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RED BLOOD CELL MICROPARTICLES: ROLE IN TRANSFUSION MEDICINE?

Rubin Olivier

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RED BLOOD CELL MICROPARTICLES: ROLE IN TRANSFUSION MEDICINE?

Thèse de doctorat ès sciences de la vie (PhD)

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par

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Titulaire d'une maîtrise en protéomique et bioinformatique de l'Université de Genève

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RED BLOOD CELL MICROPARTICLES:
ROLE IN TRANSFUSION MEDICINE ?

Lausanne, le 4 novembre 2011

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Reto Meuli

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« Celui qui étudie peut poser son regard sur les ombres et les énigmes du monde. Et s'il étudie encore et encore, il commence à comprendre de quoi sont faites ces ombres et ces énigmes, c'est un bonheur inestimable.»

Marek Halter, Le vent des Khazars

Résumé

Les microparticules sont des vésicules phospholipidiques de moins d'un micromètre relâchées dans le sang par différents types cellulaires, comme les cellules endothéliales, les plaquettes ou encore les globules blancs et rouges. Elles sont bioactives et impliquées dans de nombreux processus physiologiques incluant l'hémostase. De plus, un nombre élevé de microparticules circulantes dans le sang a été observé dans différentes pathologies.

Dans le domaine de la transfusion, les microparticules de globules rouges ont été détectées dans les concentrés érythrocytaires. Le but de cette recherche était de caractériser les microparticules de globules rouges et d'évaluer si ces dernières avaient un rôle en transfusion. Pour ce faire, une approche globale utilisant différentes techniques comme la cytométrie de flux, la protéomique, la microscopie ainsi que des tests d'hémostase de routines, a été adoptée.

Le présent travail de thèse a démontré que les microparticules de globules rouges s'accumulent dans les concentrés érythrocytaires pendant le stockage. Leur bioactivité a été démontrée de part leur rôle actif dans le processus de la coagulation. En effet, lors de test de génération de thrombine, elles peuvent non seulement supporter ce processus de coagulation, mais aussi le déclencher par un mécanisme inconnu sous certaines circonstances. Les microparticules de globules rouges présentent aussi des antigènes de groupes sanguins à leur surface, toutefois, leur implication potentielle dans l'induction d'une réponse immunitaire n'est pas connue. Bien que le mécanisme de formation et d'émissions des microparticules par les globules rouges ne soit pas complètement élucidé, il a été démontré qu'elles n'ont pas toutes le même contenu protéique et donc qu'elles pourraient avoir des fonctions différentes.

Au vu des résultats, notamment par leur implication dans la coagulation, il est fort probable que la présence de microparticules puisse affecter la qualité des produits sanguins, et causer des réactions transfusionnelles.

Résumé large public

« Les microparticules de globules rouges : rôle en médecine transfusionnelle ? »

Olivier Rubin, unité de recherche et développement, Service de Transfusion, Lausanne.

Les microparticules sont des fragments de cellules d'un diamètre de 0,1 à 1 micromètre que l'on trouve dans le sang. Elles dérivent des membranes des cellules endothéliales, de plaquettes, des globules blancs ou encore de globules rouges. Les microparticules sont impliquées dans de nombreux processus physiologiques comme dans la coagulation et une élévation de leur nombre dans le sang est liée à différents cas pathologiques.

Les microparticules de globules rouges sont le principal sujet de cette étude. Le but étant de mieux les caractériser en utilisant différentes approches et d'essayer de déterminer si elles ont un rôle en transfusion.

Dans ce travail, il a été démontré que les microparticules de globule rouges s'accumulent dans les concentrés érythrocytaires pendant leur stockage. Bien que le mécanisme de formation et d'émissions des microparticules par les globules rouges ne soit pas complètement élucidé, il a été démontré qu'elles n'ont pas toutes le même contenu en protéines et donc qu'elles pourraient avoir des fonctions différentes. Finalement, il a été démontré qu'elles participent activement à la coagulation. Ce dernier point se révèle d'une importance particulière en médecine transfusionnelle. En effet, étant donné que les microparticules de globules rouges s'accumulent pendant le stockage des concentrés érythrocytaire et qu'elles participent à la coagulation, elles pourraient donc être à l'origine de complications lors de transfusions.

Summary

Microparticles are small phospholipid vesicles of less than 1 μm shed in blood flow by various types of cells such as endothelial, platelet, white or red blood cells. They are involved in many biological and physiological processes including hemostasis. In addition, elevated number of microparticles in blood flow is often observed in various pathological situations. In transfusion context, erythrocyte-derived microparticles are also found in red blood cell concentrates. Their role is not elucidated and they are considered as a part of storage lesions. The purpose of this research was to characterize and to accumulate qualitative and quantitative data on those erythrocyte-derived microparticles from red cell concentrates. The aim was to evaluate their potential impact on blood transfusion. Therefore, a comprehensive approach based on four different techniques was employed in this study, namely flow cytometry, proteomics, microscopy and hemostasis routine tests.

This thesis provides evidences that there is release and accumulation of erythrocyte-derived microparticles in red cell concentrates during storage. It demonstrates that they are involved in coagulation in an active manner and thus are bioactive. Indeed, coagulation assays demonstrate that erythrocyte-derived microparticles support and could trigger thrombin generation under certain circumstances. Erythrocyte-derived microparticles also bear blood group antigens on their surfaces. However, it is not known whether they are able to elicit an immune response. Their protein contents vary according to the stimulus suggesting that there are different kinds of erythrocyte-derived microparticles that may have different functions. Taking together, there is little chance that presence of erythrocyte-derived microparticles in erythrocyte concentrates is totally innocuous. In conclusion, these erythrocyte-derived microparticles most likely play a role in transfusion medicine and they could cause transfusion complications.

Abbreviation

CAT	Calibrated Automated Thrombogram
CTI	Corn Trypsin inhibitor
EC	Erythrocyte concentrate
EMPs	Erythrocyte derived Microparticles
EMPs-TF	TF coated EMPs
ETP	Endogenous thrombin potential
FITC	Fluorescein isothiocyanate
MPs	Microparticles
PRP	Platelet rich plasma
PE	Phycoerythrin
RBC	Red Blood Cell
TF	Tissue Factor
TG	Thrombin generation

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General introduction

Introduction

Every year millions of lives are saved thanks to transfusion. With the development of blood banks 60 years ago, numerous efforts and progress have been made in order to secure blood products. The progress of proteomic techniques allows new perspective in blood research and transfusion sciences [1].

The three main labile blood products are erythrocyte concentrates (ECs), platelet concentrates and fresh frozen plasma. Each of those products has to be prepared and stored according to its components. Those components are subjected to modifications or degradations during storage, a process known as “storage lesion” [2].

The primary goal of erythrocytes transfusion is to sustain tissues and organs oxygenation, principally in case of hemorrhage or acute anemia. The blood bank of Lausanne (SRTS VD) relies on approximately 22’000 active donors allowing preparation of 30’671 ECs in 2010. ECs can be stored up to 42 or 49 days at 4C° in standard SAGM (Salin-Adenine-Glucose-Mannitol) or PAGGSM (Phosphate-Adenine-Glucose Guanosine-Saline-Mannitol) conservative solution. Conditions to which red blood cells (RBCs) are exposed during storage such as temperature and nature of the medium are dramatically different from *in vivo* physiological condition. During storage numerous physiological and biochemical alterations occur including an increase in the concentration of free hemoglobin, lipids, microparticles (MPs) and a pH reduction. RBCs also undergo several changes such as loss of potassium, adenosine triphosphate or 2-3-diphosphoglycerate. Their membranes become more rigid, there is a disruption of phospholipid asymmetry, lipid rafts rearrangement, loss of fragment or even release of MPs [3].

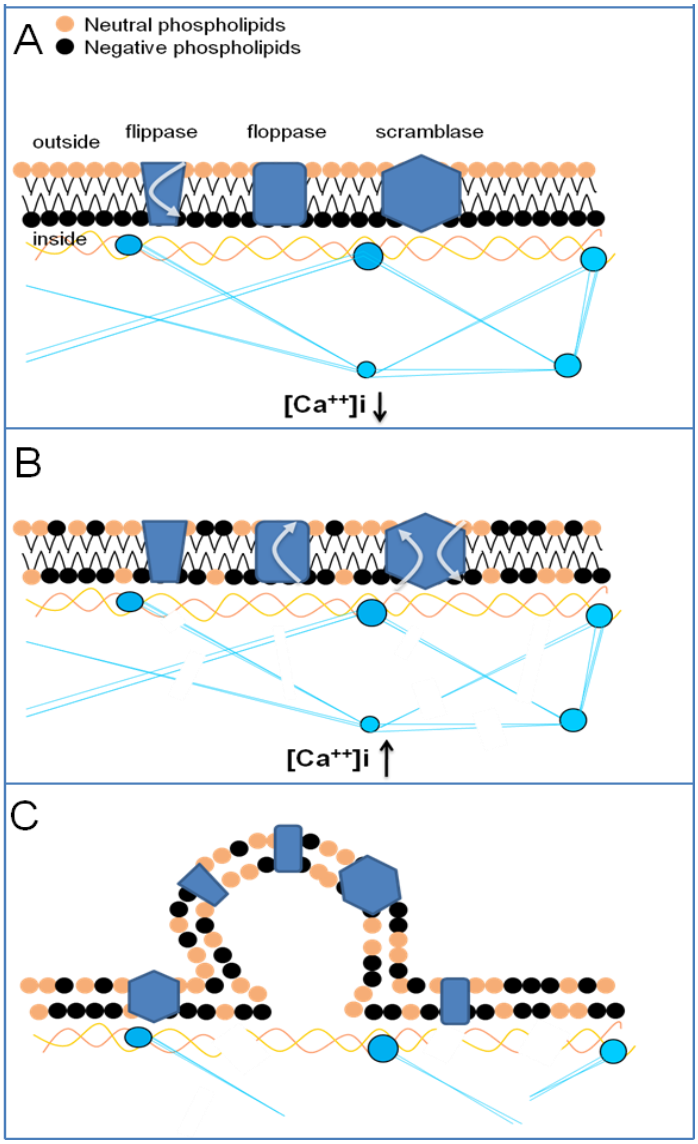
Impact of storage lesion on transfusion efficiency is not clearly understood, and relatively little is known about the putative medical consequences on recipient. Several studies have shown a link between storage time of blood products and post transfusion complications [4-6]. However, this is still controversial and some recent studies limited this link only under specific circumstances [7-10]. Even if there are no conclusive evidence that longer stored blood is worse, it is current practice that some clinical practitioners prefer to transfuse fresh blood (such as in neonatology for example). As MPs are bioactive, it could be hypothesized that erythrocyte derived MPs (EMPs) are mediators of these complications [11]. Indeed, there is a great variability in number of EMPs among ECs, it would be possible thus that a patient receive more ECs with elevate number of EMPs. In addition, the elimination rate of MPs is also unknown, some people could eliminate more rapidly than others. For these reasons, we hypnotized that EMPs could take part to post transfusion complications. Therefore progress in the knowledge of stored RBCs biology is urgently needed.

