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Regular Article

Measurement of circulating cell-derived microparticles by flow cytometry: Sources of variability within the assay

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ABSTRACT

Introduction: Circulating cell-derived microparticles (MPs) have been implicated in several disease processes and elevated levels are found in many pathological conditions. The detection and accurate measurement of MPs, although attracting widespread interest, is hampered by a lack of standardisation. The aim of this study was to establish a reliable flow cytometric assay to measure distinct subtypes of MPs in disease and to identify any significant causes of variability in MP quantification.

Materials and Methods: Circulating MPs within plasma were identified by their phenotype (platelet, endothelial, leukocyte and annexin-V positivity (AnnV+). The influence of key variables (i.e. time between venepuncture and centrifugation, washing steps, the number of centrifugation steps, freezing/long-term storage and temperature of thawing) on MP measurement were investigated.

Results: Increasing time between venepuncture and centrifugation leads to increased MP levels. Washing samples results in decreased AnnV + MPs ($P = 0.002$) and platelet-derived MPs (PMPs) ($P = 0.002$). Double centrifugation of MPs prior to freezing decreases numbers of AnnV + MPs ($P = 0.0004$) and PMPs ($P = 0.0004$). A single freeze thaw cycle of samples led to an increase in AnnV + MPs ($P = 0.0020$) and PMPs ($P = 0.0039$). Long-term storage of MP samples at -80° resulted in decreased MP levels.

Conclusions: This study found that minor protocol changes significantly affected MP levels. This is one of the first studies attempting to standardise a method for obtaining and measuring circulating MPs. Standardisation will be essential for successful development of MP technologies, allowing direct comparison of results between studies and leading to a greater understanding of MPs in disease.

Introduction

Circulating cell-derived microparticles (MPs) are sub-cellular vesicles released during cell activation or apoptosis, ranging in size from 0.1 μm to 1.0 μm [1]. Derived from the plasma membrane of a cell, they may express cell surface receptors from their parental cells. These surface markers allow identification of sub-groups of MPs, derived from platelets, endothelial cells, leucocytes, erythrocytes, vascular smooth muscle cells [2] and other tissue cells.

MPs have been implicated in several disease processes and are elevated in a variety of pathologies including many thrombotic and inflammatory conditions. They are thought to play a role in amplifying inflammatory responses in the development of atherosclerosis [3]. Indeed, elevated MP levels have been observed in several cardiovascular pathologies including stroke, hypertension and acute coronary syndromes [4]. Distinct patterns of elevated MPs may not only offer a potential unique set of biomarkers of various thrombotic diseases but significantly add to the risk assessment of primary and secondary thrombotic events in high risk individuals. The detection and accurate measurement of circulating cell-derived MPs is therefore of potentially great importance. Several techniques have been developed which aim to detect and quantify MP levels in circulating blood, including flow cytometry, procoagulant assays, and ELISA-based solid-phase capture assays [5].

Despite their enormous potential, the variety of methods used by different laboratories, coupled with the lack of standardisation of

Abbreviations: MP, Cell-derived microparticle; AnnV, Annexin-V; PMPs, Platelet-derived microparticles; EMPs, Endothelial-derived microparticles; LMPs, Leukocyte-derived microparticles; OSA, Obstructive Sleep Apnoea; FSc, Forward Scatter; SSc, Side Scatter; PPP, Platelet-poor-plasma; PRP, Platelet-rich-plasma.

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these methods, is a major impediment to the measurement and understanding of the role of MPs in disease [6,7]. Details on optimally preparing, processing and storing blood samples, along with the influence of different analytical techniques on MP counts are sparse. In particular, although flow cytometry offers a popular approach to phenotyping and qualifying MPs, there are few detailed protocols available.

In this study we aimed to identify potential causes of variability in MP quantification using a flow cytometric assay to measure annexin V+ (AnnV+), platelet-derived MPs (PMPs), endothelial-derived MPs (EMPs) and leukocyte-derived MPs (LMPs) from plasma in healthy controls and obstructive sleep apnea (OSA) patients, some of which had an increased cardiovascular risk. OSA patients were used in some experiments, as EMPs and LMPs are only found at very low levels in healthy individuals [8], and at increased levels in OSA [9].

Materials and methods

Subjects

Healthy control samples were obtained from laboratory staff. Informed consent was obtained in all cases. OSA patients were eligible if they were aged between 45 and 75 years, had proven OSA with a severity defined as more than 7.5 oxygen desaturations of >4% per hour, and no history of excessive daytime sleepiness or any other daytime symptom of OSA. All eligible patients were offered participation in the study. Plasma from 56 OSA patients was used. The study was approved by the Oxford Research Ethics Committee (REC No: 05/Q1604/159), and written informed consent was obtained from all participants.

Flow cytometric method for detection of MPs

Preparation of MP samples

The flow cytometric assay to measure MPs was adapted from the method originally described by Nieuwland et al [5]. Blood from healthy controls and OSA patients was drawn into citrate tubes (BD Vacutainer) using a 19 G needle. The first tube following venepuncture was discarded. Within 15 min of the blood being taken, tubes were centrifuged at 1550×*g* for 20 min at 20 °C, to produce platelet-poor-plasma (PPP). PPP was carefully removed, without disturbing the buffy coat. 250 μl aliquots of PPP were frozen immediately, by placing at -80 °C, and stored.

