Cell-derived microparticles in stored blood products: innocent-bystanders or effective mediators of post-transfusion reactions?

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Introduction

Plasma membrane-derived microvesicles or microparticles (MPs) are sub-cellular vesicles released upon shear stress, cell activation, injury or apoptosis. They represent factors of the extracellular vesicular compartment[1] that also includes smaller (0.03-0.1 μm) multivascular body-derived exosomes and larger (1-5 μm) apoptotic bodies which may contain fragmented DNA[1]. Exosomes are small export vesicles initially derived from the plasma membrane by an endocytosis-involving internalization of the later. In contrast, MPs are larger than exosomes and are derived directly from the plasma membrane after local cytoskeleton rearrangements and membrane budding[2]. Despite different generation mechanisms and effects, they have been considered as universal biomarkers of cell activation, injury or apoptosis, in a broad range of physiological and pathological processes.

Typically MPs range in size from 0.1 μm to 1.0 μm. They express surface markers from their parental cells that allow identification of MPs sub-groups according to their origin: from platelets (P-MPs), leukocytes (L-MPs), red blood cells (R-MPs), endothelial cells and other tissue cells. They harbor cell-derived membrane-bound and cytoplasmic proteins (e.g. chaperones) as well as bioactive lipids. However, MPs are not replicas of the maternal cells and plasma membrane, suggesting a level of selectivity in their formation and sorting of cellular proteins to release[2,3]. In fact, MPs release is a highly controlled process. According to proteomic data, plasma MPs are enriched in Ig μ-chains, J-chains, profilin I and cyclophilin A, suggesting that MPs-bound IgM may provide a mechanism for their clearance[4].

As mentioned above, MPs can be formed through several induction pathways, which determine their different molecular profiles and biologic activities. A major aspect in cell biology is communication, which occurs through a direct contact between cells or by means of soluble substances, which react with cells. The fact that MPs released by cells influence other cells is a rather new concept but is a basic mechanism to deliver a message in a highly concentrated manner or to add a missing molecule. MPs exert their effects either via stimulation of target cells by receptor interaction or by direct transfer of their contents which can include membrane proteins and lipids, cytoplasmic components of the parent cell or RNA[3,5]. By this way, MPs may facilitate cell-to-cell interactions and transfer of signals and receptors between different cell types, inducing thus signalling and response in distant cells. Although not characterized in detail, it may be hypothesized that uptake and removal by cells occurs by similar or identical molecular mechanisms[1]. The importance of cellular communication via MPs is that they contain highly concentrated signalling components and thus the massive hit of every target cells by MPs' components is likely to be more effective than the activity of individual components in solution[2]. As a result, MPs represent nowadays a novel mechanism of intercellular communication mediating inflammation, coagulation and immune responses.

The rate of steady state release of MPs in the blood of healthy individuals is usually low[1], however they are found elevated in a variety of pathologies including many thrombotic and inflammatory conditions. While it is not sure if this increase represents a contributor to or a result of the disease deterioration, it identifies MPs as potential biomarkers[6,7]. On the other hand, L-MPs participate in angiogenesis and as such, they have been suggested as novel therapeutic tools to reset the angiogenic switch in pathologies with altered angiogenesis[6].

MPs are inherent part of all blood labile products delivered to transfused patients (Figure 1). They

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are present and accumulate in cellular (red blood cells RBCs, platelets PLTs) concentrates as well as in fresh frozen plasma (FFP) during storage[8]. A clinically important issue is that MPs may have pro-coagulant activities. It has been demonstrated that P-MPs have from 50 up to 100-times higher pro-coagulant activity than PLTs[9]. MPs exhibit tissue factor activity, so they may have a role in initiating blood coagulation[4]. During MPs release, the normal asymmetric distribution of phospholipids between the two leaflets of the plasma membrane is lost, resulting in the exposure of the anionic phospholipid phosphatidylserine (PS). The later contributes to the pro-coagulant property of MPs since it serves for the assembly of coagulation factors into active complexes for thrombin generation. However, some

