VoxSanguinis
The International Journal of Transfusion Medicine

International Society of Blood Transfusion

Vox Sanguinis (2011) **100**, 179–186

ORIGINAL PAPER

DOI: 10.1111/j.1423-0410.2010.01385.x

Apheresis platelet concentrates contain platelet-derived and endothelial cell-derived microparticles

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Background and Objectives Microparticles (MP) are membrane vesicles with thrombogenic and immunomodulatory properties. We determined MP subgroups from resting platelets, activated platelets and endothelial cells in donors and apheresis platelet concentrates (PC).

Material and Methods MP were double stained with annexin V and CD61 (platelet-derived MP; PMP), P-selectin or CD63 (MP from activated platelets) and CD144 plus E-selectin (endothelial cell-derived MP; EMP) and detected by flow cytometry in platelet donors (n = 36) and apheresis PC (n = 11; Trima™).

Results PC contained MP, mainly from resting platelets [93% (90–95)], and minor fractions of PMP from activated platelets [P-selectin$^+$ or CD63$^+$; 4·8% (3·2–7·7) and 2·6% (2·0–4·0)]. Compared to donors, levels of annexin V+ MP, PMP, P-selectin$^+$ and CD63$^+$ MP were 1·7-, 2·3-, 8·6- and 3·1-fold higher in PC (all $P < 0·05$). During storage (1–5 days), levels of annexin V+ MP and PMP did not increase, although small increases in the fraction of P-selectin$^+$ or CD63$^+$ MP occurred (both $P < 0·05$). PC also contained EMP, which were 2·6- to 3·7-fold enriched in PC compared to donors ($P < 0·05$).

Conclusions Transfusion of apheresis PC also results in transfusion of HLA-carrying PMP and EMP. This might counteract the aim of reducing transfused HLA load by leucodepletion. The increases in PMP exposing P-selectin or CD63 reflect mild platelet activation during storage. We conclude that in leucodepleted platelet apheresis using fluidized particle bed technology, MP are harvested mainly from the donor by apheresis. Improvement in apheresis technology might reduce MP load.

Received: 21 May 2010,
revised 13 July 2010,
accepted 16 July 2010,
published online 4 August 2010

Key words: apheresis, endothelial cell, microparticle, platelet, platelet concentrate.

Introduction

Microparticles (MP) are cell-derived membrane vesicles that range in size between 0·1 and 1 μm and are present within blood or fractions thereof. They originate mainly from platelets (platelet-derived MP; PMP), but also from endothelial cells (EMP), granulocytes and erythrocytes [1]. The release of MP occurs upon cell activation and during apoptosis [2]. Elevated concentrations of MP have been associated with clinical conditions, including vascular diseases, diabetes, severe trauma and several types of cancers [3–9].

MP expose cell surface proteins and contain cytoplasmic components of the original cell. They have a procoagulant phenotype [10], because their phospholipid surface enables the binding of coagulation factors and the formation of tenase and prothrombinase complexes [11–15]. Besides their role in coagulation, MP are involved in intercellular

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transport and transfer of bioactive molecules, cell activation and inflammation [16–19]. EMP, in addition, carry a broad variety of proteins including HLA antigens on their surface [20].

It is well known that platelet concentrates (PC) contain MP, which may be related to the increased incidence of venous thrombosis and embolism following platelet transfusion [21,22]. MP have been detected in PC prepared from whole blood [23] and in apheresis concentrates [24] and have been related to harvest conditions and storage stress. However, the cellular origin or activation status of MP was not investigated in these studies.

As, by leucodepletion, platelet apheresis is supposed to reduce the HLA-alloantigen load in the recipient, which may be conteracted by the presence of EMP in the concentrate, and the source of MP in PC is not completely clear, we analysed the numbers of MP, PMP from resting and degranulated platelets and for comparison EMP in PC and donors.