Selection of surface markers for MP subgroups

Annexin-V-FITC (BD Pharmingen, 556420) was used as a general marker for MPs. Since not all MPs expose phosphatidylserine, both AnnV positive and AnnV negative MPs were measured in all experiments. PECAM-1 (CD31) PE (BD Pharmingen, 555446) and Integrin-α2b (CD41) PE Cy5 (BD Pharmingen, 559768) were used to identify PMPs. Common leukocyte antigen (CD45) APC (BD, 340910) was used as a marker for LMPs. A number of EMP markers were used, including VE-Cadherin (CD144) PE (eBioscience, 12-1449-80), V-CAM 1(CD106) PE Cy5 (BD Pharmingen, 551148) and E-selectin (CD62E) PE Cy5 (BD Pharmingen, 550040). Monoclonal antibodies were centrifuged at 18,000×*g* for 5 min to remove protein aggregates.

Labeling of MPs

Ann-V FITC was used to label phosphatidylserine-positive MPs in PBS-calcium (2.5 mmol/L) buffer. AnnV-FITC in the absence of calcium (PBS-Citrate (0.32%) buffer) was used as a negative control. Appropriate PE, PE-Cy5 and APC isotypes were used as negative controls. All antibodies were titrated, using either control or patient plasma, to determine optimal concentrations. 5 μl of diluted AnnV-FITC or monoclonal antibodies were added to PBS-calcium (2.5 mmol/L) to make up a total volume of 50 μl. 10 μl of sample was incubated with the appropriate monoclonal antibodies for 30 min at room temperature, protected from light. After the incubation, 900 μl of PBS-calcium (2.5 mmol/L) was added.

Establishing a MP gate

To establish a suitable MP gate on a flow cytometry plot of forward scatter (FSc) vs. side scatter (SSc), it was necessary to distinguish between MPs and small platelets. Platelet-rich-plasma (PRP) was therefore prepared by centrifugation of citrated blood at 200×*g* for 10 min. The PRP was analysed on a BD FACSCalibur (Becton Dickinson, Oxford, UK), to establish a platelet gate. This technique was described by Nieuwland and Sturk [1]. 60 μl of PRP was then incubated with 20 μl of 2 μM calcium ionophore A23187 (Sigma I0634) for 5 hours. Calcium ionophore is known to stimulate platelets to produce MPs [1,10]. The stimulated PRP was used to establish the upper limit of the MP gate and was confirmed in each experiment with 1 μm beads. 0.5 μm beads were used to determine the lowest detectable limit, and therefore used to set the threshold. The threshold was set on SSc so to exclude background noise, which is determined by running 0.1 μm filtered PBS-calcium. A fluorescence threshold was avoided in this study to allow analysis of both the AnnV positive and negative MPs. This gate was used for all other experiments.

Acquisition and analysis of MPs on the BD FACSCalibur

Fluorescent cross-talk was controlled by compensation adjustment. Compensation settings were established by acquiring single-colour stained tubes. The MP gate was checked at the beginning of each assay by measuring a tube containing 10 μl of MPs and 5 μl 1:1000 dilution of 1 μm beads (Sigma L-2778) in 950 μl of PBS-Calcium (2.5 mmol/L). The flow rate of the FACSCalibur was determined daily by acquiring a weighed tube of PBS-Ca for 10 min, then weighing the tube and calculating the volume of liquid taken up. This figure is divided by 10 to give the flow rate per min.

Sources of variability within the assay

Effect of time before initial centrifugation of blood samples

Eight consecutive blood samples were taken, from eight healthy controls. Citrated blood samples were left standing upright undisturbed for the following times, before centrifugation: 15 min, 30 min, 45 min, 60 min, 75 min, 90 min, 105 min and 120 min. The number of AnnV+ MPs and PMPs were then determined for each sample 8 times and the mean level was calculated.

Effect of washing Platelet-Poor-Plasma

Ten healthy controls had two 250 μl aliquots of PPP stored at -80 °C for 1 month and thawed on melting ice. One aliquot was washed by centrifugation at 18,000×*g* for 30 min, after which 225 μl of supernatant was removed and 225 μl of PBS-Citrate (0.32%) added. Then centrifuged again at 18,000×*g* for 30 min, 225 μl of supernatant was removed and 75 μl of PBS-Citrate (0.32%) was added. The second aliquot was labeled for MPs without any washing steps.

Single versus double centrifugation

Two citrate blood samples were taken from 18 healthy controls. One sample was centrifuged at 1550×*g* for 20 min, as per a single centrifugation protocol. The other sample was centrifuged at 1550×*g* for 20 min, followed by a second centrifugation at 13,000×*g* for 2 min. Aliquots of PPP were taken from each sample and were immediately frozen at -80 °C. Samples were then thawed, washed and analysed according to the above protocol.

Fresh versus frozen samples

Citrated blood was obtained from 10 healthy controls, and was centrifuged using the double centrifugation protocol. Two 250 μl aliquots of PPP were taken from each control. One was washed,

labeled and analysed within 3 hours and the second was placed in a -80 °C freezer, stored for one month then thawed on ice, washed, labeled and analysed.

Effect of thawing temperature on MP samples

Citrated blood was obtained from 10 healthy controls, and was centrifuged using the double centrifugation protocol. Three aliquots of PPP were frozen at -80 °C. The samples were either; thawed on melting ice, at room temperature (RT) or at 37 °C, then washed, labelled and analysed.

Effect of long-term storage at -80 °C of MP samples

56 OSA patients had 2 aliquots of PPP stored at -80 °C for MP analysis. The first aliquots were thawed and analysed within 12 months of storage. The second aliquots were stored at -80 °C for an additional 20 months before they were thawed and analysed.

