or CD15 expressing MVs.

MV Function Is Affected by Storage Time and Temperature

To validate our flow cytometry data, an annexin V capturing assay was performed to evaluate the MV count in sodium citrated PPP. Compared to the ECM data, procoagulant MV levels measured by capture onto annexin V were not significantly higher after storage for 8 hours at RT or 4°C. However, procoagulatory activity was significantly increased after 48 hours delay under both storage conditions (Fig. 5A).

TF activity was affected by time delay and storage temperature. MVs harbored a significantly higher TF activity after 24 hours storage at RT. In contrast, cooled blood storage did not significantly influence the TF activity (Fig. 5B).

Discussion

Several studies have shown that pre-analytical sample handling can influence the MV count and function (10–12,14). In this regard the ISTH and other groups did
Figure 4. Microvesicle (MV) subpopulation counts were differently influenced by the used anticoagulant, storage time and storage temperature. Blood samples were freshly drawn from healthy adult volunteers ($n = 7$) and collection tubes were stored motionless in vertical position at room temperature (RT, dotted line) or 4°C (solid line) for the indicated time points. Sodium citrate (A), heparin (B) and EDTA (C) had different effects on the MV counts overtime. Interestingly, storage temperature differently influenced the MV counts in different anticoagulants. At baseline time point the lowest MV count was observed using sodium-citrate anti-coagulated whole blood. In heparin and sodium-citrate tubes the MV count increased significantly after 8 hour storage. EDTA tubes stored at RT might be an option for the clinical routine due to the stable PMV and EryMV counts. Cooled storage of whole blood is not recommended for the enumeration of MVs. * $P < 0.05$; PMV = platelet derived microvesicles; EryMV = erythrocyte derived microvesicles; MMV = myeloid derived microvesicles.

A lot of work in the standardization of the isolation and characterization of peripheral blood microvesicles (10,14,18,19). The isolation procedure used in our study is based on their previous work. The main impulse for our work was to add important additional information on the analysis of microvesicles. We wanted to analyze, in a systemic way, the effect of long-term storage conditions combined with the used anticoagulants on MV count and function. To our knowledge, we are the first group contributing such data to the MV community. Beside the previous published results, we tried some different ways in the analysis of MVE (1). We analyzed the main MV subpopulations in human peripheral blood, trying to get an "overview" of the influence of storage time/conditions and used anticoagulants. (2) We aimed to compare under standardized isolation conditions (ISTH standard) the influence of various anticoagulants and storage time/temperature on MV populations. (3) For the first time, we added calcein AM as intracellular fluorescence stain to strengthen our data by analyzing intact microvesicles and (4) we used a flow cytometer with a 405 nm SSC adding a higher resolution which was also just recently described by Zucker et al. (16).

In the last decades, several pre-analytical blood processing steps have been described as major influencing factors for artificial MV generation ex vivo. Cells can be easily activated ex vivo by different blood collection techniques, used anticoagulant, transportation, time delay and centrifugation steps which are limiting factors for the standardized and accurate measurement of MVs in pre-clinical and clinical setting.

Different anticoagulants were used in several studies to collect blood for MV analysis. Overall, sodium citrate (3.2% or

Figure 5. Functional analysis of microvesicles (MVs) in sodium citrate PFP. (A) The phosphatidylserine dependent thrombin generation assay ($n = 7$) showed a similar increase of MVs in PFP after prolonged storage at RT or 4°C. (B) Tissue factor positive (TF+ MVs) increased during long storage of whole blood at RT ($n = 7$). Cooled storage of whole blood seems to inhibit the release of TF+ MVs up to 48 hours. * $P < 0.05$; error bars = standard deviation.

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Table 2. Known and proposed pre-analytical and analytical settings

<table>
<thead>
<tr>
<th>PRE-ANALYTICAL PARAMETERS</th>
<th>RECOMMENDATIONS</th>
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<tbody>
<tr>
<td>Blood collection tube</td>
<td>PMV: sodium citrate</td>
</tr>
<tr>
<td>for immediate</td>
<td>EMV: sodium citrate, heparin</td>
</tr>
<tr>
<td>analysis</td>
<td>MMV: sodium citrate</td>
</tr>
<tr>
<td>Blood collection</td>
<td>PMV: EDTA</td>
</tr>
<tr>
<td>tube for delayed</td>
<td>PMV: FITA</td>
</tr>
<tr>
<td>analysis &gt; 8 hours</td>
<td>MMV: sodium citrate (4°C), heparin (4°C)</td>
</tr>
<tr>
<td>Blood collection</td>
<td>Needle &gt;21 gauge without tourniquet, discard the first milliliters (10).</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>Two consecutive centrifugation steps (e.g., 2,500g for 15 minutes followed by 13,000g for 5 minutes) to obtain platelet-free plasma (10).</td>
</tr>
<tr>
<td>Storage conditions</td>
<td>Maintain tubes vertically at K1 for PMV and EMV analysis. Cooled storage for MMV analysis.</td>
</tr>
<tr>
<td>Staining</td>
<td>Centrifuge antibody solution prior to staining to remove fluorescent particles (e.g., 20,000g for 30 minutes (15)).</td>
</tr>
<tr>
<td>Cytometer Settings</td>
<td>SSC with short wavelength (405 nm) Filtered sheath fluid Washing step between each sample (&gt;2 minutes) to reduce noise</td>
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</table>

PMV = platelet derived microvesicles; EmryMV = erythrocyte derived microvesicles; MMV = myeloid-derived microvesicles.