Microparticles

Microparticles, also described as microvesicle [12] or ectosomes [13] are plasma membrane vesicles shed in blood flow by various types of cells such as platelets, red and white blood cells, or endothelial cells[14]. MPs have a size of less than 1 μm. They contain a subset of proteins derived from their original cells as well as surface receptors allowing the identification of their origin. Most studies generally agree that MPs are heterogeneous and vary in size, phospholipids, surface antigens and protein content. Release of MPs is a controlled process triggered by various stimuli including pro-apoptotic stimulation, shear stress or damage [15].

A model of MPs formation bringing translocases, lipid rafts, various protein modifications and irreversible membrane rearrangements has been established [16, 17] (Fig 1). Three translocases, namely flippase, foppase and scamblase, maintain phospholipids asymmetry on membrane. Negatively charged phospholipids are located on the inner leaflet while neutrally charged phospholipids are on the outer leaflet. In cell steady-state, only flippase whose role is to specifically translocates negative phospholipids from the outside to the inside membrane bilayer is activated. Upon activation, there is elevation of intracellular calcium concentration that inhibits flippase, and activates floppase and scramblase. Floppase externalizes negative phospholipids (mainly phosphatidylserine) from the inner to the outer leaflet while scramblase translocates phospholipids in both way aspecifically. Negatively charged phospholipids are thus externalized modifying the neutral membrane charge into negative with loss of phospholipids asymmetry. Calcium also activates proteases in which calpain cleaves association between membrane protein and cytoskeleton proteins. There are also lipid raft rearrangements and membrane stability being affected, it becomes thus less rigid allowing formation and release of MPs [18, 19].

Figure 1: Model of MPs formation.



A. During cell steady-state, maintenance of the asymmetric distribution of phospholipids by a three piece enzyme system, flippase, floppase and scamblase. There is low cytoplasmic calcium concentration, only flippase that internalize negative phospholipids is active.

B. Upon activation, intracellular calcium concentration increases, flippase is inhibited while floppase and scamblase are activated. Floppase externalize phosphatidyserine, a negative phospholipids and scamblase translocate phospholipids aspecifically through the membrane resulting in the loss of phospholipids asymmetry.

C. Increased intracellular calcium also activate protease that cleave cytoskeleton, membrane is less rigid and could bleb until formation and release of vesicles.

If first MPs have been considered as cell fragments or “dust” without any biological function [20], there are now recognized as being involved in a broad spectrum of biological activities such as thrombosis and hemostasis [14, 21], inflammation [21], vascular and immune function burnier [22], transfer of surface proteins or even angiogenesis [15].

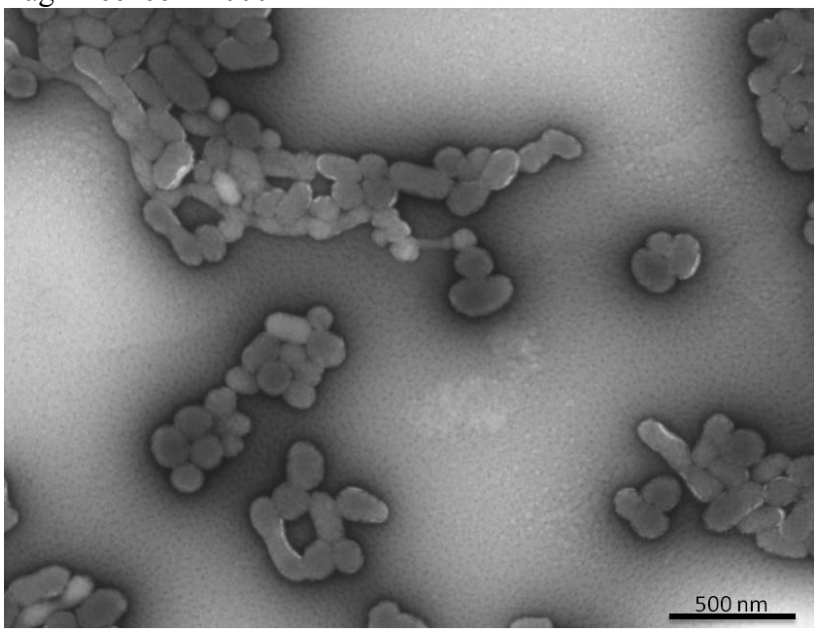
Although the presence of MPs in blood is common in healthy individual, an increase in concentration of MPs in plasma has been demonstrated under various pathological conditions such as thrombocytopenic disorders [23], thrombosis [14], cardiovascular disease [24],

diabetes [25], sickle cell disease [26], haemolytic anaemia [26] or sepsis [27]. Their release could be thus considered as a hallmark of cellular alteration. Very low number of MPs has been observed in patients presenting with Scott syndrome, a rare bleeding disorder associated with apparent normal platelet function and coagulation factor level [28].

Erythrocyte derived microparticles

The main characteristics of EMPs are similar to MPs, as described above. The size of EMPs is generally more homogeneous and around 0.15 μm [29] (Fig 2). EMPs represent around 4-8 % of total MPs [30] in platelet rich plasma (PRP), these results correspond to our observation. As for other MPs, the exact role of EMPs is not elucidated and in the context of transfusion they are considered as part of the RBC storage lesions.

Figure 2: Observation of erythrocyte derived microparticles by electron microscopy, magnificence 22'000x



EMPs has been describe as a part of RBCs senescence [2] and EMPs formation has been also proposed as a part of an apoptosis-like form in erythrocytes [31]. This "aging" process of RBCs has also been observed during storage in blood bank condition [32]. During their 120 days of lifespan, RBCs lose approximately 20% of their volume through vesicles emission whereas their hemoglobin concentration increases by 14% [33]. Vesiculation would be a mean for RBCs to get rid of specific harmful agent as denatured hemoglobin, C5b-9 complement attack complex, band 3 neoantigen, IgG that tend to accumulate in RBCs or on

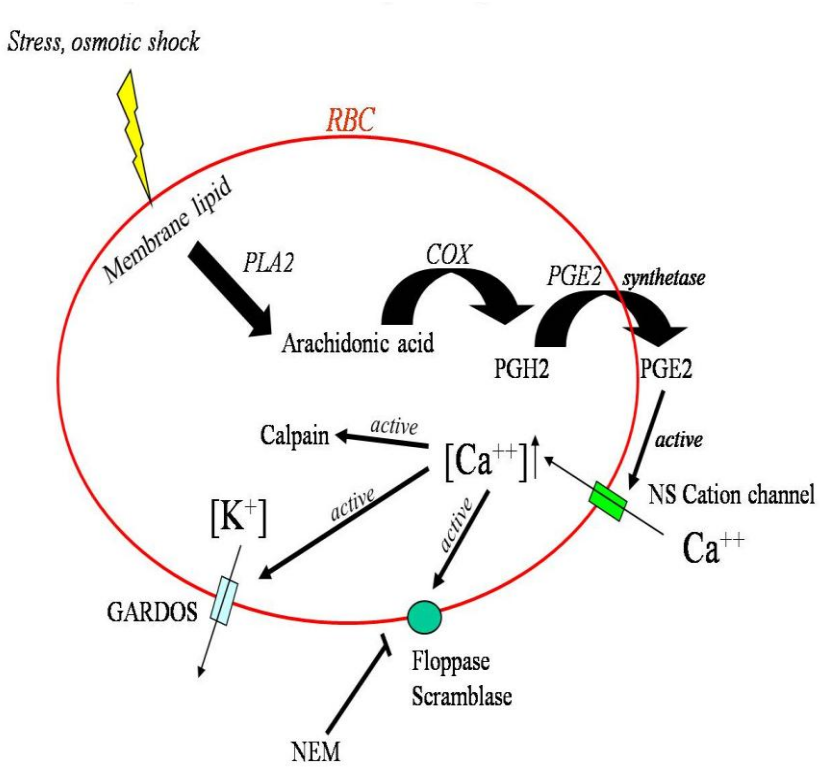
their membranes during their lifespan [3, 32, 34]. In a protective role, by the release of EMPs, RBC can clear away those molecules when they are still viable and thus, prevent early removal from blood flow. In contrast, EMPs could promote removal of erythrocytes: CD47 is an integral membrane protein present on erythrocytes surface, acting as a marker of self. Thanks to CD47, normal red blood cells are recognized as self by macrophages (through their signal regulatory protein α) and phagocytosis is inhibited. Senescent or damaged red blood cells whose CD47 expression is reduced by shedding of EMPs enriched in CD47 would be no longer recognized as self and thus be eliminated by macrophages [35].

Still in the context of RBCs aging process, two main models resulting in microvesiculation have been proposed, the eryptosis model and the band 3 clustering. The term "eryptosis" has been introduced a few years ago by F. Lang. It describes a similar mechanism of apoptosis of nucleated cells in response to various stresses but for RBCs [31] (Fig 3). Influx of ionic calcium through nonspecific cation channel leads to activation of several enzymes such as calpain or scramblase. Reasons triggering this calcium intake are largely unknown but alteration of nonspecific cation channels is often mentioned as a possible cause [36, 37]. The band 3 clustering model is characterized by protein oxidation. The oxidation of Hb contributes to hemichrome formation, which is constituted Hb derived products (likely met-Hb) linked to the inner leaflet, followed by the clustering and aggregation of band 3 multimers in the membrane [38]. Band 3 clustering forms or uncovers senescent neoantigens, probably because of relatively small structural modifications that are recognized by naturally occurring autologous IgG with subsequent complement activation [39].

Both models share final outcome, there are phosphatidylserine externalization on RBCs membrane, degradation of cytoskeleton proteins followed by modification in the

phosphorylation status of band 3. This process gives sufficient membrane flexibility resulting in EMPs formation and release.

Figure 3: Pathways leading to eryptosis



PLA2 : phospholipase A2
COX : cyclooxygenase
PGH2 : Prostaglandin H2
PGE2 : Prostaglandin E2
NS cation channel : Non specific cation channel
NEM : *N*-Ethylmaleimide

Erythrocyte derived microparticles and transfusion

During their period of conservation at 4°C prior to transfusion, storage lesion in ECs occurs resulting in modification, alteration or even degradation of various components [1, 2]. RBCs ageing in blood bank conditions clearly differ from physiological *in vivo*. Undeniably, temperature or medium in which RBCs are conserved dramatically differ from *in vivo* conditions and cause degradation or loose of efficiency of RBCs. Indeed, erythrocytes change shape from biconcave disk to rigid spheroechinocytes [40] conducting to disruption of phospholipids asymmetry and release of fragments and EMPs [2]. In addition, during the conservation of erythrocyte in plastic bags, not only physiological alterations occur, but also biochemical changes in the storage medium principally due to red cells metabolism. There is an increase in the concentration of lipids, free haemoglobin, potassium, lactate and in contrast a reduction of pH, glucose, 2,3-diphosphoglycerate, sodium or adenosine triphosphate [41]. For example diminution of glucose and increase of lactate concentration reflects red blood cells glycolysis. Those changes due to storage affect RBCs deformability, osmotic resistance and survival after transfusion [42]. In circulating blood, EMPs are rapidly removed in the liver by Kupffer cells [33]. Release of EMPs occurs throughout erythrocyte lifespan and continues during storage; consequently, the very large majority of MPs originate from RBCs and their number gradually increases with storage time [29].