Statistics

All statistics were performed using GraphPad Prism 4 Software (GraphPad Software, San Diego). The effect of time before centrifugation was assessed by a repeated measures ANOVA, followed by the post hoc test for a linear trend. The effect of thawing temperature was assessed by repeated measures ANOVA. Paired results in the effect of washing, single spin versus double spin, fresh versus frozen experiments were compared by Wilcoxon Signed Rank test. A p-value of <0.05 was considered statistically significant.

Results

Flow cytometric method for detection of MPs

The calcium ionophore stimulation of PRP was used to distinguish MPs from small platelets. The gates were set so that in PRP the major population falls in the platelet gate, and in calcium ionophore stimulated plasma the major population falls in the MP gate. Fig. 1 shows that there is some overlap between platelets and MPs on a forward-scatter vs. side scatter plot.

In PPP samples, procoagulant MPs are identified by AnnV binding (Fig. 2A), PMPs were identified by CD31+ CD41+ markers (Fig. 2B), LMPs were identified by CD45+ (Fig. 2C). CD144+, CD106+ and CD62E+ were used as markers of EMPs (Figs. 2D, E). Figs. 2B-E show all MPs, including those that are AnnV negative.

In a preliminary study, using 20 of the OSA patients, individual sub-populations of MPs were assessed for their ability to bind AnnV+. The percentage that bound AnnV in each MP subgroup varied greatly between individuals. The percentage of CD31+CD41+ PMPs that bound AnnV was 93.4% (±3.9%). The percentage of CD45+ LMPs that bound AnnV was 56.8% (16.8%). The percentage of CD144+ EMPs that bound AnnV was 52.6% (±18.9%). The percentage of CD106+ EMPs that bound AnnV was 62.0% (±29.8%). The percentage of CD62P+ EMPs that bound AnnV was 70.4% (±19.6%).

Sources of variability within the assay

Increasing time between venepuncture and centrifugation leads to increased MP levels

Fig. 3 shows that the number of plasma AnnV+MPs and PMPs increases with the length of time between venepuncture and centrifugation. There appears to be a plateau at around 1 hr post venepuncture. Repeated measures ANOVA was significant for both AnnV+MPs ($P=<0.0001$) and CD31+CD41+PMPs ($P=<0.0001$), and the post hoc test revealed a significant linear trend over increasing time in both cases ($P=<0.05$). The MP levels appear to more than double if blood samples are left for 2 hours before centrifugation, compared to immediate processing.

Washing samples results in decreased AnnV+ MPs and CD31+CD41+ PMPs

There was a significant decrease ($P=0.002$) in the number of events in the MP gate, particularly in samples with initially very high levels, following washing of PPP samples (Fig. 4A). There was also a significant decrease in AnnV+MPs ($P=0.002$) (Fig. 4B) and CD31+CD41+PMPs ($P=0.002$) (Fig. 4C) following washing of the plasma samples. There was no significant difference in the levels of LMPs and EMPs between washed and unwashed samples.

Double centrifugation causes a decrease in AnnV+MPs and CD31+CD41+PMPs

The introduction of a second centrifugation at 13,000×*g* for 2 min, prior to freezing of PPP significantly reduces the number of AnnV+MPs ($P=0.0004$) (Fig. 5A) and CD31+CD41+PMPs ($P=0.0004$) (Fig. 5B). The levels of all populations of EMPs and LMPs were not significantly different between single and double centrifuged samples.

Freezing of samples leads to an increase in AnnV+MPs and CD31+CD41+PMPs

Fresh aliquots of PPP, which had been double centrifuged, were analysed within 3 hours of venepuncture, and were compared with paired samples that had been frozen at -80 °C for 1 month (Fig. 6). Numbers of AnnV+MPs ($P=0.0020$) (Fig. 6A) and CD31+CD41+PMPs ($P=0.0039$) (Fig. 6B) increased significantly following freezing

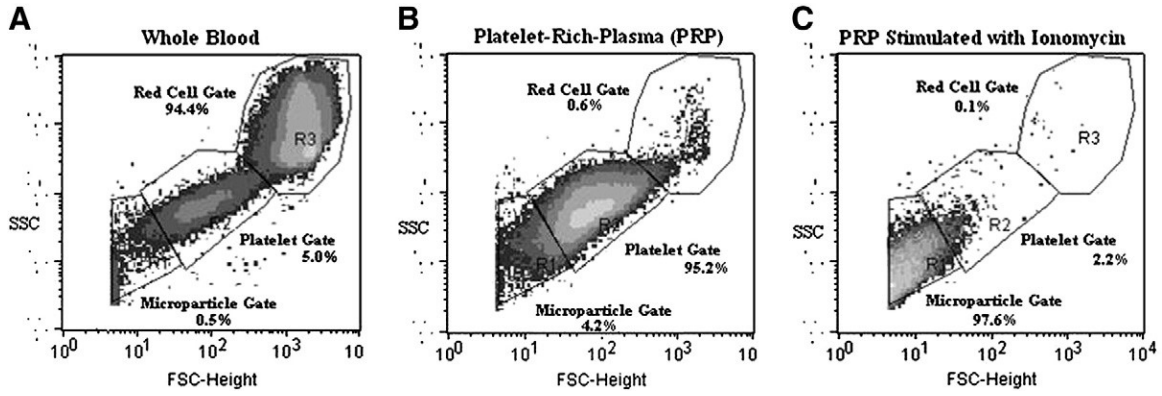

Fig. 1. FSc vs. SSc flow cytometry plots showing distinct red cell, platelet and MP gates. Plot A shows analysis of unstimulated whole blood. Plot B shows analysis of unstimulated platelet-rich-plasma. There are few MPs and mainly platelets. Plot C shows analysis of platelet-rich-plasma stimulated with Calcium Ionophore for 5 hours. Most events now appear in the MP gate. This gate was used to establish the MP gate for all future experiments.