Material and methods

Study sample

The PC donors were 33 (21–51) years old, four were men and nine were women. In addition, a group of 36 healthy persons was recruited (no smoking, no history of thrombosis, no medication). All donors had been tested negative for HIV, hepatitis B and C and had no acute infection. The Human Investigation Review Board of the Ludwig-Maximilians-University Munich approved the study. Signed informed consent was obtained from the donors allowing analysis and presentation of all laboratory data presented in this manuscript.

Platelet concentrates

PC were obtained from the peripheral blood of single donors with a Trima™ (automated blood collection system type 917000-000, software version 4.0; GambroBCT, Martinsried, Germany) and stored at 22°C (±2°C) on a shaker for a mean time of 4 (1–5) days (1 day: $n = 3$, 4 days: $n = 3$, 5 days: $n = 5$) in a Trima™ platelet set (cat 80000; Martinsried, Germany). Platelet count in the bag was 1163 (859–1374) G/l, and the bag volume was 246 (238–262) ml. After harvesting, platelets were resolved in donor plasma, to which acid-citrate-dextrose (ACD) was added in a final concentration of 0·1 ml per 1·0 ml solution.

Blood sampling and processing

Samples were collected from the donors and from the PC immediately before transfusion. To avoid activation, plasma samples were taken from donors through a peripheral drain tube without any congestion. For MP analysis, platelet-poor plasma was prepared within 15 min after blood collection by centrifugation at 1550 *g* for 20 min at 20°C. The plasma was snapfrozen in liquid nitrogen for 15 min and stored at −80°C until assayed.

Materials

Fluorescein isothiocyanate (FITC)-labelled annexin V (IQP-120F), phycoerythrin (PE)-labelled annexin V (IQP-120R), IgG1-PE (IQP-191R) and IgG1-FITC (A07795) were obtained from Immuno Quality Products (Groningen, Netherlands). Anti-CD61-PE (555754) and PE-labelled anti-E-Selectin (551145) were purchased from BD Biosciences (Heidelberg, Germany), and anti-P-selectin-PE (CD62p; PN IM 1759) and anti-CD63-PE (PN IM1914U) were from Immunotech (Marseille, France), and anti-CD144-FITC (SP 1355F) from Acris (Hiddenhausen, Germany). All antibodies and annexin V were diluted with phosphate-buffered saline (PBS; 154 mmol/l NaCl, 1·4 mmol/l phosphate, pH 7·4). Final dilutions (v/v) used for labelling of MP 1:100 (annexin V-FITC 1:100, CD61-PE and anti-P-selectin-PE), 1:200 (annexin V-PE), 1:20 (anti-CD63-PE and anti-CD144-FITC), 1:2 (anti-E-Selectin-PE). The antibodies used against P-selectin and E-selectin are specific and showed no cross-reactivity for the other antigen (data not shown).

Isolation of MP

Isolation and identification of MP was performed as described earlier [10] (see also Fig. 1). In brief, frozen plasma (250 µl) was slowly thawed on melting ice for one hour. After centrifugation at 17 570 *g* and 20°C for 30 min, 225 µl of MP-free supernatant was removed. The remaining MP pellet was diluted with 225 µl of PBS containing 10·9 mmol/l trisodium citrate (PBS/citrate buffer), resuspended and centrifuged again for 30 min at 17 570 *g* and 20°C. Subsequently, the MP-free supernatant (225 µl) was removed, 75 µl of PBS/citrate buffer was added to the MP, and the MP was resuspended. Five microlitres of the MP suspension was diluted in 35 µl CaCl2 (2·5 mmol/l)-containing PBS. For MP staining, 5 µl PE-labelled annexin V was added plus 5 µl of a cell-specific monoclonal antibody or isotype-matched control antibody. Samples were incubated in the dark for 15 min at room temperature. The reaction in all samples was stopped with 900 µl calcium buffer (2·5 mmol/l), except the annexin V control, to which citrate-containing PBS (900 µl) was added.