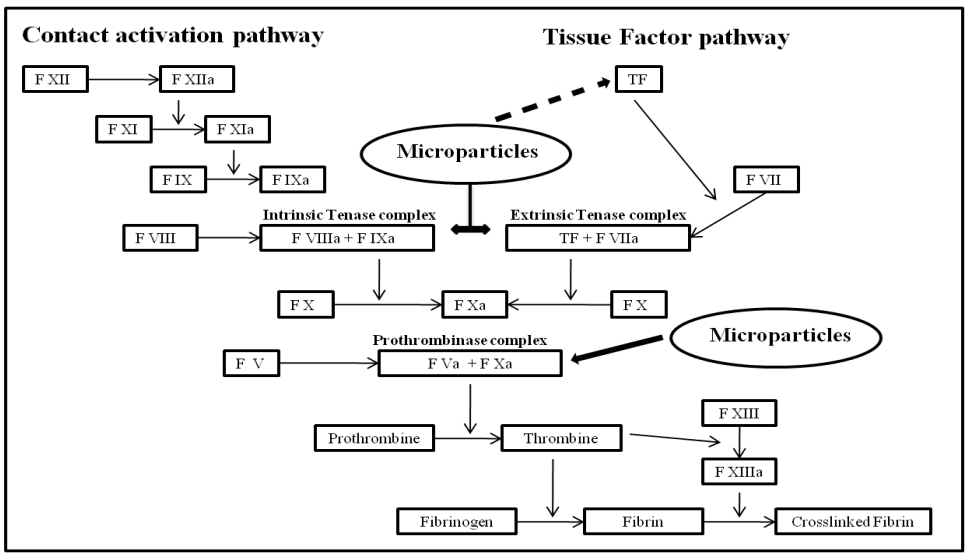
The precise consequences of storage, including EMPs accumulation in ECs, on transfusion efficiency are not understood, and relatively little is known about their possible biomedical consequences on recipients. There are accumulating evidences that receiving “older” blood is not as advantageous as receiving fresher blood. In 2008, Koch *et al* showed a link between the age of the transfused ECs and post-transfusion complications. They claimed that life expectancy at 5 years was better for patients who received fresher blood (ECs stored for less

than 14 days) as compared to older blood (stored for more than 14 days) in the course of cardiac surgery [4]. A study by Spinella *et al* concluded that ECs stored for more than 28 days was associated with an increased incidence of deep vein thrombosis and death from multi-organ failure [9]. Other studies on post-transfusion complications or mortality and storage time were also published and controversial [43-45]. However, although these studies raise important questions about transfusion safety and storage lesions, they are subjected to many discussions [46-48]. Due to the fact that transfused people are sick and polytransfused recipients are often in poor condition, it is difficult to attribute clearly an event to transfusion rather than on the clinical situation of the recipient. In a recent review, Zimrin and Hess analyzed several papers on clinical studies examining the effect of storage of red blood cells in transfusion and concluded that well designed epidemiologic studies are needed to demonstrate that a clear clinical effect may be due to ECs storage lesions [49]. Despite the lack of conclusive evidence that storing blood longer is worse, it is current practice to transfuse fresher blood to patient considered at high risk (such as in neonatology for example or cardiac surgery) and to put blood banks under pressure to prioritize delivery of fresher ECs. Blood banks are in an uncomfortable situation due to the fact that there are few arguments to evaluate the safety and efficacy of older products compared to fresher one.

Microparticles and coagulation

Blood coagulation is an essential mechanism that prevents bleeding; this highly controlled process is carefully regulated in order to maintain blood circulation in case of injury while preventing vessels obstruction by clot formation. Coagulation factors, calcium ions and procoagulant membrane surfaces are the primary components involved in coagulation activation. The presence of negatively charged phospholipid membranes and calcium ions is required for the assembly of coagulation complexes such as tenase or prothrombinase. The role of tenase complex is to convert factor X into activated factor X, in both extrinsic and intrinsic pathways. The extrinsic tenase complex is composed of tissue factor (TF) and activated factor VIIa [50] whereas the intrinsic tenase is composed of co-factor VIII and activated factor IX [51, 52]. The function of prothrombinase, constituted of activated co-factor V and activated factor X, is to convert prothrombin into thrombin. Activated factors IX and X can convert their substrate without their respective cofactors, however negatively charged membranes catalyses the reaction, for example $3x10^5$ fold faster in the case of prothrombinase [53]. The importance of negative phospholipids is to facilitate complex association, speeding up their association by around 1000 fold for efficient haemostasis [54]. As MPs expose negative membrane, they could contribute to coagulation (Fig 4). It has been demonstrated that platelets-derived MPs have from 50- up to 100-times more procoagulant activity than platelets [55]. MPs membranes support more efficiently thrombin formation than platelet membrane when corrected for unit of surface, at least *in vitro* [16, 52]. As they present negatively charged membrane as well, EMPs or other MPs could be involved in this process [26, 56].

Figure 4: Scheme of the coagulation cascade showing at what level microparticles participate



Although controversial, some authors claim that MPs support clotting not only through their negatively charged membranes, but also by expressing an inactive form of tissue factor (TF) on their surfaces. The origin and mechanism of action of blood-born TF is still subject to debate, nevertheless authors have suggested that MPs may be a reservoir of blood-born TF[57]. Furie *et al* delineated a "*microparticles accumulation pathway"* as part of the coagulation cascade [52]. According to this model, some monocyte-derived MPs expressing TF in an "inactive" form bind to a forming clot in vessels and display active TF that helps to amplify coagulation. In an other hand, Connor *et al* proposed an activated factor X assay to measure clotting time of various samples containing MPs and their results show that there is a correlation between the number of MPs and clotting time; interestingly enough, the assay was insensitive to the presence or the absence of TF [58]. Other studies mention the presence of TF on endothelial-derived MPs [59], platelet-derived MPs [60]**,** or even on erythrocyte-derived MPs [30] , while some others did not detect any MPs-exposing TF [26]. All these pieces of evidence are difficult to explain, in particular because results often depend on the study design, methods of MPs isolation, and various pre-analytical factors; therefore one

cannot exclude that discrepancies might result from different experimental methodologies [61].

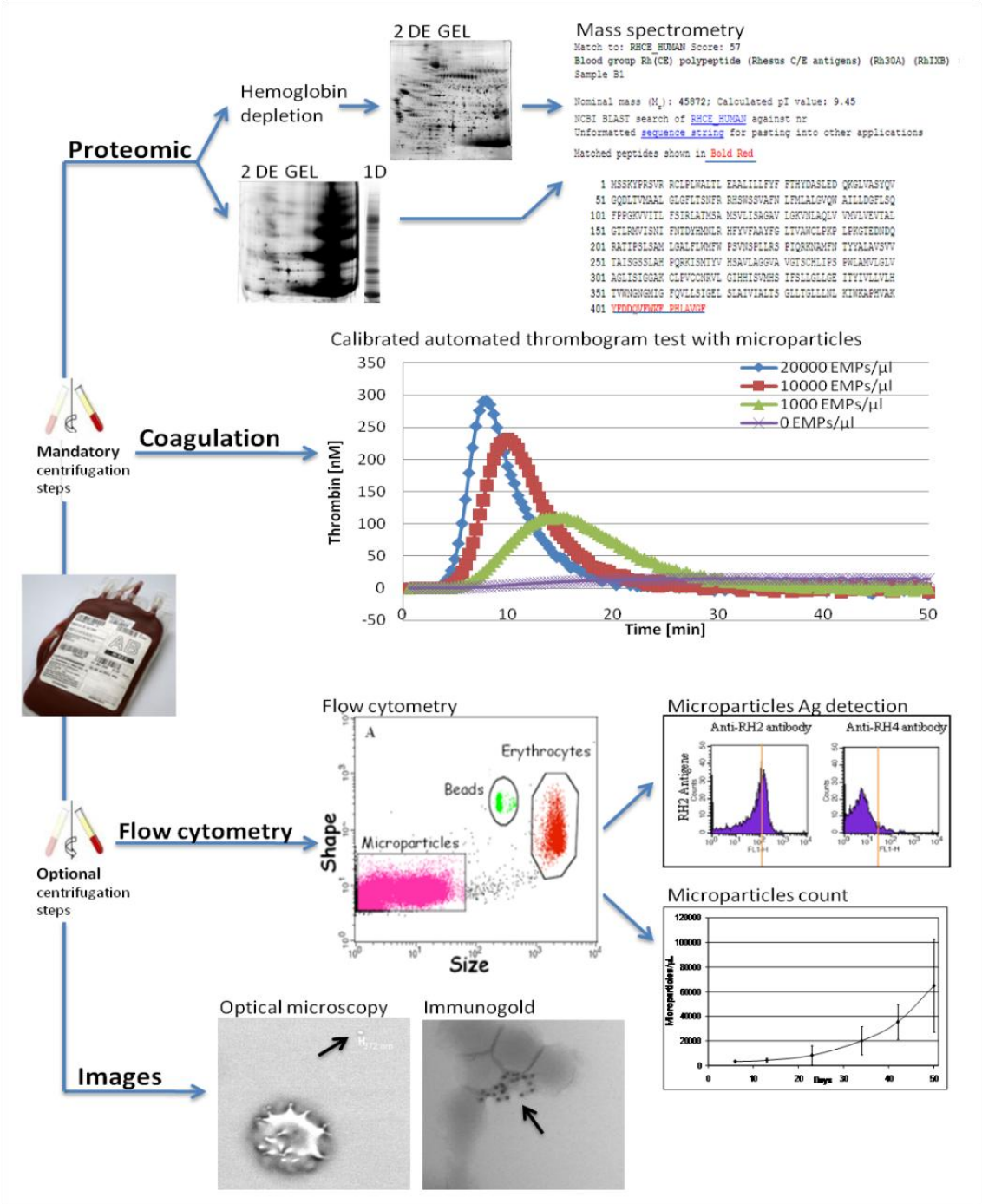
As stated before, MPs may have procoagulant activities; therefore, we may reasonably suggest that transfusion of “older” ECs containing a higher number of MPs could increase the risk of adverse reactions, by inducing a hypercoagulable state leading to thromboembolic complications. Inversely, in many situations requiring blood transfusion, a hypercoagulable state may be useful to diminish or even helping to stop the bleeding.

Outline of this thesis

As there is little data on EMPs, the main goal of this thesis was to characterize EMPs from ECs and to evaluate if they have a role in transfusion medicine. As mentioned above, MPs are bioactive and involved in various pathophysiological processes and their presence has been demonstrated in blood product. There is thus little chance that their presence is totally innocuous. Therefore, a comprehensive approach based on four different techniques has been employed in this study, namely flow cytometry, proteomics, imaging and hemostasis routine tests (Fig 5).