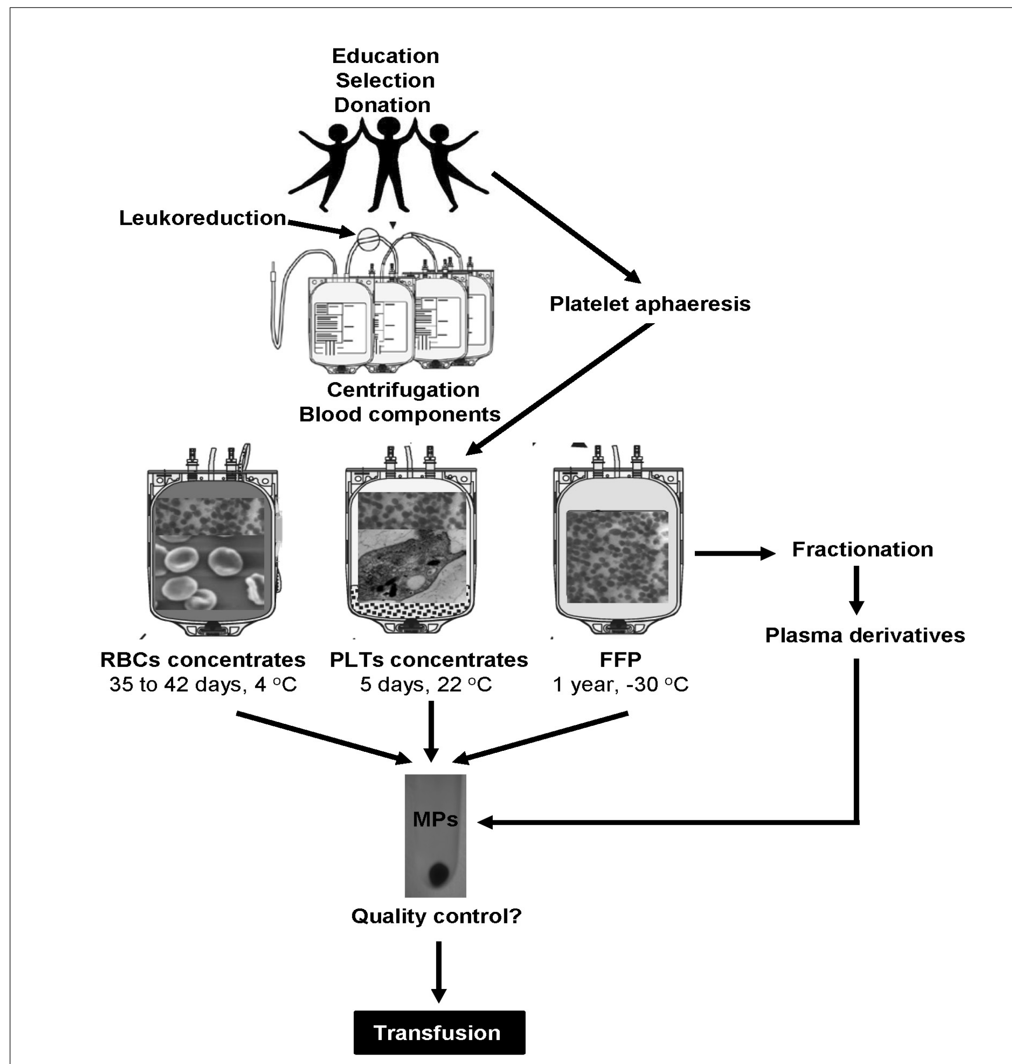

Figure 1 - Microparticles (MPs) are inherent part of all blood labile products and are concomitantly delivered by transfusion to recipients. A growing body of literature has demonstrated an increased incidence of adverse clinical outcomes associated with the transfusion of a large number of units or, potentially, with increased storage time of the units. Since MPs accumulate in blood labile products during storage, transfusion of more or "older" units will offer to the recipient higher number of MPs. The transfused MPs have the potential to increase the risk of adverse reactions, by inducing a hypercoagulable state leading to thromboembolic complications. Inversely, in other situations requiring blood transfusion, a hypercoagulable state may be useful to diminish or even helping to stop the bleeding. In both cases, MPs accumulation, cellular origin and composition might be factors of blood labile products quality control.

observations also suggest the existence of MPs without PS externalization, like in the case of P-MPs derived from PLTs-poor plasma[10] suggesting that they possess other activities aside from pro-coagulant phospholipid one. In recent years, a growing body of literature has demonstrated an increased incidence of adverse clinical outcomes associated with the transfusion of a large number of units or, potentially, with increased storage time of the units. These events include increased risk of infection, renal failure, respiratory failure, multiple organ failure, and death, particularly in physiologically compromised patient populations[11,12]. Since MPs accumulate in blood labile products during storage, transfusion of more or "older" units will offer to the recipient higher number of MPs. The transfused MPs could increase the risk of adverse reactions, by inducing a hypercoagulable state leading to thromboembolic complications. Inversely, in other situations requiring blood transfusion, a hypercoagulable state may be useful to diminish or even helping to stop the bleeding. Many clinical studies suggest that transfusions might be immunosuppressive, although these observations are not generally accepted[13,14]. However, a clinical study indicated that transfusions of RBCs might be responsible for a diminished survival in cancer patients[15].

This review outlines the current knowledge on MPs present in blood labile products used for transfusion: their formation, the effect of storage and their probable activities towards both the "storage lesion" progression as well as the post-transfusion effects. Although the current clinical studies offer only indirect manifestations of MPs activity in the recipients, a lot of data suggest that MPs co-transfused with stored cells and plasma may exert various favorable or adverse effects pre- and post-transfusion, but are definitely not bystanders in any step of this clinically important process.