Flow cytometry

MP were analysed in a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) using Cell Quest Software

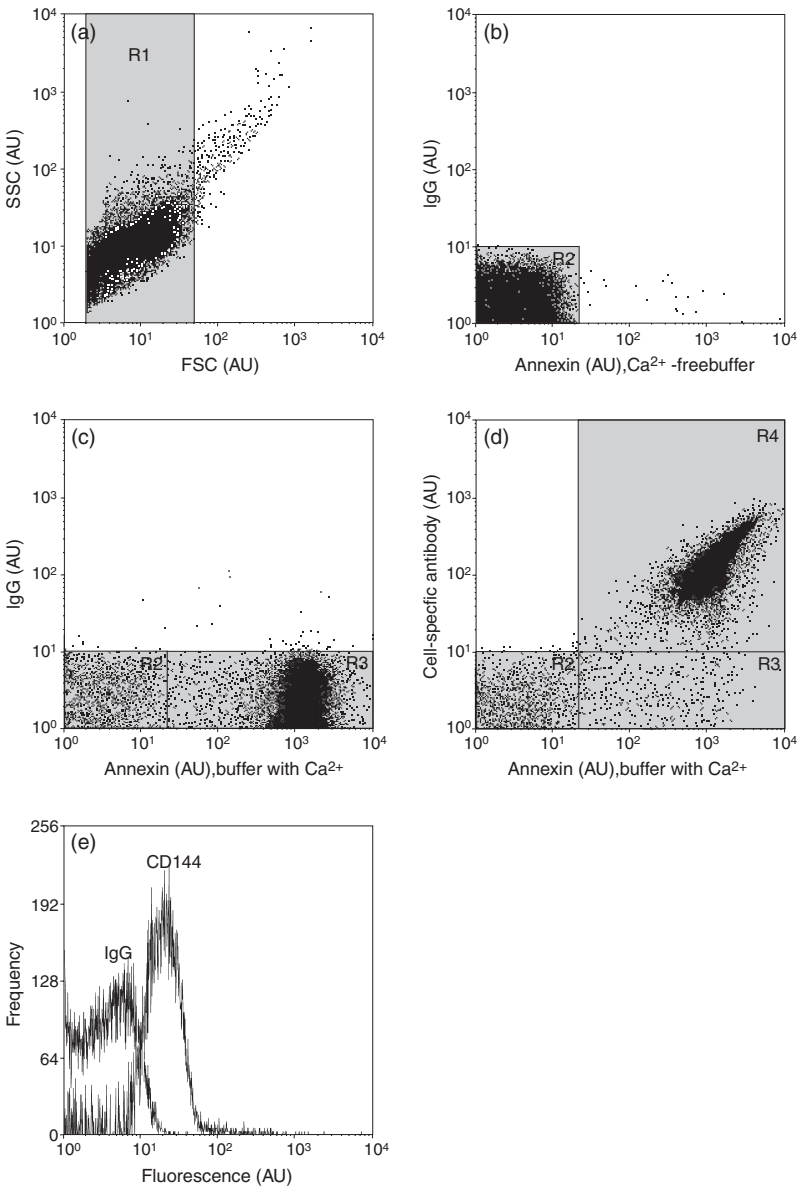

Fig. 1 Identification of microparticle (MP). Platelet-poor plasma was prepared from fresh blood samples, and MP were isolated by high-speed centrifugation as outlined in Methods, washed twice, resuspended and analysed by flow cytometry. First, the MP were identified by their dimension as described by Nieuwland *et al.* [10] (a). As a negative control, MP were double stained with control antibody IgG plus annexin V in a Ca^{2+}-free buffer (b). After addition of Ca^{2+}, annexin V will bind with high specificity and sensitivity to MP exposing PS (c; R3). Double staining with annexin V and a cell-specific antibody identifies the MP subpopulations (d; R4). Figure e shows the frequency distribution of IgG- and CD 144-labelled MP. The absolute MP count was determined by measurement in a defined volume (for calculation: please see Berckmans *et al.* [1]).