An extensive overview of the current knowledge on MPs from RBCs and their putative implication in transfusion medicine are introduced in Chapter 2. In order to obtain better characterization of EMPs, qualitative and quantitative experiments conducted on EMPs from stored ECs by various methods are presented in Chapter 3. Chapter 4 highlights the importance of pre-analytical factors and their impacts. There is also a discussion on various methodological aspects of experiments on EMPs. Implications of EMPs in coagulation and the putative side effect on transfusion role are discussed in Chapter 5. Experiments concerning the participation of EMPs to coagulation (or thrombin generation) using Calibrated Automated Thrombogram are presented in Chapter 6. Finally, ethical considerations on blood donation are discussed in chapter 7 (in French).

Figure 5: General schema of experimental approach on erythrocyte derived microparticles during the thesis (adapted from Rubin et al) [62].



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Analysis and clinical relevance of microparticles from red blood cells

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Purpose of review
The mechanisms involved in the formation of red blood cell (RBC) microparticles *in vivo* as well as during erythrocyte storage are reviewed, and the potential role of microparticles in transfusion medicine is described.
Recent findings
Microparticles release is an integral part of the erythrocyte ageing process, preventing early removal of RBCs. Proteomics analyses have outlined the key role of band 3–ankyrin anchoring complex and the occurrence of selective RBC membrane remodelling mechanisms in microparticles formation. The presence of several RBC antigens, expressed on microparticles, has been demonstrated. The potential deleterious effects of RBC microparticles in transfused recipients, including hypercoagulability, microcirculation impairment and immunosuppression, are discussed.
Summary
Formation and role of RBC microparticles are far from being completely understood. Combining various approaches to elucidate these mechanisms could improve blood product quality and transfusion safety. Implementation of RBC microparticles as biomarkers in the laboratory routine needs to overcome technical barriers involved in their analysis.

Keywords
microparticles, proteomics, red blood cells, transfusion

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Abstract

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Summary

Formation and role of RBC microparticles are far from being completely understood. Combining various approaches to elucidate these mechanisms could improve blood product quality and transfusion safety. Implementation of RBC microparticles as biomarkers in the laboratory routine needs to overcome technical barriers involved in their analysis.

Introduction

Microparticles, also described as microvesicles [1] or ectosomes [2], are heterogeneous populations of phospholipid vesicles of less than 1 mm, released in circulating blood by erythrocytes, platelets, white blood cells or endothelial cells [3]. The production of microparticles is a highly controlled process, triggered by various stimuli, including cell stimulation and apoptosis [4]. If microparticles have been first described as cell dusts [5], they are now recognized as being involved in a broad spectrum of biological activities, such as thrombosis and haemostasis [3,6], inflammation [6], vascular and immunefunction [7], apoptosis [4] or even intercellular communication by the transfer of surface proteins [8]. Microparticles are detected in healthy individuals and their increase has been observed in a variety of diseases with elevated thrombotic risk, vascular involvement or metastasis [9,10,11].Proteomics analysis has been recently applied to the study of red blood cell (RBC) microparticles (reviewed in [12]) and allows identification of numerous different proteins in biological samples. Proteomics has been successfully used in the field of transfusion medicine [13–15] and allowed the deciphering of various blood diseases [16]. Nevertheless, and whatever methods are employed to characterize microparticles, preanalytical issues remain of major importance to correctly assess microparticles in blood [17]. The lack of standardized methods has impaired microparticles analysis implementation in the clinical setting [18].

Formation and clearance of red blood cell microparticles

The normal erythrocyte has a unique membrane and cytoskeleton organization with redundant surface area and sufficient flexibility to undergo extensive deformation during its transit through the spleen [19]. Under physiological conditions, the asymmetric distribution of RBC membrane phospholipids is maintained by the cooperative action of translocases (flippase, floppase and scramblase), concentrating negatively charged aminophospholipids, like

phosphatidylserine on its inner leaflet[20].

Microvesiculation

Rapid externalization of phosphatidylserine after cell activation or apoptosis modifies the neutral membrane charge into negative with loss of phospholipid asymmetry, leading to a cascade of events which disrupts the interactions between membrane and cytoskeleton proteins, specifically spectrin and protein 4.1R. The stability of the membrane is thus directly affected, becoming less rigid and allowing the formation and release of microparticles [21,22]. During their lifespan, RBCs lose approximately 20% of their haemoglobin (Hb) content, and similar surface area through vesicles emission, thus decreasing their favourable surface-to-volume ratio [23]. Microvesiculation occurs throughout the erythrocyte lifespan and is triggered by different types of stimuli [23], such as shear stress, complement, attack,n oxidative stress and pro-apototic stimulations [4]. As shown during complement activation, microvesiculation prevents immediate killing of RBCs by eliminating the membrane attack complex C5b–9; this survival strategy is especially used when expression of complement regulatory proteins on the cell surface are decreased [24]. It remains obscure how the spleen facilitates microparticles formation during the second half of the erythrocyte lifespan; interestingly, improvement of RBC osmotic fragility due to increase of surface area has been described in patients with hereditary spherocytosis after splenectomy [25,26].

Erythrocyte ageing models

Microparticles formation has been described as an inte-gral step of RBC senescence [23]. Two major models of erythrocyte ageing have been proposed so far: the eryptosis and the band 3 clustering. The eryptosis model, which is similar to apoptosis of nucleated cells, could be regarded as the RBC response to various stresses, whereasmthe band 3 model may explain

the physiology of RBC ageing [27]. In the eryptosis model, intracellular flux of calcium through possibly altered nonspecific cation channels leads to activation of several enzymes, such as scramblases, calpains and transglutaminase 2. This results in phosphatidylserine externalization, degradation and cross-linking of cytoskeletal proteins, followed by modifications in the phosphorylation status of band 3 [28,29,30]. The band 3 clustering model is characterized by protein oxidation [31]. The oxidation of Hb contributes to hemichrome formation, which is constituted Hb-derived products (likely met-Hb) linked to the inner leaflet, followed by the clustering and aggregation of band 3 multimers in the membrane [32]. Band 3 clustering forms or uncovers senescent neoantigens, probably because of relatively small structural modifications that are recognized by naturally occurring autologous IgG with subsequent complement activation [33–35]. Both models share the similar final outcome that leads to modifications of band 3 and induces perturbations of the inner leaflet microenvironment, which could alter the tight balance between the membrane and cytoskeleton forces, thus resulting in microvesiculation. It has been shown that modifications of the band 3–ankyrin anchoring complexes could enhance lateral compression forces of the cytoskeleton, making the RBC membrane prone to distortion. Thereby, band 3 anion exchanger should be considered as a key protein in the regulation of erythrocyte structure and function [23], through binding cytoskeleton proteins, glycolytic enzymes and Hb [36].

Microparticles composition

Reviews of proteomic analysis of microparticles have been published elsewhere [17,37,38,39]. The composition of RBC microparticles may vary according to the stimulus and differs from their parental cell by nearly complete absence of cytoskeletal-linked molecules, decrease of membrane proteins content, presence of more metabolic proteins and Hb and exposure of removal signal molecules such as phosphatidylserine and autologous IgG

[23,40]. Furthermore, enrichment of several erythrocyte membrane components has been described, especially band 3, glycophorins, complement receptors, glycosyl-phosphatidylinositol (GPI)-anchored proteins and lipid-raft markers, suggesting selective membrane lipids and protein sorting. In lipid-raft origin of RBC microparticles, the oxidation of cytoskeleton proteins may promote lateral movement of stomatin oligomers in the membrane, which serve as nuclei for lipid-raft aggregation and extension, leading to the budding of membrane lipid-patches detached from cytoskeleton and segregation of molecules, including stomatin and GPI-anchored proteins [39,41].

Clearance mechanisms

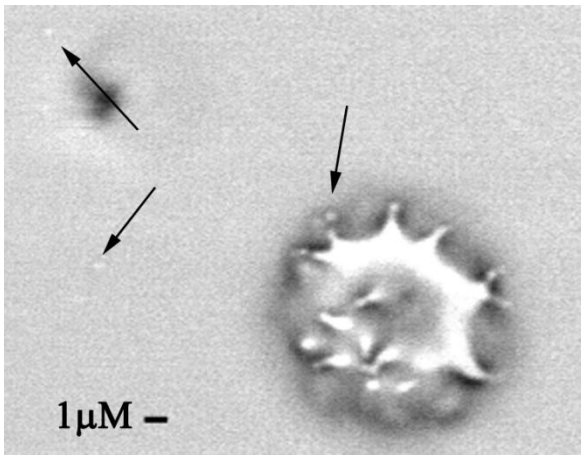
Once generated in circulating blood, phosphatidylserine exposing RBC microparticles are rapidly and efficiently removed by binding to the macrophage's scavenger receptors of the organ in which they originate [40]. Alternatively, RBC microparticles carrying senescent neoantigen-specific autoantibodies are recognized by Fcg-receptors and eliminated through the mononuclear phagocyte system [42]. According to the limited capacity of RBCs for self-repair, vesicles formation, which is an ATP-free mechanism even faster than translocation back of phosphatidylserine [40], may not only help to remove membrane-damaged molecules, postponing the phagocytosis of otherwise functional erythrocytes, but also prevents the exposure of potentially dangerous molecules [39]. Once microvesiculation capacity is exceeded, old erythrocytes are most likely phagocytosed.

Red blood cell microparticles and transfusion medicine

Under blood bank conditions, RBCs undergo progressive structural and biochemical changes commonly referred to as 'the storage lesion' [39]. As shown in Fig. 1, erythrocytes show progressive cell shape transformation from biconcave disk to rigid spheroechinocyte [42],

accompanied by the release of microparticles from the tips of spicules [6,43]. In addition, there is a depletion of ATP, pH acidification, haemolysis and microparticles accumulation observed in the medium [41]. Although differences have been reported between in-vitro and in-vivo RBC senescence such as denaturation of spectrin, changes in carbohydrate part of the membrane and increased mean cellular volume, the storage lesion shares similar features with the cell ageing process [38,44]. RBC membranemodification during storage is triggered by ATP depletion and oxidation and is centred on changes in band 3 leading to membrane detachment and disorganization that probably affect RBC deformability, osmotic resistance and survival after transfusion [45,46].

Figure 1: Observations of microparticles by microscopy give information about their size and shape



By confocal differential interference contrast microscopy, it is possible to observe forming microparticles (arrows) from spicules of echinocytes (magnification 4000 x)

Microparticles in erythrocyte concentrates

RBC microparticles formation represents a continuousprocess of membrane remodelling, which occurs early during blood banking [41,47], and prevents the exposure of phosphatidylserine on RBC [48]. Almost all microparticles found in erythrocyte concentrates originate from RBCs and their number gradually increases with storage time [37,42,49]. The level of vesiculation in erythrocyte concentrates may vary not only with the length of storage

but also according to the product and the storage solution: RBC microparticles are increased in washed blood products [50,51], whereas they could be lowered with additive solutions that manage effectively the oxidative stress [44]. The level of circulating microparticles could significantly increase, as already observed in transfusedpatients presenting with paroxysmal nocturnal haemoglobinuria [50].