3.8%) is the most widely used anticoagulant for the analysis of MVs (20). Acid-Citrate-Dextrose (ACD) (21,22), Citrate-Theophylline-Adenosine-Dipyridamol (CTAD) (14), EDTA (13,23,24) and heparin (25,26) have been used to investigate blood MVs with divergent results. Avoiding the activation of platelets seems to be an important pre-analytical step. Different anticoagulants seem to differently activate platelets during collection and preparation. Activation of platelets results in release of alpha and dense granules and as well as PMVs (27,28). By the chelation of free calcium, sodium citrate inhibits the degranulation of platelets and leukocytes (29) and furthermore seems to prevent the vesiculation process (30). However, sodium citrate had the lowest MV count after blood collection compared to EDTA and heparin. Especially EDTA is known to induce a P-selectin-dependent platelet activation which might result in falsely high MV counts (31). Interestingly, in our study sodium citrate and EDTA showed comparable PMV counts after immediate blood processing without time delay. However, choosing the proper collection tube for MV analysis is essential. As shown by Philippe et al. (32), even different sodium citrate tube brands seem to influence the platelet reactivity and therefore might influence the MV count and function. To our knowledge, there is no study which directly assesses the influence of different sodium citrate tubes on MV count and function.

Storage time and conditions are important pre-analytical factors for the investigation of MVs. Ideally, blood is immediately processed after collection to avoid changes in MV count and function (20). However, in the clinical routine, the logistics of blood sample transport, transport duration and in-house processing times are variables which are difficult to standardize and control across different laboratories. Therefore, the assessment of storage time and temperature are crucial for standardized sample handling for MV analysis. According to the Clinical and Laboratory Standards Institute (CLSI) standards, cooled storage of whole blood samples is not recommended due to possible activation of FVII and FVIII, loss of von Willebrand factor (vWF) and platelet disruption (33). In our study, storage at RT or 4°C had different influences on MV subpopulation counts and MV function. Cooled storage seems to increase, but not significantly (P = 0.11), the release of MVs 4 to 8 hours after blood collection in sodium citrate and heparin tubes. During storage, whole blood samples should be stored at RT without agitation (10). As described above, sodium citrate is the recommended anticoagulants for MV analysis (20). Interestingly, in our study EDTA preserved the PMV and EryMV count up to 48 hours after blood collection. In contrast, sodium citrate displayed stable PMV and EryMV counts up to 8 hours. When blood is not processed immediately, Connor et al. (13) suggested EDTA as an alternative for MV enumeration in peripheral whole blood. The stable PMV and EryMV counts might suggest an advantage of EDTA for the long term storage for MV enumeration but has to be evaluated in a larger preclinical and clinical setting. The proposed pre-analytical setting is summarized in Table 2.

In the last decade, several studies investigated the impact of TF⁻ MVs in peripheral blood under various clinical conditions (34–36). The enumeration of TF⁻ MVs by FCMD is yet not fully established and validated (15,37). Therefore, we used a TF-based activity assay to evaluate the concentrations of TF expressed on circulating MVs and a phosphatidylserine (PS)-dependent thrombin generation assay after different storage times and temperatures. Although the cooled storage induced the release of MVs as seen via flow cytometry and thrombin generation, TF-dependent activity remained stable for 48 hours. Interestingly, storage at RT seems to induce the release of TF⁺ MVs in whole blood. The mechanism behind this process is unknown, but may be due to unspecific activation of monocytes overtime.

These new approaches need to be validated in a clinical laboratory setting before being applied in patient studies. Several review articles discussed the must of the standardization of microparticle analysis and the advantage of flow cytometry technique in the measurement of submicron particles in comparison to other techniques (38,39). LaCroix et al. (40) made steps toward a standardized laboratory setting by comparing
matched samples in different laboratories equipped with different cytometers - with divergent results. It seems that the MP measurement is highly dependent of the used cytometer (optimized for FSC or SSC) and dependent of the calibration strategy. In this regard Zucker et al. (16) could demonstrate that the 405 nm SSC is more sensitive than the 488 nm SSC when silver and/or gold nanoparticles were used; the discrimination and the CVs of this particles gave better results. Recently, Poncet et al. (41) showed the feasibility of different calibration protocols for FSC- and SSC-based flow cytometers. This calibration protocols open the opportunity for multicenter laboratory studies. As also recently shown by Arraud et al. (12), fluorescence triggering for MV analysis seems feasible and, interestingly, results in higher MV counts compared to the scatter triggering. This might be an important step toward standardization of fluorescence triggering. These steps need to be done to establish and validate a clinical laboratory setting. In this article Arraud et al. also detected significant amounts of particles not positive for annexin V. These results are only partly in accordance with our results. When calcine AM+/AnnV- events were analyzed we could not detect significant CD41, CD235a or CD15 positive events at time point 0. At time point 48 hours we also detected CD41 positive events on single calcine positive events but not for CD235a or CD15. Single Annex V positive events might be attributed to membrane debris and/or protein aggregates which in turn additionally can be stained for surface molecules and therefore might detect false positive particles. Although we carefully designed the calcine staining in our protocol, we cannot exclude “unspecific” staining of calcein. Therefore we primarily excluded calcine+/AnnV- events in our analysis.

In conclusion the primary aim of our study was to compare whole blood drawn with different anticoagulants at several time points and under various temperature conditions. We therefore designed a protocol including MV isolation, staining and flow cytometry measurement which, with some precautions, can be easily performed in the daily routine. The used anticoagulants, storage time and storage temperatures differently influence the analysis of MVs. To date, sodium citrated tubes are commonly used and recommended for MV enumeration and functional analysis. EDTA tubes might be an option for the clinical routine due to the stable PMV and EryMV counts. Cooled storage of whole blood is not recommended for the enumeration of MVs. Although it induced the artificial release of MVs overtime, functional assays as FXα conversion via TF on MVs might benefit from cooled blood storage by inhibition of the release of TF-V-MVs.

Literature Cited