Methodological approaches

In the past few years, the number of research works on blood MPs has dramatically increased, reflecting the interest on their emerging roles and activities in various diseases as well as in transfusion medicine. The most common approaches to investigate MPs are flow cytometry, coagulation assays (thrombin time, activated partial thromboplastin time etc.), proteomics and ELISA-based solid-phase capture assays[8,16]. They all aim to gain insights on the biological mechanisms involved in MPs generation and function. Most investigators have designated to characterize blood MPs using flow cytometry and some technical protocols have been reviewed in a recent forum[17]. Flow cytometry allows the detection, enumeration and assessment of cell origin of MPs after labeling with various markers (cell-specific and annexin V). A severe limitation of the method is that it fails to detect MPs smaller than 200 nm. Microscopy approaches, including transmission electron microscopy, represent the gold standard for the determination of both MPs size and structure[18]. Moreover, MPs composition and protein topology can be resolved by immune-electron microscopy approaches[18-20]. Proteomic techniques have lightened the variation in MPs proteome as a function of their origin, time of storage or stimulus that triggers their generation[4,21,22]. In addition to analyzing MPs antigens, several investigators have assessed the coagulant function of blood MPs using functional assays[23-25]. In capture-based assays a probe, antibody or annexin V, specifically binds a subset of MPs from plasma. Captured MPs can then be quantified/characterised by using a second probe for example peroxidase conjugated antibody[26] or by measurement of the concentration of negatively charged phospholipids (e.g. their pro-coagulant activity)[27]. Enumeration of total MPs population and information on the size distribution of MPs is unable with captured based assays. There is furthermore interference by soluble antigens. On the opposite, a clear advantage of capture based assays is that they allow measurement of MPs directly in plasma.

Unfortunately, MPs methodologies are poorly standardized and therefore the choice of MPs assay protocol will significantly influence the results obtained[8]. The last meeting of the Scientific and Standardization Committee of Vascular Biology has focused on MPs. The session was divided into two sections: the Educational session, devoted to MPs function, generation and role in disease, and the business session, focused on MPs determination and standardization (standardized strategies, functional assays and novel methodologies, available at: http://isth.org/default/index.cfm/ssc1/2011-ssc-subcommittee-minutes/2011-vascular-biology-minutes/). The potential causes of variability in MPs measurement and

analysis are several and are mostly arisen by the fact that blood cells are highly sensitive to environmental factors and respond by releasing MPs. Pre-analytical variables are a major source of variability and potential artifacts in MPs analysis. Uncontrolled pre-analytical parameters may lead to false interpretation of results. The bulk of new data simultaneously raise questions regarding the roles of MPs, the answers to which depend on forthcoming analytical improvements in the standardization of methods used[28]. MPs analysis by proteomics and microscopy need PLTs poor plasma to reduce the plasma protein content and to increase the MPs concentration. Multiple washing steps during high-speed centrifugation isolation will certainly cause some loss of MPs in the pellet. In proteomics and other methodologies it is important to work with purified, isolated MPs but unfortunately, there are no standard isolation protocols. Most groups apply centrifugation conditions from 18,000 x *g* to 250,000 x *g*[4]. The problem is that during centrifugation of cells like PLTs some MPs may be selectively depleted, while forced filtration of MPs holds the risk of fragmentation into smaller vesicles[1]. In any case, PLTs should be pelleted shortly after collection and before freezing stage. Blood collection conditions (e.g. diameter of the needle used for venipuncture and duration of placement of the tourniquet), time before processing, plasma preparation methods, working and storage duration and temperature, the type of anticoagulants used, number/time and other parameters of centrifugation and washing steps needed to yield platelet-free plasma, freeze-thaw cycles, needle-to-analysis time and analysis protocols, cell and cellular fragments contamination, labeling of MPs, fresh *vs.* frozen plasma, all of them might artificially affect the MPs analytical approaches and must be carefully standardized before any assessment of MPs biological and clinical effect[1,28,29]. To mention some more, in stored RBCs concentrates the MPs count varies not only with storage time but also with temperature, MPs dilution buffer, vortexing and agitation[28]. PLTs release MPs in response to shear stress[30] and storage[31]. Furthermore, freeze-thaw cycles of PLTs-reach plasma results in increased number of PS-positive MPs[10]. Despite difficulties encountered in analyzing MPs and disparities of results obtained with a wide range of methods, MPs generation processes and effects are nowadays better understood.

MPs in stored RBCs concentrates

Stored RBCs undergo a series of time-dependent -yet early enough recognizable- physiological, structural and biochemical alterations, which are only reversible to some extent[32]. In the RBC storage lesion context, depletion of ATP, pH acidification, physiologically important disturbances in energy metabolism and S-nitrosohaemoglobin content, rheological properties (shape, deformability, aggregability, intracellular viscosity), oxidation stress and finally, in cellular aging process have been widely characterized[32-35]. Altered membrane surface and cytoskeleton contribute to the RBCs damage and clearance[36,37]. RBCs lose their membrane stability leading to haemolysis and MPs formation. Although the clinical importance of the RBCs storage lesion is poorly understood, some of the irreversible deteriorations of the stored RBCs, like haemolysis, potassium release and MPs accumulation, are associated with reduced post-transfusion survival/efficacy and increased risk of adverse reactions in the recipients[38,39].