(Becton Dickinson, San Jose, CA). Forward scatter (FSC) and side scatter (SSC) were set at a logarithmic gain. MP were identified based on their size and density and their capacity to bind a cell-specific monoclonal antibody and annexin V. Cell-specific labelling with monoclonal antibodies was corrected for identical concentrations of isotype-matched control antibodies, and annexin V measurements were corrected for autofluorescence. The concentration of MP/l plasma was estimated according to Berckmans *et al.* [1], and all samples were analysed for one minute. As a positive control, MP from platelets exposed to thrombin-receptor activating peptide (TRAP; 15 μmol/l) were used.

Statistical analysis

Results are reported as median (interquartile range), except when stated otherwise. Independent variables were analysed by the Mann–Whitney *U*-test and Fishers's exact test, dependent variables by the Wilcoxon test. Statistical analysis was performed with the statistical package for the social sciences (SPSS) for Windows 17.0 (SPSS Inc., Chicago, IL). All *P*-values are given as two-sided values, a *P*-value below 0·05 being regarded as significant. Because of the exploratory nature of our analyses, no adjustment for multiple testing was undertaken.

Results

Platelet concentrates

The platelet count was 4·8-fold higher in the PC when compared to donors [1176 (1072–1229) vs. 247 (216–292); $P < 0{\cdot}05$]. Age and gender of the platelet donor did not influence MP results (data not shown).

MP in donors and PC

PC contained high levels of MP, which mainly originated from resting platelets [93% (90–95)]. Only minor fractions of PMP from activated platelets were present [P-selectin: 4·8% (3·2–7·7) or CD63: 2·6% (2·0–4·0)].

In the donors, the majority of MP [75% (60–85)] originated from platelets (PMP, $CD61^+$), whereas about 6·3% (3·5–11·2) originated from endothelial cells ($CD144^+$). The fractions of PMP exposing platelet activation (secretion) markers were 3·2% (1·2–6·0) for CD63 and 1·4% (0·6–2·6) for P-selectin, showing that only minimal activation of platelets had occurred.

In PC (pooled data independent of the storage time), the average percentages of both PMP ($CD61^+$) and EMP ($CD144^+$) were 93% (90–95) and 10·0% (9·2–12·1; both $P < 0{\cdot}05$). Finally, PMP exposing CD63 or P-selectin were 2·6% (2·0–4·0; $P > 0{\cdot}05$) and 4·8% (3·2–7·7; $P < 0{\cdot}05$; data shown in Figs 2 and 3).

The ratio of MP per platelet was 2·3-fold lower in the PC for annexin V-positive MP [8 (6–13) vs. 18 (12–31); $P < 0{\cdot}05$] and 1·8-fold lower for PMP [$CD61^+$; 7·4 (5·1–12·4) vs. 13·1 (7·9–23·4); $P < 0{\cdot}05$], whereas CD63-, CD62P-, CD62E and CD144-exposing MP did not differ. Thus, during the preparation of PC relatively more platelets than PMP are isolated from the donor blood than MP (see Fig. 4).

In our institution, the platelets are resuspended in plasma from the donor, which is diluted 1:9 with ACD. The addition of plasma to the platelets may transfer MP directly from the donor into the concentrate. Therefore, we calculated the ratio of all annexin V+ MP and CD61- and CD144-exposing MP, which reached the concentrate via platelet apheresis and directly by the plasma bypass, respectively. For the three groups, the main part of the MP went into the concentrate via apheresis, the apheresis to plasma ratio was equal for MP (1·6:1 = 39%), PMP (1·6:1 = 39%) and EMP MP (1·7:1 = 35%, NS). Thus, we conclude that only a minor part of the MP reached the PC directly via the donor plasma, and direct transfer does not explain the enrichment of MP in the PC.

Impact of storage time on MP concentrations in the PC

The levels of all annexin V-positive MP as well as the subgroups of CD61-, CD62E- and CD144-exposing MP were not dependent on storage time, which excludes relevant MP production during storage by resting platelets. In contrast, the fractions of PMP exposing either P-selectin (CD62P) or CD63 increased during storage (Figs 2 and 3), indicating progressive production of MP by degranulated platelets.