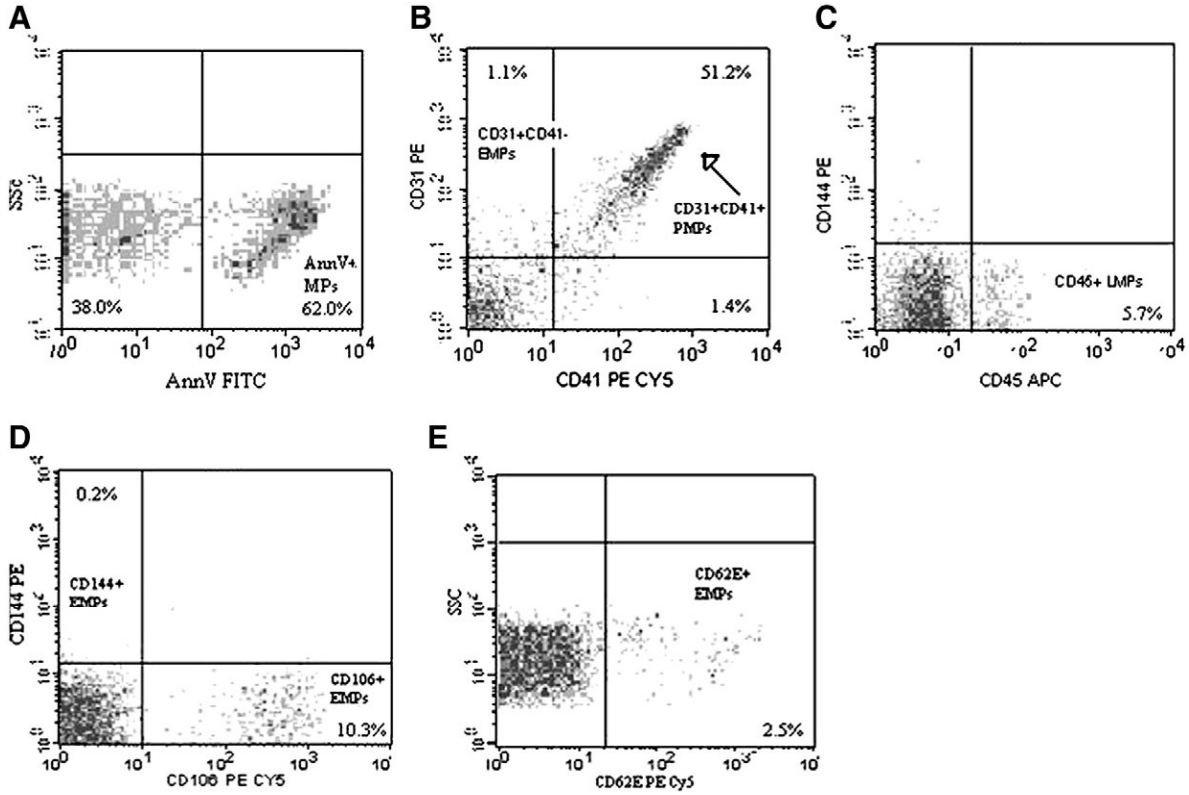

Fig. 2. FACs plots of MAb labeled PPP. Fig. 2A shows AnnV staining of MPs expressing phosphatidylserine. Fig. 2B-2E include both AnnV + and AnnV- MPs. Fig. 2B shows CD31-PE and CD41-PE Cy5 staining, defining CD31 + CD41 + PMPs and CD31 + CD41- EMPs. Fig. 2C shows CD45-APC staining, defining CD45 + LMPs. Fig. 2D shows CD144-PE and CD106-PE Cy5 staining, defining CD144 + EMPs and CD106 + EMPs. Fig. 2E shows CD62E-PE Cy5 staining, defining CD62E + EMPs.

of the sample. All other populations of LMPs and EMPs did not show a significant change in level between fresh and frozen samples.

Temperature of thawing does not affect MP levels

Fig. 7 shows the effect of thawing temperature on the levels of AnnV+ and PMPs detected There was no significant difference found in the number of AnnV+ (Fig. 7A) and CD31 + CD41 + PMPs (Fig. 7B) between samples that had been thawed on ice, at RT or at 37 °C.

Long-term storage of samples at -80° results in decreased MP levels

56 OSA patient PPP samples were analysed for all MP subgroups firstly within 12 months of storage at -80 °C, and then after an additional 20 months at -80 °C (Fig. 8). All types of MPs were significantly reduced (AnnV + MPs P = 0.0002, CD31 + CD41 + PMPs P = 0.0004, CD45 + LMPs P = 0.0008, CD144+ EMPs P = <0.0001, CD106 + EMPs P = 0.0021) after an additional 20 months storage, except for CD31 + CD41- EMPs.

Discussion

This study found that minor protocol changes in a flow cytometric MP analysis method, such as, time before processing, washing of PPP, speed and number of centrifugations, freezing and long-term storage of samples, all lead to significant differences in MP levels.

Flow cytometric method for detection of MPs and validation

The overlap between platelets and PMP (Fig. 1) is expected, as the smallest platelets form a continuum in size with the largest PMPs [11]. This is a potential limitation of detecting MPs by flow cytometry.