The exact mechanism of R-MPs release has not been elucidated; however it is very likely that there may be multiple mechanisms that initiate MPs release[40,41]. These mechanisms are tightly controlled and associated with different types of cell stimulation[42]. MPs formation from RBCs *in vivo* occurs throughout the RBCs lifespan as a part of the normal physiological aging process, but is accelerated in the second half, if a functional spleen is present[43]. As a result, there is a loss of the 30 and 20% of the RBCs volume and surface, respectively, at the end of their lifespan. MPs are rapidly removed *in vivo* by the reticuloendothelial system[44]. RBCs microvesiculation is triggered by different types of stimuli such as shear stress, complement attack, oxidative stress, calcium influx and pro-apoptotic stimulations[45]. The composition of R-MPs may vary according to the stimulus, or between *in vitro* and *in vivo* conditions, and differs from their parental RBCs by nearly complete absence of cytoskeleton-linked molecules, decrease of membrane proteins content, presence of proteins involved in cell metabolism and most importantly, exposure of removal signals[45]. It should be noted that many stimuli can be additive or even synergistic.

The storage-associated progressive transformation of discocytes to echinocytes and finally to spherical and degenerative shaped cells, is closely associated with membrane loss in the form of MPs[46]. R-MPs formation represents a continuous process of stored RBCs membrane remodeling, which occurs early during blood banking[36,47]. Firstly reported in stored RBCs units by Rumsby and colleagues in 1977[48], the vast majority of MPs collected from the supernatant of RBCs units originate from RBCs and contain haemoglobin (Hb)[18]. Since their number gradually increases with storage time[8,18], the older units contain significantly higher number of MPs compared to the fresh ones. The level of vesiculation in RBCs concentrates may vary not only with the length of storage but also according to the product and the storage solution: lower R-MPs accumulation has been found under RBCs storage in additive solutions that manage effectively the oxidative stress[19] as well as after pre-storage leukoreduction of whole blood or packed RBCs units[49]. Notably, R-MPs accumulation varies also importantly from donor to donor[8].

The identity of the storage parameters that slow or promote MPs generation is still elusive. According to theoretical models, cytoskeletal defects induced by low ATP levels can account for discoid RBCs transformation to echinocytes and release of membrane MPs as well as for further loss of cellular ATP[50]. ATP depletion, RBCs aging, degradation of the spectrin-bilayer anchoring through the band 3-ankyrin complex or calcium loading result in cytoskeleton rigidity and compression forces to the attached fluid membrane that may lead to buckling and MPs formation[40,51]. In support, *in vitro*, both echinocytosis and microvesiculation are promoted by intracellular calcium elevation and ATP depletion but during routine storage this correlation is poor[52]. The composition of R-MPs in RBCs concentrates is similar to those generated *in vivo*, except for the increased levels of stomatin, making it likely that a raft-based process is responsible for RBCs microvesiculation at low temperatures[47]. Considering that the cytoskeleton is essential for membrane stability in RBCs, MPs formation has been further associated with the storage-induced local deformation of the spectrin network, tyrosine phosphorylation of band 3 and unstable adhesion of the cytoskeleton to the membrane of stored RBCs that favors budding[40,41,44,47,53]. Storage reduces the formation of the spectrin-actin-protein 4.1 complex[54] and furthermore shifts the redox potential toward oxidative stress[37,52], leading to oxidation and proteolysis of several cytoskeleton proteins[19]. The effect of cytoskeleton protein oxidation damage to the formation of cytoskeleton-depleted[8] R-MPs has been previously reported in stored RBCs[55].

In fact, R-MPs collected from units of stored RBCs are devoid of most of the RBCs integral membrane proteins or cytoskeletal components, with the exception of actin and band 3, found in aggregated or degraded forms[18,21,44,47]. The significant difference observed in membrane composition between RBCs and R-MPs outline that MPs are generated by specific processes[40], which allow sorting of lipids, as well as of membrane and cytoplasmic proteins[21]. Numerous components that are predominantly seen in senescent RBCs like C5b-9 complement attack complex, removal signals like PS, IgGs and band 3 neoantigen, as well as oxidized/damaged material, including Hb, methaemoglobin, and activated caspases have been detected in their surface and cytosol respectively[18,19,21,45,56]. R-MPs-mediated extrusion of those non functional or potentially harmful agents and death signalling mediators from RBCs apparently contributes to cellular homeostasis by preventing the premature removal of viable RBCs[57]. At the same time, the release of MPs leads to loss of critical surface area[56]. This depletion contributes to echinocytosis, increased osmotic fragility and cellular deformability defects that threaten post-transfusion viability of stored RBCs[39,58,59]. According to the absence of a removal mechanism inside the stored RBCs units, R-MPs become more heterogeneous over time, exhibiting a gradual increase in their size and in their content of proteasome components, as well as a decrease in PS exposure[18,21,47,57,60], suggesting that MPs structure or nature of MPs formation may vary with storage time.

Although the "loading" of R-MPs with toxic cellular components contributes to the survival of parent cells, it simultaneously renders MPs highly pro-inflammatory and pro-thrombotic[21], increasing thus the risk of adverse post-transfusion reactions. As a result, the degree of vesiculation and irreversible transformation of stored RBCs have been used as measures of storage quality[61]. Proteome information

on R-MPs components involved in coagulation is little. However, phospholipid scramblase 1, plasminogen precursor, fibrinogen beta chain precursor, complement component C9 precursor and beta-2-glycoprotein 1, have all been detected in R-MPs collected from the supernatant of stored RBCs[21]. Notably, MPs pro-coagulant proteins were not found on stored RBCs membrane, suggesting that they are either of plasmatic origin or enriched on MPs microdomains[21]. In the context of pro-coagulant property of MPs, it has been proposed that R-MPs may work synergistically with P-MPs in mediating some transfusion-related thrombotic complications[62]. In support, recent studies reported that the pro-coagulant state in sickle cell disease is partially explained by the factor XI-dependent pro-coagulant properties of circulating R-MPs[63].