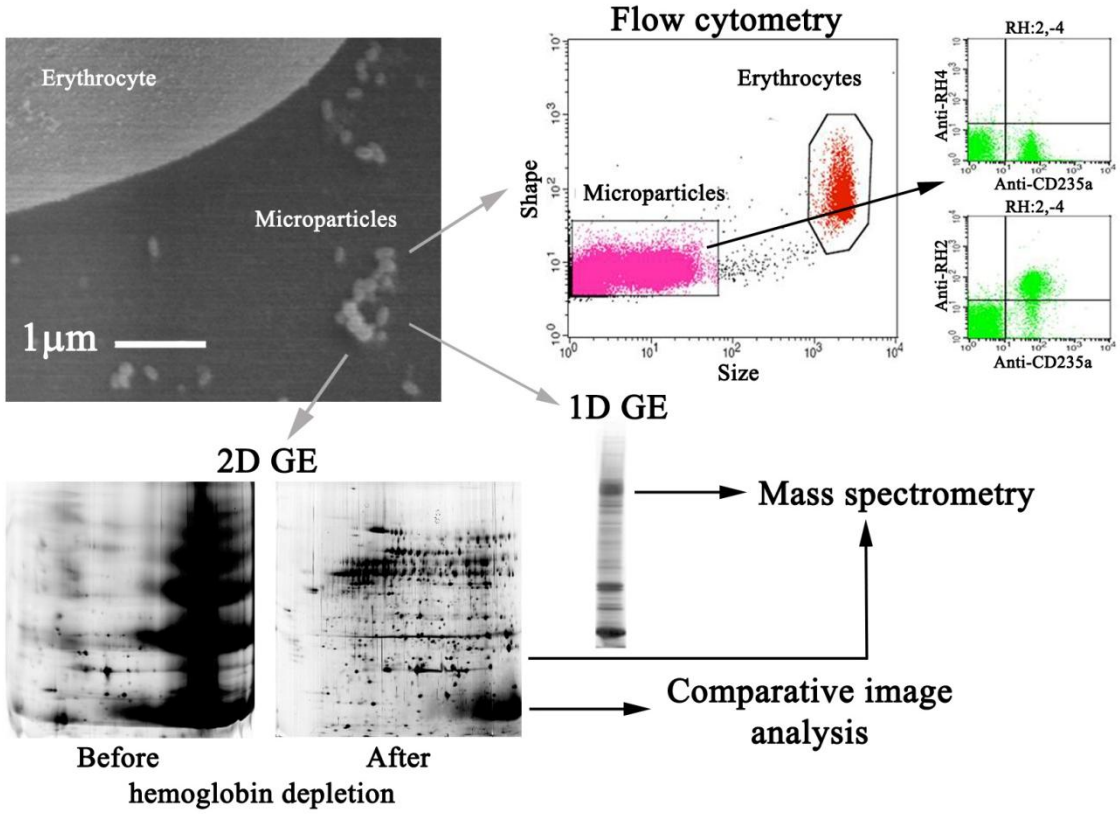
The composition of RBC microparticles in erythrocyte concentrates is nearly similar to those generated in vivo, except for the increased levels of stomatin, making it likely that a raft-based process is responsible for microvesiculation at low temperatures [41]. As those generated in vivo, the vesicles found in erythrocyte concentrates are devoid of most of the RBC integral membrane proteins or cytoskeletal components, with the exception of actin and band 3, found in aggregated or degraded forms [6,39,41]. Proteomics data confirm exposure of phosphatidylserine, binding of immunoglobulins and partial activation of complement on their surface. The significant difference observed in membrane composition between RBCs and microparticles outline that vesicles are generated by specific processes, which allow sorting of lipids and proteins [39]. Nonetheless, the pathways responsible for membrane lipids remodelling before microvesiculation remain mostly unknown. According to the absence of immunologic removal, microparticles in erythrocyte con- centrates become more heterogeneous over time with a gradual increase of their size and of their content of proteasome components, and a decrease of phosphatidylserine exposure [38,42], suggesting that either microparticles structure or nature of vesicle formation may vary with storage time.

Microparticles and blood group antigens

Half of the RBC transmembrane proteins carry various blood group antigen specificities [52]. Some of them, such as the rhesus (Rh) proteins, contribute to membrane stabilization through their link with protein 4.1R [53]. Blood storage is associated with the generation of soluble

ABH antigens in the bag, parallel to the decrease of their expression on erythrocytes [54]. Various studies have shown that blood group antigens are located on microparticles [55,56]. The identification of Rh peptides by mass spectrometry and Rh antigens by flow cytometry (Fig. 2) indicates that the protein is present within the membrane and that the antigenic part is located at the outer side of the microparticles membrane [37]. Our flow cytometry analyses demonstrated the presence of several antigens such as RH2 on microparticles (Fig. 2) as well as RH1, RH3, RH4, RH5, FY1, FY2, JK1, JK2 and KEL1 (data not shown). Despite their cytoskeletal dissociation, Rh antigens on microparticles keep the property to react with the corresponding antibodies. However, it is not known whether they are able to elicit an immune response. Another striking feature is the suppression of RBC antigens during autoimmune haemolytic anemia and during transfusion of incompatible blood [57,58]. It has been reported that antibody binding could induce disruption of membrane organization as well as vesicle formation with consequently the loss of the corresponding blood group antigens [59]. The exact mechanism of antibody-induced antigen suppression, which allows transfused RBC to escape haemolysis, is not known but it is tempting to speculate that selective loss of antigen might occur through RBC vesiculation.

Figure 2: General scheme presenting the most used analysis methods of microparticles



The upper left corner of the figure shows part of a red blood cell and microparticles (scanning electron microscopy; magnification, Â37 000). By flow cytometry technique, it is possible to sort microparticles from erythrocytes according to size and shape. Panels (A) and (B) show the region of microparticles sorted according to their fluorescence. Microparticles issued from a RH:2,-4 typed erythrocyte concentrate were tagged by RH2 antibodies (B), but not with RH4 antibodies used as negative control (A) (tagged microparticles are on right and nontagged on the left) (adapted from [37]). Microparticles were double labelled with anti-CD235a (glycophorin A) which binds to the vast majority of erythrocyte microparticles (histograms show 'MP region' according to their fluorescence; the tagged microparticles are on the right and nontagged on the left of panel A and B). Flow cytometry analysis also may reveal other red blood cell antigens on microparticles (such as Duffy or Kell). Microparticles proteins can be separated by different techniques such as SDS-PAGE (one-dimensional gel electrophoresis) or two-dimensional gel electrophoresis combined either with image analysis or mass spectrometry. Using this approach, various microparticles proteins were identified. Of note, two-dimensional gel electrophoresis of microparticles is hampered by the presence of large amounts of haemoglobin, which can be removed by off-gel electrophoresis, allowing a resolution sufficient for comparison image analysis. GE, gel electophoresis. Adapted with permission from Rubin et al. [12].

Clinical relevance

A large proportion of Hb is enclosed in RBC microparticles [60,61] and, with free Hb, participates in the increase of extracellular Hb during storage. Hb composition of microparticles resembles that of the oldest RBC and is enriched in denatured and high molecular weight fractions [39,42]. Due to their small size and the lack of most of the cytoskeletal proteins, it is expected that RBC microparticles could localize near the endothelium and will probably scavenge nitric oxide as effectively as cell- free Hb [62,63]. This reduction of nitric oxide bioavailability will affect adversely the microcirculation by promoting platelet aggregation and endothelial adhesion, impairing vasodilatation and generating reactive oxygen species [60].

Moreover, phosphatidylserine exposure on RBC microparticles surface offers a binding site for prothrombinase and tenase enzyme complexes [12,64] and is associated with increased activity of factors VIII, IX and XI [65], thus participating in thrombin generation and amplification. Hypercoagulable state associated with enhanced microparticles levels has been observed in chronic haemolytic anemia, including in sickle cell patients [65,66]. Although the relationship between RBC microparticles concentration and increased risk of arterial or venous thrombosis events after blood transfusion is not evident, this question has been raised recently in cancer patients [67].

The RBC microparticle potential role of cell-to-cell communication has not been investigated so far. RBC microparticles, as other cell-derived microparticles, could transfer molecules and modify cell phenotype such as illustrated by the transfer of GPI-anchored proteins CD55 and CD59 from vesicles found in erythrocyte concentrates to erythrocytes and granulocytes of paroxysmal nocturnal haemoglobinuria transfused recipients [50]. Moreover, the recruitment

of RBC microparticles by nucleated cells allows the transfer of removal signals such as phosphatidylserine to innocent bystanders [68], labelling them with an apoptosis marker. These data offer a perspective of further investigations in the field of cell-to- cell communication capacity of RBC microparticles.

In addition to activating the classical complement pathway through IgG on their membrane, storage vesicles may affect the innate immune response by inhibiting the production of pro inflammatory cytokines such as tumour necrosis factor-a and interleukin-8 [49]. To what extent this anti-inflammatory signal may explain some of the immunosuppressive effects of blood transfusions remains to be clarified.

Although vesiculation contributes to erythrocyte homeostasis in eliminating sorted oxidized proteins [42], theimpact of RBC microparticles on the microcirculation coupled with their immunomodulation and thrombogenic activities could potentiate adverse clinical outcomes observed in susceptible recipients transfused with older blood [69,70]. Undeniably, these observations raise important questions about the clinical impact of RBC storage lesions; however, this topic remains a matter of debate and needs strong epidemiologic data to clarify its role in transfusion medicine.

Conclusion

Extensive proteomic analyses of RBC microparticles have enabled a better comprehension of erythrocyte changes during ageing and blood storage and outlined the pivotal role of band 3–ankyrin anchoring complex. Indisputably, vesicle formation represents an integral part of the erythrocyte ageing process. RBC microparticles could be considered as the ‘Dr Jekyll and Mr Hyde’ in the field of transfusion: on one hand, improving RBC survival in transfused

recipients by allowing the elimination of toxic molecules and removal signals; on the other hand, enhancing the deleterious events like microcirculation impairment, thrombosis and immunosuppression in susceptible patients.

Despite the currently available proteomics and immunochemical data, the exact and complete mechanisms of erythrocyte microvesiculation remain only partially elucidated. Combining comparative and qualitative proteomic analysis with other different approaches including immunochemical data or flow cytometry will help to discover the precise components involved in RBC microparticles formation and may identify the potential role of microvesicles in transfusion medicine. Therefore, proteomic analysis will provide a tool for the development of methods that could enhance RBC quality and survival after transfusion. Nevertheless, the use of microparticles as biomarkers in clinical routine or blood banking needs the technical barriers involved in their analysis to be overcome.

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3

Microparticles in stored red blood cells: an approach using flow cytometry and proteomic tools

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Vox Sanguinis

Background and Objectives Microparticles (MPs) are small phospholipid vesicles of less than 1 μm, shed in blood flow by various cell types. These MPs are involved in several biological processes and diseases. MPs have also been detected in blood products; however, their role in transfused patients is unknown. The purpose of this study was to characterize those MPs in blood bank conditions.

Materials and Methods Qualitative and quantitative experiments using flow cytometry or proteomic techniques were performed on MPs derived from erythrocytes concentrates. In order to count MPs, they were either isolated by various centrifugation procedures or counted directly in erythrocyte concentrates.

Results A 20-fold increase after 50 days of storage at 4°C was observed (from 3370 ± 1180 MPs/μl at day 5 to 64 850 ± 37 800 MPs/μl at day 50). Proteomic analysis revealed changes of protein expression comparing MPs to erythrocyte membranes. Finally, the expression of Rh blood group antigens was shown on MPs generated during erythrocyte storage.