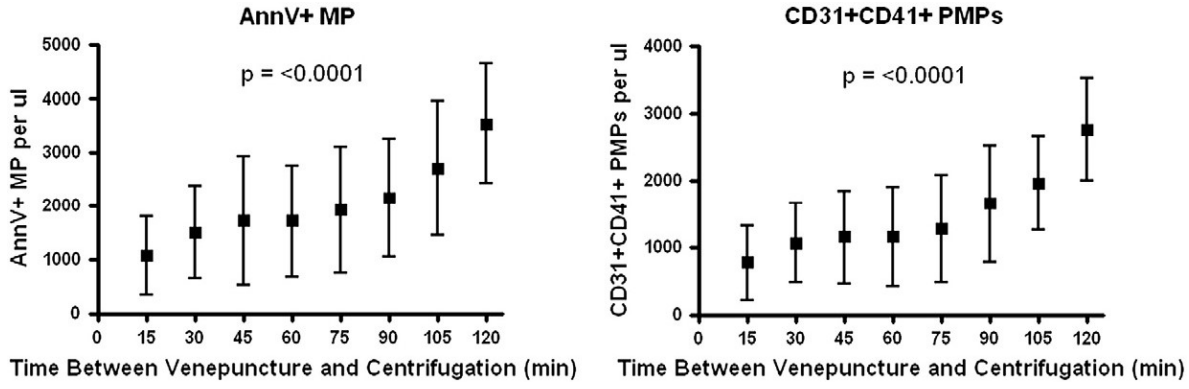

Fig. 3. The number of AnnV + MPs and PMPs compared to the length of time between venepuncture and centrifugation of blood samples. 8 citrate tubes were taken from 8 healthy controls and left for increasing length of time prior to centrifugation and freezing. Each sample was analysed 8 times and the mean for each sample was plotted. The graph shows the mean and 95% CI for the eight controls at each time point.

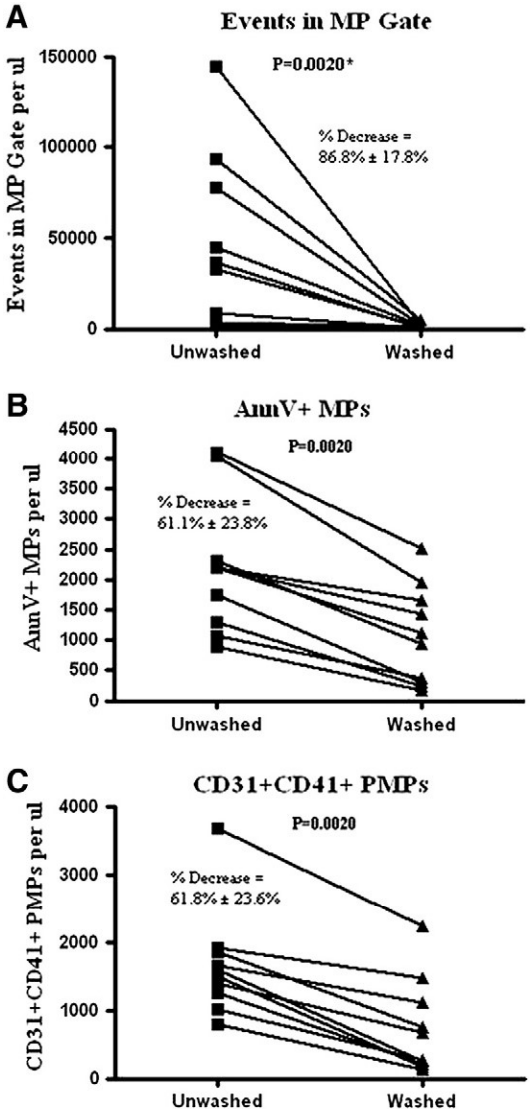

Fig. 4. The number of Events in MP Gate (A), AnnV + MPs (B) and CD31 + CD41 + PMPs (C) for unwashed and washed samples. Unwashed samples were thawed and stained. Washed samples were thawed and spun at 18,000 g for 30 min at RT, removing the supernatant, twice.

Within this study a number of markers were used to define subpopulations of MPs (Fig. 2). Although not present on all MPs [12,13], AnnV is considered a useful marker to determine general levels of MPs, particularly those found in procoagulant conditions. Preliminary data in this study, suggests that different subgroups of MPs appear to bind AnnV at varying levels. For example, around 90% of PMPs were also AnnV+, whereas only around 50% of CD144+ EMPs were also AnnV+. AnnV binding to MPs is also very variable between individuals, so it is important to measure both AnnV+ and AnnV- MPs.

Lactadherin is another marker that binds phosphatidylserine, but does not require the presence of calcium, so could prove to be a useful marker of MPs [14]. Bio-maleimide, a general MP marker, has been shown to give comparable results to AnnV. It is considerably cheaper and can be stored for longer, making it a useful marker for large scale analysis of total MP levels [15].

We used CD31 and CD41 as markers to define PMPs [13,16], both of which have been used previously to observe levels of PMPs in stroke patients [16]. CD45 is a marker for LMPs and has been used to define LMPs in cardiovascular disease [4] and pulmonary hypertension [17].