There are indications suggesting that stored R-MPs can enhance the inflammatory response observed in patients who receive RBCs of prolonged storage. Indeed, the concentration of PS-positive MPs increases steadily in packed RBCs over time in storage[18,64], in particular from the younger cells[65] and may modify PLTs-leukocytes, PLTs-endothelial cell and RBCs-leukocytes interactions in transfused patients[62]. Interestingly, the ability of stored RBCs supernatant to prime neutrophils is present on MPs[66]. It has been recently reported that MPs collected from older units of stored RBCs contribute to neutrophil priming and/or activation in a murine model of blood banking and haemorrhagic shock[67]. R-MPs express the Duffy antigen receptor for chemokines, which has been shown to shuttle chemokines to active receptors[68]. Other studies have also showed that R-MPs interact with platelets to increase inflammatory chemokine bioavailability *in vitro*[64]. It is therefore plausible to suggest that RBC-derived MPs can bind to platelets in tissue beds under stress and release chemokines, which then prime neutrophils and exacerbate the inflammatory response. Tung and colleagues have proposed P-MPs and R-MPs of prolonged storage PLTs or RBCs supernatants respectively, as potential mediators of TRALI in a sheep model[69]. Regarding R-MPs, their high component in complement and IgGs[18,21] might also activate neutrophils via neutrophil Fc receptors, which is consistent with MPs proinflammatory potential. In light of studies showing that the majority of CD40L in blood is MPs-bound, it is very important the finding that the soluble CD40L which accumulates during PLTs and RBCs storage has the capacity to activate adherent polymorphonuclear leukocytes causing endothelial damage and possibly TRALI in predisposed patients[70].

Furthermore, R-MPs produced during storage of RBCs express their parent cell's blood group antigens, including the Rhesus antigens[8,44,71] and could therefore potentially be immunogenic if present in large numbers. Interestingly, another study demonstrated the immunosuppressive effect of R-MPs *in vitro*[72]. Phagocytosis of R-MPs by macrophages inhibits their activation and decreases interleukin-8 and tumor necrosis factor alpha secretion. Consequently, if R-MPs bear the capacity to down-regulate the innate immune system, producing a long lasting anti-inflammatory signal[72], MPs transfused with RBCs units may account for some of the immunosuppressive effects of the transfusions. The property of MPs to down-regulate the activity of macrophages may be relevant for those that travel to the spleen. Whether a related down-regulation on specific immune cells might occur *in vivo* is speculative; however, considering controversy about the immunosuppressive activity of blood transfusion, investigations in this field are welcomed.

In another pathway, MPs release leads to increased consumption of nitric oxide, an important signalling modulator of blood flow[73] and deleterious effects such as susceptibility to PLTs activation, inflammation, poor control of blood flow and ROS generation[45]. Importantly, the effects of MPs on nitric oxide bioavailability could be more severe than those of cell-free Hb as R-MPs are not cleared by haptoglobin[74].

Finally, R-MPs could transfer neutral molecules or removal signals to other cells modifying their phenotype. In transfused patients presenting with paroxysmal nocturnal haemoglobinuria, transfer of glycosylphosphatidylinositol-anchored proteins, like CD55 and CD59, from R-MPs to deficient cells after RBCs transfusion has been observed[75]. Notably, R-MPs may also transfer PS to the surface of nucleated cells and falsely "mark" them as apoptotic[76]. So, the role of R-MPs in transfusion medicine is dual: on one hand, they improve the survival of transfused RBCs in recipients by allowing

the elimination of toxic molecules and removal signals; on the other hand they might enhance the deleterious events of microcirculation impairment and immunosuppression in some patients[45].

Although R-MPs are the predominant species in RBCs concentrates, P-MPs and L-MPs are also generated in stored non-leukoreduced packed RBCs. Neutrophils, monocytes/macrophages, and lymphocytes are all candidates for MPs release. They have a role in maintaining or disrupting vascular homeostasis. When they carry tissue factor or coagulation inhibitors, they participate in haemostasis and pathological thrombosis. Both pro-inflammatory and anti-inflammatory processes can be affected by L-MPs, thus ensuring an appropriate inflammatory response. L-MPs also play a dual role in the endothelium by either improving the endothelial function or inducing an endothelial dysfunction. L-MPs modify the endothelial function and promote the recruitment of inflammatory cells in the vascular wall, necessary processes for the progression of the atherosclerotic lesion[6]. Leukocyte-derived MPs may play a role in the inflammatory process possibly as part of signalling pathways of cellular crosstalk involving soluble cytokines and mediators of direct cell-cell contact[77].

The time course of generation of R-MPs, P-MPs and L-MPs vary considerably in the units of non-leukoreduced stored RBCs. The degree of neutrophil activation caused by the RBCs supernatant has been found correlated to the P-MPs levels, while the thrombin generation to the P-MPs and R-MPs levels, suggesting that multiple subpopulations of MPs in the non-leukoreduced units of packed RBCs may variably contribute to the pro-inflammatory and pro-coagulant potential of labile blood product as a function of storage time[62].