Conclusions Our work provides evidence that storage of red blood cell is associated with the generation of MPs characterized by particular proteomic profiles. These results contribute to fundamental knowledge of transfused blood products.

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Key words: blood product storage, microparticles, proteomics, red blood cells, transfusion.

Abstract

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Conclusions

Our work provides evidence that storage of red blood cell is associated with the generation of MPs characterized by particular proteomic profiles. These results contribute to fundamental knowledge of transfused blood products.

Introduction

Millions of blood products are transfused worldwide every year; many lives are thus directly concerned by transfusion. Since the beginning of blood transfusion, numerous efforts have been made to secure blood products and gain knowledge about their molecular structures. The progress of proteomics allows re-examining important issues in blood research [1] and transfusion science [2] with the tools of large-scale biology [3].

The three main labile blood products used in transfusion are erythrocyte concentrates (ECs), platelet concentrates and fresh-frozen plasma. Each of these products has to be stored according to its particular components. However, during storage, modification or degradation of those components may occur and are known as storage lesions. Among these lesions, the generation of microparticles (MPs) has been identified [4–6].

Ageing erythrocytes in blood bank conditions differ from in vivo, therefore, it has been suggested that erythrocyte physiological ageing process may be accelerated by storage conditions [7]. Indeed, during storage, several biochemical and physiological changes occur in ECs, including an increase in the concentration of free haemoglobin, lipids, MPs and a pH reduction. Concerning red blood cells (RBCs), they lose adenosine triphosphate, 2,3-diphosphoglycerate or potassium and their membrane undergoes various modifications, such as more rigidity, disruption of phospholipids asymmetry, protein clustering, lipid raft rearrangement, loss of fragments or even release of MPs [8–10]. The exact effects

of storage lesions on transfusion are still unknown.

Microparticles are small phospholipid vesicles of less than 1 µm in size, also known as microvesicles [11] or ectosomes [12]. They are released from a variety of cells, such as platelets, RBCs and white blood cells, or endothelial cells [13]. They contain a subset of

proteins derived from their parent cells. However, MPs are heterogeneous and vary in size, phospholipid and protein composition. Release of MPs is a highly controlled process prompted by various stimuli, such as shear stress, complement attack, proapoptotic stimulation or damage [14].

Microparticles have long been considered as cell fragments or ‘debris’ without any biological function. Although their true biological function is still unknown, there are more and more indirect evidence that MPs are involved in a broad spectrum of biological activities, such as haemostasis [15], thrombosis [13], inflammation [15], transfer of surface proteins [16] or even angiogenesis [17].

An increase in the number of MPs in plasma has been demonstrated under various pathological conditions, such as heparin-induced thrombocytopenia [18], thrombotic thrombocytopenic purpura [19], diabetes [20,21], acute coronary syndromes [22], cardio vascular disease [23] or sepsis [24]. Despite their potential important activities, only few studies are available on MPs in blood products. It has been demon- strated that the number of MPs increases with the age of blood products [25] and that there is a link between the risk of transfusion complication and the age of the transfused blood products [26,27]. Thus, an appealing hypothesis is that a high number of MPs in ECs is linked to adverse transfusion reactions. In addition, Koch et al. recently demonstrated a link between the age of transfused RBCs and post-transfusion complications [28], indicating that progress in the knowledge of stored RBCs biology is urgently needed. In order to have a better understanding of MPs in stored ECs, qualitative and quantitative experiments using flow cytometry or proteomic techniques were developed and performed. The goals of this study were: (i) to test centrifugation methods for the isolation of MPs from ECs; (ii) to count MPs directly in ECs and in their supernatant fraction; and (iii) to gain insight into the protein content of MPs as compared to RBC membranes, and (iv) to evaluate if erythrocyte-derived MPs also express Rhesus antigens on their surface.

Materials and methods

Erythrocyte concentrates

Whole blood was collected and prepared at the Lausanne or Bern blood banks, according to standardized procedures. Only ECs that did not satisfy quality criteria for transfusion were used for this study, notably those collected from donors presenting with elevated levels of alanine transaminases (ALAT). Briefly, 450 ± 50 ml of whole blood was drawn by venipuncture and collected in blood bags (Fenwal, Lake Zurich, IL, USA) containing the anticoagulant solution (citrate–phosphate–dextrose). Leucocytes and platelets were then removed by filtration. After separation of plasma from erythrocytes by centrifugation, RBCs were finally suspended in 100 ml of preservative solution sodium–adenine–glucose–mannitol. For the experimental purpose of this study, ECs were stored up to 50 days at 4°C instead of 42 days, the usual expiration date for concentrates in sodium–adenine– glucose–mannitol solution.

Flow cytometry analysis and microparticles counts in erythrocyte concentrates

Samples were analysed on a FACScalibur flow cytometer with CellQuest pro software (BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometer was daily calibrated with CaliBRIT™ 3 kit (BD Biosciences) containing different fluorescent beads. Size events were defined using flow cytometry size beads of 1–1.4 μm (Spherotech, Lake Forest, IL, USA). For the different windows used, the flow cytometer was set on a logarithmic scale.

Fluorescein isothiocyanate (FITC) anti-human CD47 (BD Pharmingen, San Diego, CA, USA) and phycoerythrin (PE) anti-human CD235a (or glycophorin A) (BD Pharmingen) were the two antibodies used to tag erythrocyte MPs. Moreover, experiments have been done with FITC human annexin V (BD Pharmingen) that tags negative phospholipids present on MPs surface.

Microparticles counts were determined in the supernatant of a stored EC at 4°C for 38 days, after various centrifugation conditions (870 g, 1850 g, 2550 g and 3250 g). Four samples of 50 ml from the EC were spun down twice for 20 min, and 100 µl of supernatant was then mixed with 3 µl of FITC anti-human CD47 or 3 µl PE anti-human CD235a or both for double staining. After 20 min of incubation on an orbital shaker in the dark at 4°C, 400 µl of phosphate-buffered saline (PBS) was added and flow cytometry analysis was carried out within 1 h in a Trucount™ tube (BD Biosciences). Isotypic controls were performed with PE immunoglobulin G2b (IgG2b) or FITC IgG1 (both from Diaclone, Besançon, France). MPs were also determined in the supernatant of 13 different ECs stored from 2 to 50 days at 4°C after two centrifugations at 1850 g for 20 min. Measurements were done in triplicates. The flow cytometer settings for counting MPs in supernatants were as follows (detector: voltage): FSC: E00, SSC: 360, FL1: 500–600, FL2: 500–600 and FL3: 570.

Finally, to avoid pre-analytical variability due to centrifugation conditions, MPs counts were determined in ECs from seven different blood donors (without centrifugation). For each EC, six measurements were performed from day 2 to day 50 of storage at 4°C. Red blood cells were carefully mixed with the storage solution, and 5 µl of the RBC suspension was mixed with 4 µl of FITC anti-human CD47 for 5 seconds. The mixture was then incubated for 20 min on orbital shaker in the dark. Lastly, 4 µl of the solution was diluted to 1 ml with 0.9% NaCl in a Trucount™ tube, and was directly analysed by flow cytometry. Isotypic controls were performed with FITC IgG1 (Diaclone). The flow cytometer settings for counting MPs in concentrates were as follows (detector: voltage): FSC: E00, SSC: 300, FL1: 650, FL2: 520 and FL3: 600.

Proteomics

Proteomic analysis was done on MPs and erythrocyte membranes of the same 42-day stored ECs. MPs were obtained after three centrifugations (1850 g twice and 3200 g once, 20 min at 4°C) and the supernatant containing MPs was collected. Then, three ultracentrifugations at 120 000 g for 90 min at 4°C were done, each time pellets were suspended in PBS. To obtain erythrocyte membranes, RBCs were washed in PBS 10× and spun at 1850 g for 20 min at 4°C three times. Collected pellets were then washed in deionized water and after another centrifugation at 1850 g for 20 min at 4°C, pellets were collected and prepared for future analysis.

To determine the quantity of sample to load, protein concentration of each sample was measured according to the Bradford's method [29]. For sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), 30 μg of proteins was loaded onto a 4–12% NuPAGE Novex Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, CA, USA). The migration was carried out at constant voltage (200 V).

For mass spectrometry (MS) analysis, SDS-PAGE was run as previously described, but 300 μg of proteins was loaded. Upon electrophoresis completion, the gels were rinsed twice with deionized water and stained with colloidal Coomassie blue (National Diagnostics, Atlanta, GA, USA) overnight. The gels were destained with deionized water. Bands of interest were excised from the gels and transferred into an Eppendorf. In-gel proteolytic cleavage with sequencing-grade Trypsin (Promega) was automatically performed in the robotic workstation Investigator ProGest (Perkin Elmer Life Sciences) according to the protocol of Shevchenko et al. [30]. Digests were evaporated to dryness and resuspended in 3 μl of α- cyano-hydroxycinnamic acid matrix (5 mg/ml in 60% (v/v) acetonitrile : water), of which 0.7 μl was deposited in duplicate on a target plate. Matrix-assisted laser desorption/ionization MS (MALDI-MS)/MS analysis was performed on a 4700 Proteomics Analyser (Applied

Biosystems, Framingham, MA, USA). After MALDI – Time of Flight (TOF)/MS analysis, internal calibration on trypsin autolysis peaks and subtraction of matrix peaks, the 10 most intense ion signals were selected for MS/MS analysis. Non-interpreted peptide tandem mass spectra were used for direct interrogation of the Uniprot (Swissprot + TrEMBL) database using Mascot 2.0 (http://www.matrixscience.com). The mass tolerance for database searches was 50 p.p.m. MASCOT was set up to only report peptide matches with a score above 14. With the parameters used, the threshold for statistical significance ($P < 0{\cdot}05$) corresponded to a total (protein) MASCOT score of 33. Proteins scores above 80 were automatically considered valid, while all protein identifications with a total MASCOT score between 33 and 80 were manually validated. Validation included examination of the peptide root mean square mass error and of individual peptide matches. Peptide matches were validated only if at least an ion series of four consecutive y ions were matched, in addition to ions belonging to other series. Generally, only proteins matched by at least two peptides were accepted.

Western blotting

From 20 to 50 μg of proteins from the samples described before (MPs and erythrocyte membranes) were loaded onto a 4–12% NuPAGE Novex Bis-Tris polyacrylamide gel (Invitrogen). After migration, carried out at constant voltage (200 V), proteins were transferred to polyvinylidene fluoride membranes using a Novex blot module (Invitrogen) for 1 h 45 min at fixed voltage (30 V), according to the manufacturer's instruction. After transfer, blotted membranes were soaked overnight in blocking solution with PBS, 0·1% Tween-20 (v/v), 5% milk and 1% BSA (w/v). Four Western blots were done, each one with a different antibody, namely, anti-human CD235a (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-human actin (Sigma, Saint Louis, MO, USA), anti-human stomatin (Proteintech Group, Chicago, IL, IL) and anti-human CD47 (from Santa Cruz or from AbCam, Cambridge, UK).