Discussion

We present a subgroup analysis of MP in apheresis PC. We found relatively high levels of MP in PC, which were several-fold increased compared to the blood of donors. Most MP originated from unstimulated platelets and their levels did not change during storage time. Only PMP from degranulated (CD62P/CD63-positive) platelets slightly increased during progressive storage time, but even then they still were only a small fraction of the total PMP. In addition, we show for the first time that a significant fraction of MP in the PC was of endothelial origin. For measurement of EMP, we used a combination of antibodies directed against two proteins, whose expression is strictly limited to endothelial cells, i.e. E-selectin (CD62E) and VE-cadherin (CD144) [25,26].

Because it is unlikely that the EMP present in the concentrates originate from endothelial cells within the PC, we assume that these EMP originate from the donor blood. The direct transfer by resuspension of the platelets in donor plasma could not explain the observed MP levels in the concentrates. Therefore, we suspect that during apheresis EMP circulating in the donor blood enter the apheresis device, become partially enriched and find their way into the PC. The enrichment of EMP was 2·6-fold, which is on average half the value of the platelets (4·8-fold). As *in vitro*, MP sedimentation needs extremely high and long-lasting acceleration, the low g force and the short contact time in the apheresis device seem to enrich MP only half as intense as platelets. Alternatively, we cannot exclude that complexes of (activated) platelets with MP may have been present within the donor blood *before* apheresis, which