As mentioned above, the non-leukoreduced units of packed RBCs contain more MPs compared to the leukoreduced ones[49]. Activation of PLTs during the storage leads to PLTs-leukocytes interaction, leukocytes apoptosis and MPs-associated increase in the pro-coagulant activity of the supernatant[78]. Leukoreduction however, does not eliminate MPs present in plasma; neither totally suppresses the membrane vesiculation of stored RBCs. As a result, leukoreduction can only mitigate the putative MPs-associated adverse effects of transfusions.

MPs in stored PLTs concentrates

Platelets transfusions are life-saving medical procedures for patients undergoing major surgery, massive bleeding, for patients having not fully functional PLTs and for those suffering from diseases such as cancer or thrombocytopenia. PLTs concentrates still present one of the major challenges to the blood bank, owing to the scarce availability deriving from the short shelf life of PLTs concentrates under standard blood bank conditions. The usual process to collect PLTs is platelet-aphaeresis but it is possible to produce PLTs concentrates by differential centrifugation of whole blood at room temperature. PLTs concentrates have a short period of life (5 days or 7 days after virus inactivation) and are quite susceptible to environmental changes. They are stored between 20 °C to 24 °C with constant agitation. There are two main reasons that PLTs shelf life is limited to a small number of days. The first one is the risk for bacterial contamination, a risk that can be mitigated by bacterial testing of the product or by treatment of PLTs with pathogen inactivation processes[79]. Although the quality of PLTs concentrates is -to some extent- compromised by the currently applied pathogen reduction technologies[80], there is hope that the storage limitation imposed by bacterial risk will one day being gone. The second reason is that over the storage period, PLTs begin to show evidence of a loss of quality that raises concerns about the efficacy of a transfusion that would be performed with such a product.

Collectively, the loss of PLTs quality over storage is known as the platelet storage lesion, or the platelet storage deficit[81,82]. PLTs storage lesions include activation, proteolysis and changes in morphology, membrane glycoproteins and surface receptor expression[83]. The normal platelet discoid shape is found to be lost after 5 to 7 days of storage at 22 °C. At this storage time, mainly spherical or fragmented PLTs remain. During PLTs storage, granule release and PLTs activation occurs, as indicated by the accumulation of β-thromboglobulin and platelet factor 4 in the storage medium, and the increase in surface levels of P-selectin, respectively. There is a significantly storage-dependant decrease of platelet aggregation response to a number of agonists used alone, such as adenosine diphosphate, epinephrine, collagen and arachidonic acid[84]. The reduction of

glycoproteins, particular GPIb (the subunit of the GPIb-IX-V complex responsible for the von Willebrand factor interaction) on the platelet surface during storage, is most likely due to proteolysis[85]. Recent studies[86] suggested that protein kinases might be involved in the development of platelet storage lesion and provide a potential target for inhibition in order to reduce it. Furthermore, PLTs proteomic studies have documented several protein changes occurring over storage, that are suggestive of a renewal process regarding proteins involved in cytoskeleton integrity or signalling pathways[87,88].

PLTs undergo processes resembling apoptosis, like PS exposure, pro-caspase 3 processing and MPs release[89]. P-MPs release is induced *in vitro* by various stimuli, including epinephrine, adenosine diphosphate, thrombin, collagen and calcium[42]. It has been suggested that there may be more than one mechanism for P-MPs formation[90,91]. Parameters governing P-MPs release are among others shear stress and biological factors like cytoskeletal reorganization, plasma membrane blebbing and PS exposure. However, PS exposure is not always followed by MPs release, since intracellular calcium threshold required for MPs formation to occur is higher than for PS exposure. Activation of μ-calpain, that is involved in cytoskeleton reorganization and regulation of phosphatases activities, very likely plays a crucial role in P-MPs generation[42]. There is also a hypothesis that MPs release after pro-apoptotic stimulation is the cell's early defense mechanism preceding the point of no return in apoptosis. Caspases and calpain might participate in membrane vesiculation through the ceramide pathway that is a marker of cellular stress. Furthermore, ceramide promotes and stabilizes membrane microdomains such as lipid rafts, which commit specific interactions with the cytoskeleton, regulating thus membrane curvature. It has been shown that cytochalasin D, an actin depolymerizer, inhibits MPs release from activated PLTs[92]. Whole membrane remodeling and lateral organization, changes in calcium homeostasis and disturbances in membrane ion fluxes[93], are other factors. From clinical aspect, it is very important to establish or exclude a correlation between *in vitro* PLTs activation and MPs accumulation with potential adverse effects *in vitro* and *in vivo*, in particular with the increase in anti-coagulant and pro-coagulant activity of stored PLTs[89].