All antibodies were used at a dilution of 1 : 500. The goat anti-rabbit and goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Dako, Baar, Switzerland) were both used at a dilution of 1 : 10 000. Subsequent visualization was performed using enhanced chemiluminescence (GE Healthcare, Uppsala, Sweden). The signal was finally captured using X-ray film.

Expression of Rhesus antigens

Flow cytometry was used to determine the presence of Rh antigens on MPs generated during storage. MPs were first isolated from the supernatant of an EC after two centrifugations at 1850 g at 4°C for 20 min. The supernatant was then spun down at 18 000 g for 5 min to pellet MPs. Pellet was dissolved in 100 µl of PBS. One µl of the primary antibody (anti-D, anti-C, anti-c, anti-E and anti-e, respectively) was later added and mixed on orbital shaker for 90 min. IgM anti-D were obtained from Orthobiotech (Bridgewater, NJ, USA), whereas IgM anti-C, IgM anti-c, IgM anti-E and IgM anti-e were obtained from Biotest (Dreichen, Germany). One µl of the secondary antibody was then added, and after 1 h on orbital shaker in the dark, 400 µl of PBS was added, and samples were analysed by flow cytometry within an hour. Secondary antibody directed against primary IgM was FITC anti-human IgF(ab) from Chemicon (Melbourn, Australia). To demonstrate the presence of various Rh antigens on MPs, blood samples expressing different Rh phenotypes, such as DCCee, DccEE or dccee, were selected. The antithetical phenotype was used with each antibody as a negative control.

Results

Microparticle counts

Using flow cytometry, MPs clearly were distinguished from RBCs by their size as well as by the negatively charged phospholipids on their outer membrane detected by annexin V. Indeed, the great majority of MPs were annexin V-positive, while merely a few percentage of erythrocytes were positive. MPs were also identified using either anti-CD47, anti-CD235a or both antibodies, without any differences in their numbers according to the choice of the antibody (Figs 1 and 2). In both methods used, an increase in the number of MPs during storage of ECs was observed (Figs 2 and 3). The number of MPs was clearly related to centrifugation conditions (Fig. 2). In the measurements performed directly in the concentrate, the number of MPs increases about 20-fold after 50 days of storage at 4°C and considerably varies among different samples; it starts from 3370 + 1180/μl after 6 days, up to 64 850 + 37 800/μl after 50 days of storage (Fig. 3). The intra-assay coefficient of variation was evaluated. With both methods, the coefficient was less than 15%, even after 50 days of storage (data not shown). Nonetheless, and without evident explanation, we observed a huge individual variation of the MPs counted among different donors.

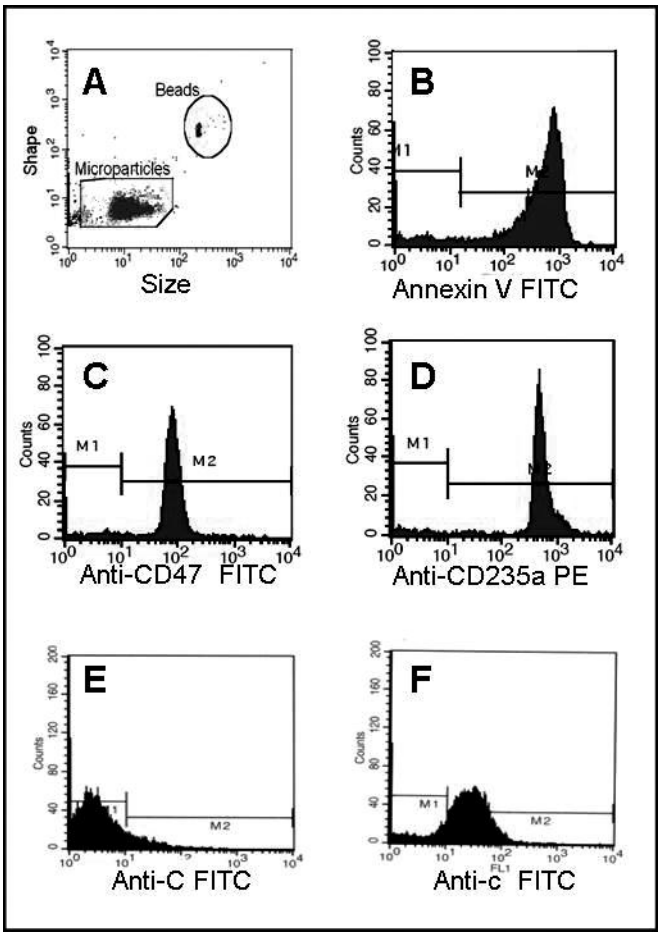

Fig. 1 Flow cytometry analysis of microparticles from a supernatant after centrifugation of erythrocyte concentrate. (a) Events that were sorted according to their size and shape. Two different regions were determined; microparticles and beads. A precise number of beads were used to determine the number of microparticles in each sample. Histograms represent events from the microparticle region (b–e) of (a) according to their fluorescence (due to an FITC- or PE-labelled antibody). M1 represents non-stained events whereas M2 shows stained event. (b) Microparticles stained with annexin V; (c) microparticles stained with anti-CD47 FITC; and (d) microparticles stained with anti-CD235a. FITC, fluorescein isothiocyanate; PE, phycoerythrin. In (e) and (f), microparticles were derived from an erythrocyte concentrate from a donor typing dccee. In this example, microparticles were negative for anti-C antibody (e), while they were positive for anti-c antibody (f).

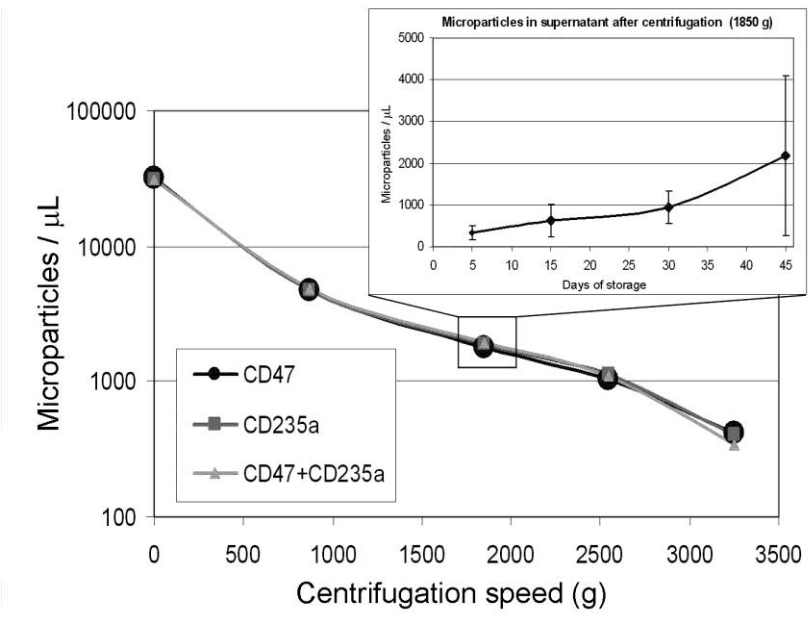

Fig. 2 Count of microparticles in supernatant of erythrocyte concentrates (ECs). The number of microparticles measured decreased with increasing centrifugation speed (test on one EC stored for 38 days). Note that the numbers measured were identical with the two antibodies used in this study (anti-human CD235a or CD47). The inset shows an increase in the number of microparticles in supernatant of ECs during storage (after centrifugation at 1850 g, here anti-human CD235a was used).

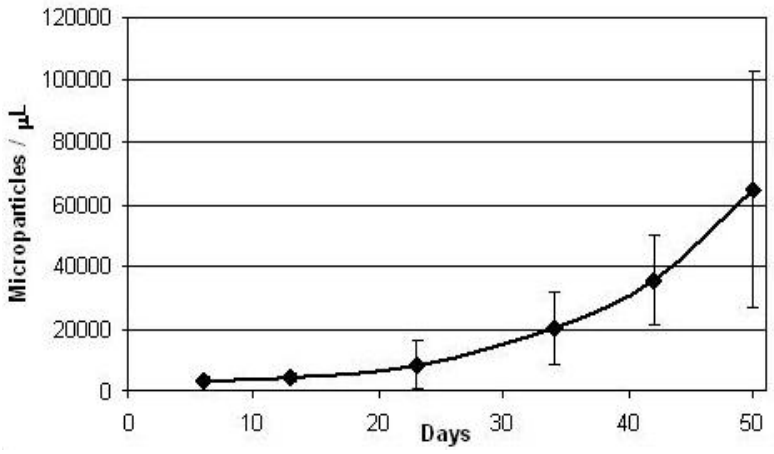

Fig. 3 Count of microparticles directly in erythrocyte concentrates during storage (without centrifugation). Data are expressed as the mean ± SD experiment (n = 7). At day 5, 3371 ± 1188 microparticles/µl were counted, whereas at day 50, their numbers were 64 858 ± 37 846 microparticles/µl. Anti-human CD47 was used to stain microparticles

Proteomics

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis showed a number of large as well as discrete bands obtained after electrophoresis of RBC membranes (Fig. 4, lane A) and MPs (Fig. 4, lane B). A major difference was observed in the 25–35 kDa region; thus, in order to perform protein identification, bands of interest stained with Coomassie blue were excised and proteins were identified by MS. Table 1 lists the proteins identified in the three excised bands of lane A (erythrocyte membranes) and lane B (MPs). Not surprisingly, abundant proteins such as carbonic anhydrases or peroxiredoxins were identified both in RBC membranes and MPs with good sequence coverage. A set of 14-3-3 proteins was also identified in both samples: 14-3-3 proteins are abundant and ubiquitous proteins [31] that act as regulators of a number of processes, such as modulation of protein kinase activities, signal transduction [32]. Remarkably, 14-3-3ζ has been shown to be implicated in GPIb-IX-V translocation to the cytoskeleton during platelet activation [33]. It was thus not surprising to find 14-3-3 proteins in RBC ghosts. The gel band that appeared to be quantitatively the most different between lanes A and B was identified as stomatin, identified in MPs with a score of

1131, a sequence coverage of 76·4% from 46 peptides, whereas it was identified in RBC membranes with a score of 156, a sequence coverage of 14·9% from four peptides, indicating that stomatin was largely enriched in MPs compared to erythrocyte membranes.

Interestingly, some of the identified proteins did not have molecular weights that corresponded to their respective position on the gel. For example, haemoglobin subunits α and β, which have a molecular weight of about 15–16 kDa, were observed in the region corresponding to 25–35 kDa on the gel. However, it has been documented that denatured and cross-linked haemoglobin strongly binds to the cytoskeleton during RBC storage in blood banking conditions [34]. It is thus highly probable that the haemoglobin subunits identified were present as homogeneous or heterogeneous dimers. Additionally, Band 3 (a major membrane protein) as well as Rhesus protein were identified in MPs only, from three and two sequenced peptides, respectively. For Band 3, the identified peptides correspond to the cytoplamic domain of the protein (spanning the region 117–180), which means that the Band 3 fragment that appears on the gel in the 25–35 kDa region belongs to the cytoplasmic domain of the protein. The Rhesus protein was identified from two peptides covering the 17 last amino acids on the C terminus of the protein sequence. Of interest, Mascott allowed the identification of Rh peptides (Table 1; lane B of Fig. 4). However, it was not possible to discriminate between RhD and Rh(CE) proteins, a well-known problem in proteomics [35]. Nevertheless, the presence of various Rh antigens at the surface of MPs was confirmed by flow cytometry (see below).