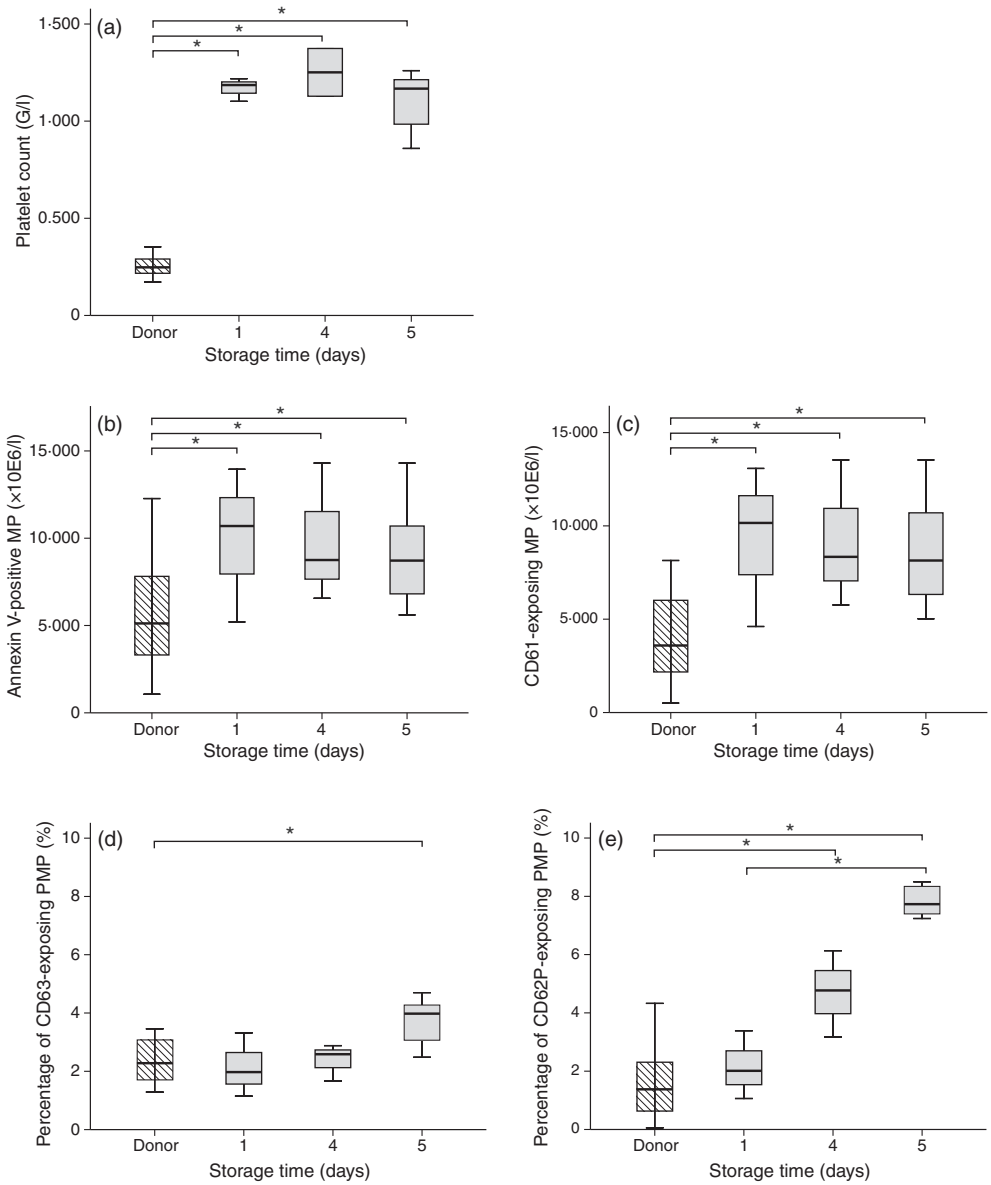

Fig. 2 Platelet counts and PMP levels in donors and platelet concentrates (PC). The figures show the levels of (a) platelets, (b) annexin V-positive MP and (c) PMP ($CD61^+$). Figures (d) and (e) show the percentages of PMP from activated platelets (CD63, CD62P).*$P < 0{\cdot}05$. Hatched boxes: donor plasma, grey boxes: stored PC.

dissociate in time *after* apheresis. Unfortunately, there are no available methods to distinguish between EMP originating from endothelial cells and EMP originating from circulating endothelial cells (CEC). Therefore, we cannot exclude that the EMP we observed in the PC originate rather from CEC in the donor than from circulating EMP.

Similarly to EMP, the CD62P/CD63-negative PMP in PC most likely originate directly from the donor, because their enrichment was comparable to EMP (2·3-fold). As PMP levels remained constant during storage, it excludes a continuous production of PMP by resting platelets at least inside PC. With progressive storage time, we found a modest change in MP composition. Whereas the level of all MP, the EMP and PMP from resting (CD62P/CD63-negative) platelets stayed constant, our data show raising levels of the small fraction of CD62P- and CD63-exposing PMP, which may be related to the degranulation in a minor fraction of harvested platelets, which is in line with earlier reports showing an increase in the levels of CD62P-positive platelets during storage [27,28]. Direct measurement of

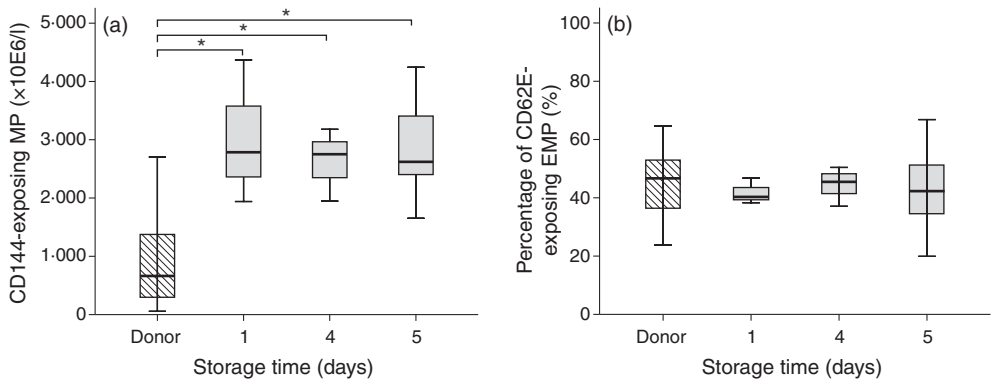

Fig. 3 Endothelial cell-derived MP (EMP) levels in donors and platelet concentrates (PC). The figures show the levels of all EMP (a) and the percentage of activated EMP (b) Storage time has no influence on the levels of EMP. **P* < 0·05 between indicated groups. Hatched boxes: donor plasma, grey boxes: stored PC.