PLTs-derived MPs are the most abundant MPs in the human circulation. In common to the subtypes of MPs, they expose PS and tissue factor, they promote coagulation and are considered as PLTs activation markers. MPs derived from activated PLTs contain membrane surface proteins (including GPIIIa, GPIIb and P-selectin) as well as enzymes and chemokines (including CXCL4, -7 and -5)[22]. Elevated numbers of P-MPs have been reported in several diseases associated with arterial thrombosis, like cardiovascular and rheumatic diseases[94] and diabetes type II. Their high thrombogenic potential renders P-MPs critical for the vascular diseases. The clinical relevance of P-MPs in supporting coagulation was documented in leukemic and thrombocytopenic patients. Despite having low PLTs counts, these patients do not bleed probably because of the high levels of circulating P-MPs. Apart from their role in coagulation P-MPs are also involved in the transfer of bioactive molecules as well as in cell activation, inflammatory processes and immunomodulation.

Preparations of PLTs that are stored under blood bank conditions and used for transfusion purposes, appear to be enriched in MPs with high coagulant activity[90]. In fact, during the standard storage of PLTs concentrates there is an obvious increase in P-MPs. The release of MPs is further augmented under storage of PLTs at 4°C[95]. Storage-dependent MPs generation is probably the result of PLTs activation that is amplified by interaction of PLTs with the bag wall[31]. In contrast to MPs studies in aphaeresis PLTs concentrates[96] or concentrates prepared from whole blood[31], in some studies, levels of PS-positive MPs and P-MPs were not found increased during storage for 1-5 days. However, in that case there was a small increase in the fraction of MPs derived from degranulated PLTs[97]. In similarity to the R-MPs collected after prolonged storage of RBCs[19] P-MPs collected from PLTs concentrates supernatant after 5-7 days of storage contain functionally active caspase 3 and can induce apoptosis in human macrophages[98]. Furthermore, P-MPs can bind and activate neutrophils *in vitro* via P-selectin-PSGL-1 interaction[99]. As mentioned above, soluble CD40L released from stored PLTs concentrates has been proposed as a potential mediator for TRALI[70]. The contribution

of shear stress to MPs release was documented in a study reporting that storage of PLTs concentrates on a 6-rpm elliptical rotator resulted in a significantly higher MP release as compared to 2-rpm circular tumbler rotator[83]. Interestingly, there are reports showing that pre-storage leukoreduction induces significantly higher release of P-MPs after storage as compared with unfiltered PLTs[100]. Apheresis PLTs concentrates contain MPs from resting PLTs, activated PLTs and endothelial cells that are enriched compared to the blood of donors[97]. MPs of endothelial origin in PLTs concentrates can arise either from circulating endothelial cells or circulating MPs in the donor. The half time of P-MPs in patients plasma is about 5-6 hours after transfusion of aphaeresis PLTs concentrates[101].

The clinical relevance of P-MPs in transfusion products is a subject for further investigation in different (thrombocytopenic) patient groups receiving PLTs concentrates. Dependent on the cause of thrombocytopenia, P-MPs might be helpful in restoring haemostasis and prevention of overt bleeding in patients after surgery or liver biopsy (in case of severe liver cirrhosis). Unfortunately, there is no single laboratory test that can accurately predict the behaviour of PLTs when transfused. However, contemporary scientific approaches to the platelets storage lesion may identify new targets for the development of quality control assays. Several laboratories have recently begun to apply proteomic approaches to the study of platelet storage[102].

An illustration of the influences of MPs on coagulation *in vivo*[103,104] is provided by Scott syndrome. Scott syndrome is a rare inherited haemorrhagic disorder linked to the lack of scramblase activity. Scramblase is an enzyme responsible for the transportation of phospholipids randomly across the cell membrane. In the case of deficient scramblase, when PLTs are activated, phospholipid surfaces are not translocated to the outer leaflet and there is neither PS exposure nor MPs release. Thus, there is no catalytic surface for interacting coagulation factors usually provided by PLTs and P-MPs. As a consequence, coagulation is extremely affected. Even if it is not possible to draw clear conclusions about the role of MPs in adverse effects of transfusion, a lot of additional data is being compiled that is expected to elucidate important aspects of transfusion medicine.

MPs and the haemostatic potential of plasma

It is less difficult to store plasma, because it is free of blood cells. Two common processes are used to obtain plasma for transfusion: centrifugation of whole blood (and freezing within 8 hours for fresh frozen plasma [FFP] or 24 hours for frozen plasma [FP] after collection) and plasmaphaeresis. FFP role and administration is still a debate subject in transfusion medicine. A major indication for FFP transfusion is to improve haemostasis in patients with acquired multiple coagulation factor deficiencies[105]. Furthermore, severe bleeding after injury requires transfusion of blood products, including FFP. Hemorrhage remains a major cause of potentially preventable death and transfused trauma patients are frequently coagulopathic. It is likely that the ability of FFP to generate thrombin and form a clot is the key to its ability to improve haemostasis *in vivo*. Most clinical uses of FFP, currently recommended by practice guidelines, are not supported by evidence of effectiveness from randomized clinical trials, particularly for the prophylactic use of FFP[106]. Since in many settings the clinical effectiveness of FFP has not been demonstrated, it is difficult to predict the *in vitro* characteristics of FFP that might influence its therapeutic efficacy.