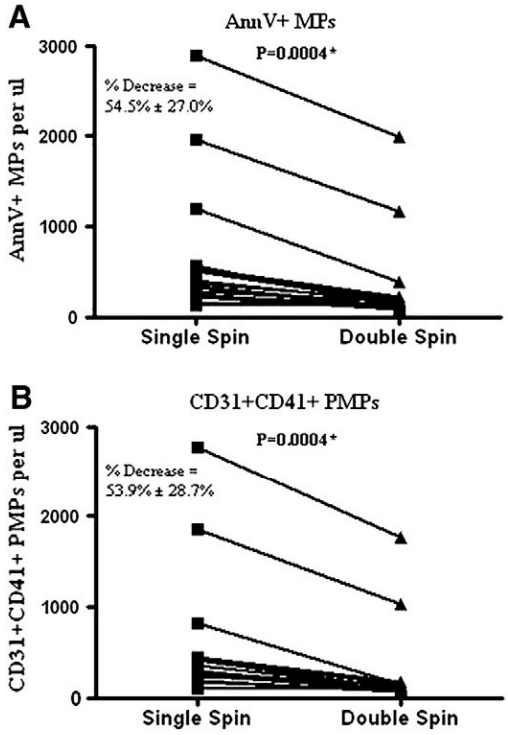

Fig. 5. The number of AnnV + MPs (A) and CD31 + CD41+ PMPs (B) for single spun and double spun samples. Single spun samples were spun at 1550 g for 20 min, before freezing at -80 °C. Double spun samples were spun at 1550 g for 20 min, followed by centrifugation at 13,000 g for 2 min, before freezing at -80 °C.

Several subgroups of EMPs exist, which can be distinguished by different surface markers and these subgroups are thought to be associated with different pathological mechanisms. Thus CD62E+

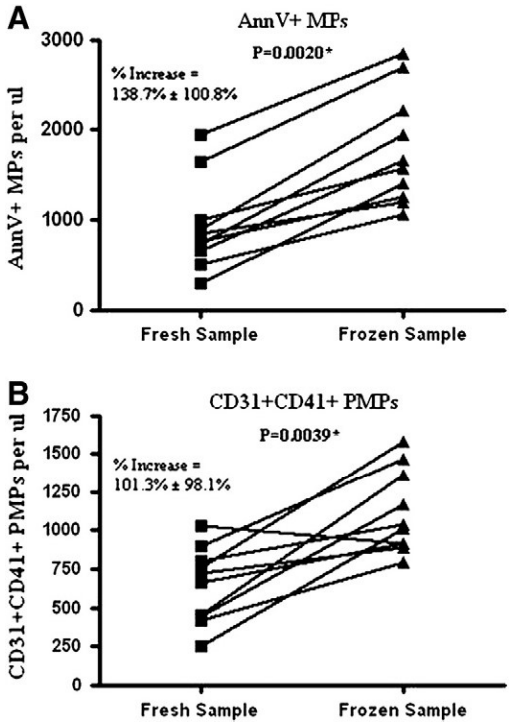

Fig. 6. The number of AnnV + MPs (A) and CD31 + CD41+ PMPs (B) for fresh and frozen samples, when using a double spin. Fresh samples were run within 3 hours of venepuncture. Frozen samples were centrifuged, frozen stored at -80 °C for 1 month, then thawed and analysed.

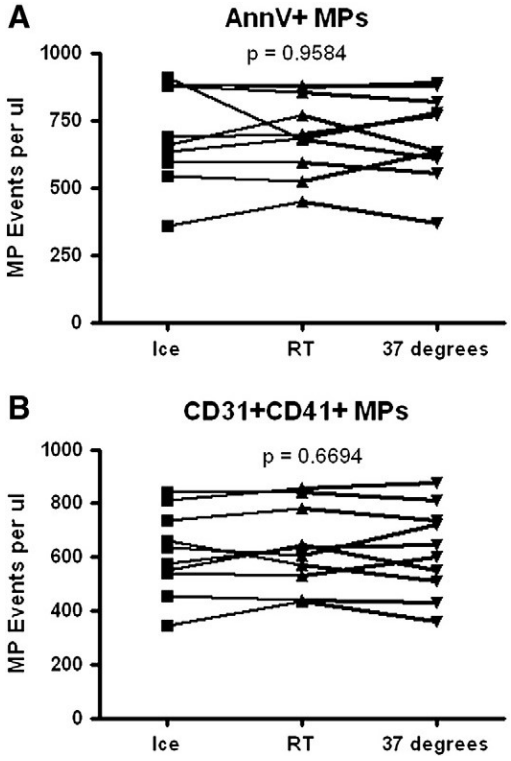

Fig. 7. The effect of thawing temperature on the numbers of AnnV + MPs (A) and CD31 + CD41 + PMPs (B) detected. Samples had been single spun, 3 aliquots were taken and frozen at -80 °C. For each control, one aliquot was thawed on ice, one at RT and one at 37 °C.

EMPs are suggested to be markers of early endothelial cell activation, while raised numbers of CD144+ and CD31 + CD41- EMPs may reflect structural damage of endothelial cells [17]. In addition, *in vitro* experiments have shown that using different stimuli to induce EMP release leads to the release of phenotypically distinct EMPs. Activation by TNF-α results in elevation of CD62E + EMPs, whereas endothelial destruction leads to release of CD31+ EMPs [11,18]. A number of endothelial surface markers were explored in this study, and multiple EMP markers may be necessary to provide information that reflects the nature of endothelial injury in different disease states [18]. Although CD31 + CD41- was used as one alternative marker for EMPs in this study, it is acknowledged that the use of negative staining may not be sufficiently specific to identify MP subgroups. For example, LMPs could also be CD31 + CD41-. Therefore, cell-specific markers should ideally be used for MP identification.

Linearity analysis was performed on all MP subgroups and the R^2 values were all greater than 0.93 (data not shown), suggesting that this assay is able to detect MPs present at variable levels in plasma. This is important as MP levels can vary greatly between patients and controls.