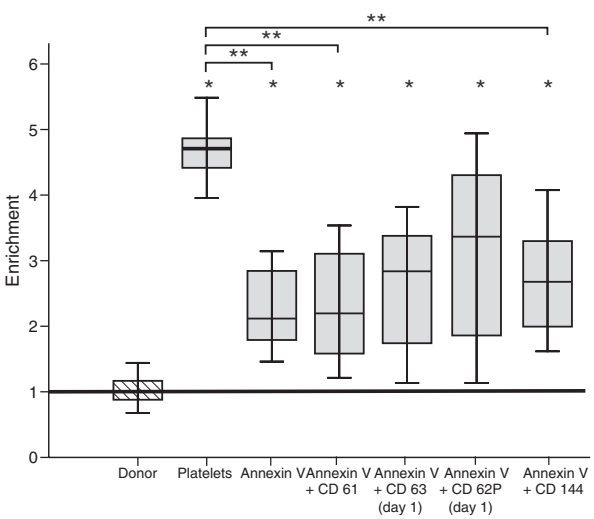

Fig. 4 Platelet and microparticle (MP) levels in the platelet concentrates (PC) in relation to the donor group (horizontal line). Apheresis enriches platelets and MP in the concentrate. Level of MP in the PC is significantly higher than in the donor. However, the enrichment is lower than that of platelets. **P* < 0·05 to donor group; ***P* < 0·05 between indicated groups. Hatched boxes: donor plasma, grey boxes: stored PC.

platelet activation was not necessary in our study, because MP exposing platelet activation markers, e.g. P-selectin, closely reflect platelet activation [29].

Our observation is in contrast to two former MP studies in PC prepared from whole blood [23], but also in apheresis concentrates [24], where increasing PMP levels were observed during storage time. However, these two studies identified MP only by their dimension using beads, which attributed to their much higher light scattering properties compared to MP of similar diameter will lead to inappropriate setting of flow cytometry thresholds and therefore may produce incorrect results. As these two studies did not perform CD62P/CD63 staining, the fraction of PMP from resting platelets remains unclear. We suspect that more gentle PC preparation of our Trima™ reduces platelet activation and degranulation and thus explains the difference.

Our data show that PC transfusion does not only result in the donation of platelets but also transfusion of MP. Apart from their role in coagulation, MP are also involved in transport and transfer of bioactive molecules, cell activation, inflammatory processes and may modulate inflammation and adaptive immunity [16–19,30]. As EMP carry cytoplasmic components and cell surface antigens of the donor, the effect of transfused EMP on patients might be perturbing: EMP surface expression of cell adhesion molecules, such as E-selectin and ICAM-1, is associated with increased binding and activation of both monocytes [31] and leucocytes [32]. In addition, EMP decrease endothelial nitric oxide release *in vitro* [33,34] and increase arterial stiffness, stimulate activation and apoptosis of cells [35] and may also serve as potential carriers of immunogenic and pathogenic compounds. As EMP carry HLA antigens in high density [20], transfer of EMP into the recipient might counteract the aim of reducing transfused HLA load by leucodepletion. However, the clinical consequences of our observation remain to be investigated.

We conclude that at least in leucodepleted platelet apheresis using modern fluidized particle bed technology, MP are mainly harvested from the donor by apheresis. Considering the possible consequences of uncontrolled application of EMP following platelet transfusion, reduction in MP load by improvement in apheresis technology might be rewarding.

Acknowledgement

This work is part of the doctoral thesis of Michael Iberer.

Conflict of interest

All authors declare no conflict of interest.

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