Pro-coagulant MPs are not only pathogenic markers of thrombotic disorders and vascular damage but also efficient effectors in the haemostatic response[107]. The haemostatic potential of FFP, is probably the average result of several parameters including coagulation factor activity, presence or absence of inhibitors and the pro-coagulant activity of MPs. Quantitative or qualitative declines of these plasma components may result in reduced plasma haemostatic potential. Coagulation factors, calcium ions and pro-coagulant negatively charged phospholipid surfaces are the primary components involved in the assembly of coagulation complexes, such as tenase or prothrombinase, and coagulation activation. MPs of FFP are considered essential for clot formation and haemostasis as they expose PS that provide binding sites for activated clotting factors facilitating thus complex association[108]. Flow cytometry showed that the most numerous MPs in plasma prepared after an overnight hold of whole blood at 4 °C were derived from RBCs and most of them bind annexin V. It has been found that FFP

MPs have minimal effect on the prothrombin time and fibrinogen, von Willebrand factor antigen, factor VIII and anti-thrombin III levels, but they exhibit a major effect on endogenous thrombin potential[108], possibly contributing to the therapeutic benefit of FFP infusion. In other studies, P-MPs levels have been found higher than those of R-MPs in FFP[109].

The number and cellular origin of MPs in FFP is different compared to that observed in peripheral blood[108]. R-MPs or P-MPs accumulation in FFP depends on whether leukodepletion was performed at whole blood or following separation to plasma[110]. In the leukodepleted preparations of FFP, filtration decreases the number of P-MPs but increases the number of R-MPs in the resultant product[110]. Longer blood storage before plasma separation and freezing contributes to P-MPs generation and thus to functional haemostasis[109,111]. Plasma units prepared after an overnight hold of whole blood at 4 °C prior to processing contained significantly increased levels of MPs in comparison with plasma produced within 8 hours of blood donation[111]. This was coincidental to stronger and more rapid clot formation as assessed by thrombelastography. Furthermore, this finding is in line with reports of increased P-MPs in PLTs concentrates during storage. On the other hand, in both preparations P-MPs count of the thawed plasma has been found significantly decreased after 5 days of storage at 1-6 °C, in association with an analogous decline in haemostatic potential and clot forming ability[109], contributing thus to lower haemostatic potential of thawed product.

Cryoprecipitate is prepared by controlled thawing of frozen plasma to precipitate high molecular weight proteins, which include factor VIII, von Willebrand factor and fibrinogen. The precipitated proteins are separated by centrifugation, re-suspended in a small volume of plasma and stored frozen at –20 °C. Cryoprecipitate is not available in most western European countries but it is still used in the US and UK. FFP and cryoprecipitate contain a significant number of PLTs. When PLTs concentrates are prepared, the resulting supernatant plasma contains more PLTs than if the plasma was harvested directly from whole blood. The concentration of P-MPs in FFP after thawing is proportional to the number of PLTs originally present. It has been shown that P-MPs are highly concentrated by cryoprecipitation. High levels of P-MPs in cryoprecipitate are considered to contribute to its therapeutic effects in bleeding patients[112].

The potential clinical relevance of MPs in stored plasma used for massively transfused patients remains to be evaluated. The clinical impact of the MPs within FFP is unknown as few studies have been performed in order to establish their *in vivo* biological activity. *In vivo*, the ability of MPs to support and localize haemostasis to the site of injury is not fully known, although in a FVIII-deficient mouse model it has been shown that infusion of leukocyte derived MPs generated *in vitro* temporarily reversed abnormal haemostasis[113]. To definitively establish the relative merits of infusing MPs of different cellular origins into patients would require prospective clinical studies. However, both the level and cellular origin of the MPs could give an indication as to any potential biological activity when transfused[42].

Conclusions

The important issue raised by the presence of MPs in blood labile products is to determine the factors contributing to their formation as well as their exact involvement in recipient's reactions to transfusion. To answer them, intensive research on MPs biology, standardization of analytical and pre-analytical approaches as well as large clinical studies focusing on the precise effects (if any) of MPs in blood recipients, are needed. Microparticles are potential candidates for intracellular communication and are important mediators of inflammation and regulation of immune responses. The mechanistic basis of MPs biological effects, the knowledge of how MPs are involved in the coagulation cascade, in the inflammation and in the immunomodulation is critical in order to decipher the complexity of the hypercoagulable states and the effect of transfusion in routine clinical practice. The *in vivo* correlates and the clinical risks *vs.* benefits associated with transfusion of aged blood components need to be evaluated. At this point, no data exist as to whether these *in vitro* findings change clinical outcomes, for better or worse. This is an active area of investigation, with competing studies suggesting a risk and others suggesting that storage duration risk is confounded by the number of units transfused and the associated severity of illness. Although the attention of blood

transfusion services was drawn to other important issues such as emerging infectious risks, reducing the costs and transfusion reactions documented in haemovigilance systems, there is nothing more important to our patients than making sure that the transfusions we provide are stored by ways that do not impair their principal functions. Improvement of knowledge on MPs such as their formation, levels in blood and blood labile products as well as MPs proteome under various conditions may help to get the very best from transfusions.

Keywords: microparticles, microvesiculation, transfusion reactions, blood products.

Contributions

Anastasios Kriebardis and Marianna Antonelou contributed equally to this review article.

The Authors declare no conflicts of interest.

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