Sources of variability within the assay

Our results clearly demonstrate that the longer the time-delay between venepuncture and centrifugation, the higher the number of MPs detected (Fig. 3). This is likely to be due to platelets and other blood cells continuing to release MPs *in vitro*, after venepuncture. Therefore it is very important that samples are processed within strict time-frames and that handling of samples should be identical in every subject in order to get interpretable results.

There was a significant decrease in the number of events in the MP gate following washing (86.8% ± 17.8%) (Fig. 4). This may be due to the loss of other particles, such as chylomicrons [19], during the washing step, which would have otherwise been detected in the microparticle gate. There was also a significant decrease in the number of AnnV + MPs (61.1% ± 23.8%) and CD31 + CD41 + PMPs (61.8% ± 23.6%), following washing. It is possible that during washing, MPs are sticking together and therefore multiple vesicles are only detected as a single event, artificially affecting the MP count. Alternatively, it is possible that the pelleting step of 18,000 g might not be sufficient to sediment all MPs. However, MP levels were not

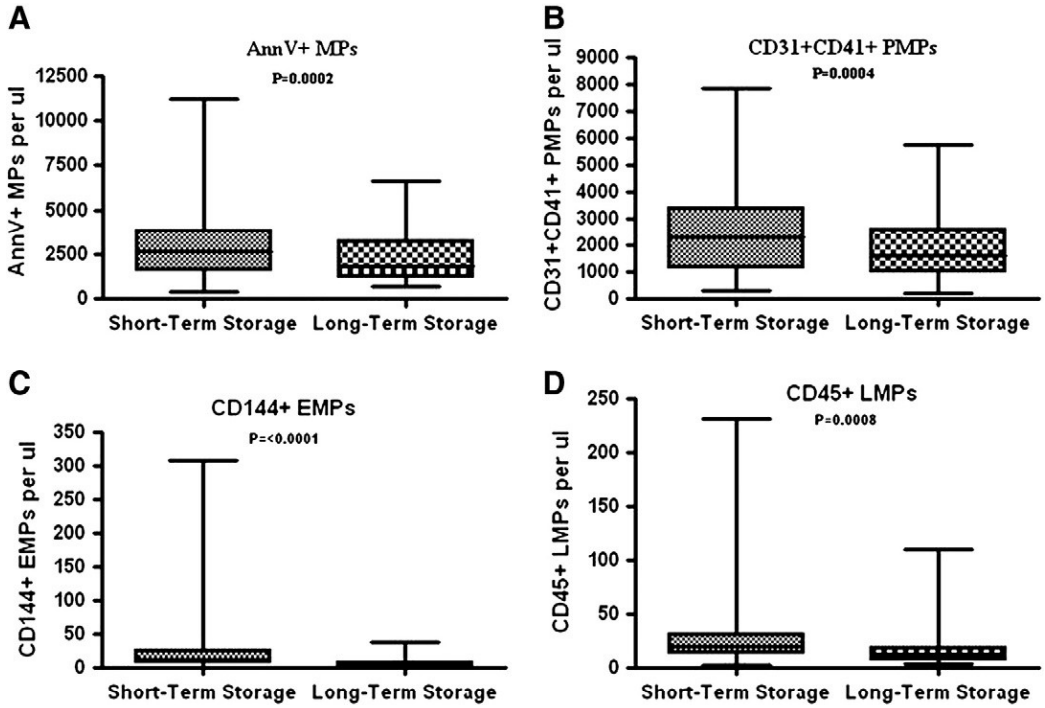

Fig. 8. The effects of long term storage on the numbers of AnnV + MPs (A), CD31 + CD41 + MPs (B), CD144+ EMPs (C) and CD45+ LMPs (D). The short-term storage samples were all processed within 12 months of freezing, the long-term storage samples were processed an additional 20 months later.

analysed in the supernatant following washing, so this cannot be confirmed. It would be interesting to assess the effect of washing on MP levels, at a higher centrifugation rate (eg 100,000×*g* for 60 min [5]). No significant difference was detected in the levels of EMPs and LMPs following washing. One possible explanation could be that the washing process removes non-specific events from the MP gate, thereby enabling detection of smaller MP populations, such as LMPs and EMPs.

Of the published MP flow cytometric techniques, no consensus exists for the speed and timing of centrifugation of the whole blood. This study compared two commonly used centrifugation protocols [5]. Our data shows that a single centrifugation step results in significantly higher levels of AnnV + MPs and CD31 + CD41+ PMPs (Fig. 5). It is possible that a single centrifugation is not sufficient to remove all platelets from the plasma and those platelets that remain, continue to release MPs during the subsequent freeze/thawing. Van Ierssel *et al* found that an additional high-speed centrifugation, reduces platelet count in plasma by 65% [20]. The data in our study suggests that a high-speed centrifugation step is necessary to remove platelets from plasma prior to freezing, to avoid artificial PMP production during freeze/thawing. Not surprisingly levels of LMPs and EMPs were not significantly different, as circulating leucocytes or rare endothelial progenitor cells would be removed by a single centrifugation at 1550×*g*.

Freezing of PPP samples is essential if MP measurement is to be feasible in large studies or for routine analysis. If researchers were restricted to the use of fresh samples for MP analysis, it would increase sample-to-sample variation and prevent laboratory collaborations. In this study, a significant increase in AnnV + MPs and CD31 + CD41+ PMPs was observed following freezing of PPP samples (Fig. 6). The additional PMPs observed may be produced by any remaining platelets in the plasma [21], or by larger PMPs breaking down to produce more of the smaller PMPs. However, it was previously found that freezing of samples does not affect the FSc vs SSc distribution of MPs [13]. The samples in this study were not snap-frozen, as suggested in some protocols, and this could have compounded the differences found. A previous study found that certain sub-groups of EMPs are increased following freezing [20]. We suggest that results from samples processed fresh and those frozen prior to analysis should not be directly compared.

Protocols for MP analysis recommend varying thawing temperatures. It was previously found that PMPs were significantly reduced when thawed on ice, compared to those thawed at RT or at 37 °C [22]. However, our data in Fig. 7 shows no statistically significant difference in the numbers of AnnV + MPs and CD31 + CD41+ PMPs in double centrifuged samples, which were thawed at different temperatures. Other subpopulations of MPs were not analysed in this experiment. The lack of concordance between the previous studies results and ours may be due to differences in the methodology, such as the snap-freezing of samples and the initial centrifugation protocol.

The above experiments were performed on healthy samples, with very low (although detectable) levels of LMPs and EMP subtypes. This may account for the lack of significant change between the pre-analytical variables in these subgroups of MPs. Ideally these experiments would be repeated using samples from patients with known elevations in LMPs and EMPs. However in a clinical situation it would be difficult to obtain multiple samples, which would need to be treated differently in regards to the pre-analytical variables.

This study shows that most subgroups of MPs are significantly reduced by long term storage at -80 °C (Fig. 8). This may be due to MPs becoming smaller when frozen for a long time period, to a size that cannot be detected by our flow cytometer, less than 0.5 μm in size. The samples in this study were not snap frozen, which may have affected their stability. Also samples were only single-centrifuged prior to freezing, which may have resulted in artificial generation of MPs from any remaining platelets. It is possible that these MPs degrade more easily than *in vivo* generated MPs. CD31 + CD41- EMP levels were not significantly altered by long-term storage, but this may simply reflect their initial very low levels, making it difficult to observe the effect of long-term storage. In large studies, blood samples are collected over a long time periods and MPs are analysed in batches. Our results suggest that care should be taken when analysing samples that have been stored at -80 °C for variable time periods, particularly those that have only had a single centrifugation, or those which have not been snap frozen. Ideally all samples within a study would be analysed after storage at -80 °C for an equal length of time.

From the results obtained in this study, we make the following recommendations:

1) Blood samples should be processed within strict time-frames and sample handling should be identical between patient and control groups.
2) PPP samples can be washed prior to analysis, to remove non-specific particles, to concentrate samples, and to enable detection of smaller MP sub-groups. However it should be noted that washing of samples may result in the loss of some MPs.
3) A double centrifugation step is recommended to ensure removal of platelets from PPP prior to freezing, to decrease PMP production during subsequent freeze/thawing.
4) Samples processed fresh and those frozen prior to analysis, should not be directly compared.
5) If long-term storage of single-centrifuged samples at -80 °C is necessary, then ideally all samples should be stored for as near an equal length of time, as is feasible.

Microparticle analysis standardisation

There is an urgent need for standardisation of MP analysis [23]. Several groups are working to improve the standardisation between laboratories measuring MPs. One obstacle is the lack of an available reference sample for MP analysis. The Scientific and Standardisation Committee (SSC) Subcommittee on Vascular Biology are working on developing a reference sample for MPs to improve the consistency of MP analysis between laboratories [24].

MegaMix beads (Biocytex, 7801) are a mixture of fluorescent beads with defined diameters; 0.5 μm, 0.9 μm and 3.0 μm, that allow for standardization of instrument settings on different flow cytometers. They can be used to determine if an instrument is capable of resolving small particles. The MegaMix beads showed that the FACSCalibur used in this study is capable of clearly resolving particles of 0.9 μm and 0.5 μm in size. A recent study found that using MegaMix beads on three different Beckman-Coulter Cytomics FC500 flow cytometers in different laboratories, gave CV% values of PMP measurements as less than 12% [25]. However, when MegaMix beads were used to set MP gates on BD flow cytometers, PMP measurements were much more variable. This may be due to the gate that is defined by MegaMix beads not capturing the whole PMP population on BD flow cytometers.[24] The use of calibrated beads may provide a strategy for standardizing flow cytometry analysis of MPs, but this appears be dependent on the instrument used.

Limitations

Whilst flow cytometry is the most commonly used method for MP analysis, as it is capable of distinguishing MPs from different cellular origins, it has limitations. Flow cytometry cannot be used to detect MPs smaller than 300 nm in diameter, depending on the threshold of the machine [2,23], which varies from machine to machine. The use of isotype controls in the flow cytometric analysis of MPs may also be a source of variation. It has been found that the diversity in staining intensity of isotype controls, can lead to a large CV% and errors in the

quantification of MPs [26]. Fluorescence-minus-one (FMO) control gating may be a more accurate strategy for MP analysis, and will be evaluated in future studies. In this study, absolute counts of MPs were determined using the weighed tube flow-rate method. The flow rate can be affected by bubbles or clots in a sample. To avoid this, calibrated counting beads can be added to each sample to determine absolute counts, although this adds an additional cost to MP analysis.

Conclusions

The results of this study show that MP levels detected by flow cytometry are subject to change by several variables, such as time before processing, number of centrifugations, if samples are frozen or not, and how long they are stored at -80 °C. It is essential to consider these factors when measuring MPs in disease groups, to ensure that any differences in levels are due to the clinical condition, and not variations in the technique used to measure them. Standardisation of methodologies will be essential for successful development of MP technologies, allowing direct comparison of results between studies and leading to a greater understanding of MPs in disease.

Conflict of interest statement

None.

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