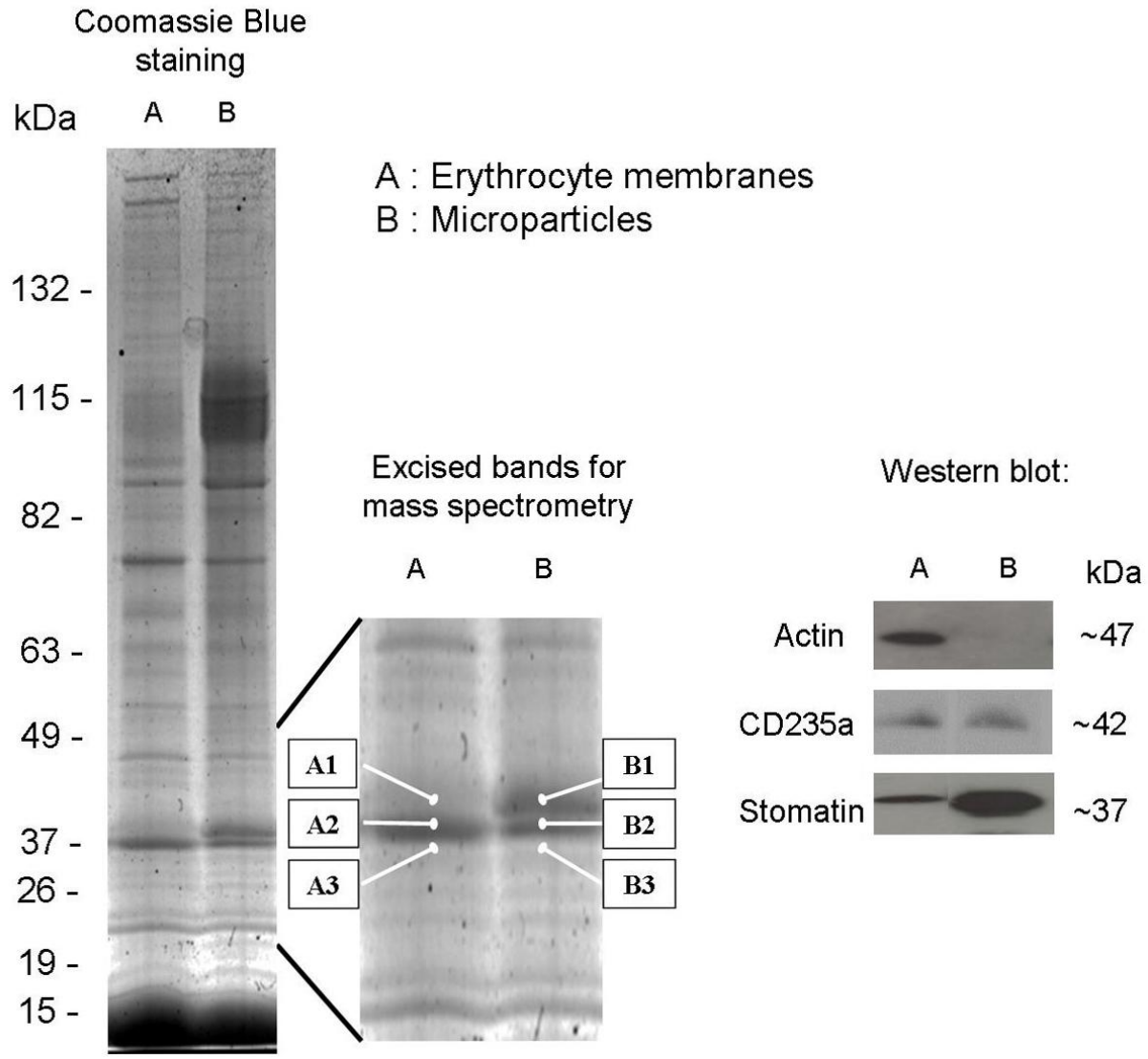

Fig. 4 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of erythrocyte membranes and microparticles, both were from a 42-day stored erythrocyte concentrate. The gel was stained with Coomassie blue (for better compatibility with mass spectrometry). Inset shows enlargement of Coomassie blue-stained gel used for the preparation of the bands submitted to mass spectrometry analysis. Western blot analysis points out the variation of protein expression. Thus, by comparing erythrocyte membranes and microparticles, a clear reduction of actin and an accumulation of stomatin were observed on microparticles. The staining of CD235a (glycophorin A) was similar in both gels.

Western blots

Western blotting confirmed the presence of CD235a, actin and stomatin on RBC membranes as well as on MPs, with a clear reduction of actin and an accumulation of stomatin on MPs

(Fig. 4). However, using this technique and the antibodies available, neither CD47 nor Rh-proteins could be detected after SDP-PAGE. With blot techniques, no quantitative difference was observed between MPs samples from ECs stored for 5 or 42 days.

Rhesus systems

The presence of blood group antigens of Rhesus system on MPs surface was investigated by flow cytometry using specific antibodies. In each assay, positive and negative samples were selected for the corresponding antigen (Fig. 1e,f). By this approach, the presence of C, c, D, E or e antigens was observed on MPs when they were present on the RBCs from which they derived. However, it was not possible to exclude that a population of MPs was Rh negative.

Table 1 Proteins identified in the region 25–35 kDa of RBC membranes (lane A of Fig. 4) and microparticles (lane B of Fig. 4). Reported are the sequence coverage (%) and the number of sequenced peptides

AC	Entry name	Protein name	MW (Da)	RBC (sequence coverage %)	RBC (identified peptide)	MPs (sequence coverage %)	MPs (identified peptide)
P31946	1433B_HUMAN	14-3-3 protein β/α	28 082	25·2	7	16·7	4
P62258	1433E_HUMAN	14-3-3 protein epsilon	29 174	34·9	11	NO	NO
P61981	1433G_HUMAN	14-3-3 protein γ	28 303	NO	NO	13·4	4
P27348	1433T_HUMAN	14-3-3 protein θ	27 764	14·3	5	NO	NO
P63104	1433Z_HUMAN	14-3-3 protein ζ/δ	27 745	44·5	11	8·2	2
P02730	B3AT_HUMAN	Band 3 anion transport protein	101 792	NO	NO	6·8	3
Q4TWB7	Q4TWB7_HUMAN	β-Globin chain (Fragment)	11 487	93·3	15	NO	NO
P07738	PMGE_HUMAN	Bisphosphoglycerate mutase	30 005	45·9	13	37·8	10
P00915	CAH1_HUMAN	Carbonic anhydrase 1	28 870	72·8	27	65·5	20
P00918	CAH2_HUMAN	Carbonic anhydrase 2	29 246	72·7	25	71·9	15
P07451	CAH3_HUMAN	Carbonic anhydrase 3	29 557	36·2	8	17·7	4
A0N071	A0N071_HUMAN	δ-Globin chain (haemoglobin δ)	16 055	55·1	10	55·1	9
P27105	STOM_HUMAN	Erythrocyte band 7 integral membrane protein	31 731	14·9	4	76·4	43
P17931	LEG3_HUMAN	Galectin-3	26 188	11·6	3	NO	NO
P78417	GSTO1_HUMAN	Glutathione transferase ω-1	27 566	33·2	10	7·9	2
P69905	HBA_HUMAN	Haemoglobin subunit α	15 258	71·1	9	71·1	9
P68871	HBB_HUMAN	Haemoglobin subunit β	15 998	83·0	16	93·9	15
Q16775	GLO2_HUMAN	Hydroxyacylglutathione hydrolase	28 860	8·8	2	NO	NO
P30041	PRDX6_HUMAN	Peroxiredoxin-6	25 035	25·9	6	16·1	4
P18669	PGAM1_HUMAN	Phosphoglycerate mutase 1	28 804	8·3	2	NO	NO
Q06323	PSME1_HUMAN	Proteasome activator complex subunit 1	28 723	16·5	4	NO	NO
P25788	PSA3_HUMAN	Proteasome subunit α type 3	28 433	6·7	2	NO	NO
P25789	PSA4_HUMAN	Proteasome subunit α type 4	29 484	12·3	4	NO	NO
P60900	PSA6_HUMAN	Proteasome subunit α type 6	27 399	9·3	2	NO	NO
O14818	PSA7_HUMAN	Proteasome subunit α type 7	27 887	21·4	4	NO	NO
P00491	PNPH_HUMAN	Purine nucleoside phosphorylase	32 118	59·5	16	17·6	4
Q0KG01	Q0KG01_HUMAN	RhD protein	45 052	NO	NO	4·1	2

AC, accession number; MW, molecular weight in Da; NO, not observed.

Discussion

There is no standardized method to count MPs. Several approaches have been proposed in the literature, the majority dealing with platelet MPs from whole blood or platelet concentrates. Centrifugation speeds, for MPs characterization, varied from 200 g to 13 000 g [36]. In our hands, complete elimination of RBCs from supernatant at low centrifugation was obtained after two centrifugation at 1850 g for 20 min. Annexin V is frequently used to detect phosphatidylserine, a negatively charged phospholipid known to be present on the outer leaflet of apoptotic cells as well as on MPs of various origins [18]. In this study, MPs were counted using flow cytometry with different antibodies, notably anti-CD235a and anti-CD47. These antibodies were chosen because both are reactive towards RBC membrane molecules known to be present on MPs [14,29].

Counting MPs directly within the homogenized ECs appeared as the simplest approach, avoiding handling, centrifugation and washing. The drawbacks were due to the presence of a great number of RBCs, the need of quite large amount of antibodies and an intra-sample variability in the number of MPs counted. In addition, samples cannot be stored, contrasting with supernatants containing MPs, which can be kept at 4°C or even be frozen before being evaluated by flow cytometry. Disadvantages of working with supernatants were related to the handling procedures and, more importantly, to the influence of centrifugation conditions. After centrifugation, residual RBCs were eliminated. However, a number of MPs appeared to be pelleted together with RBCs (Fig. 2). In any case, an increase in the number of MPs in ECs during storage was observed, even if the number of MPs counted differs according to the method. From our results, it is really clear that the number of MPs counted in EC was dependent on the centrifugation protocols.

Whereas it cannot be excluded that MPs from platelets, white blood cells or endothelial cells be present in the starting EC, this increase in total of MPs count can be attributed only to the shedding of MPs from RBCs present in the concentrate. Noteworthy, the increase varied quite importantly from donor to donor. The reason of such a variation is unknown, but factors like ABO blood group, age, fasting or sex of blood donor may have a role and should be investigated. Finally, the most important parameter associated with the number of MPs in ECs was the duration of storage at 4°C. A set of experiment with three ECs satisfying quality criteria for transfusion (normal ALAT level) was done and gave very similar, if not identical MPs count when compared to ECs with elevated ALAT level (data not shown).

Microparticle tend to aggregate at high concentration, either related to the methods used for their isolation or to their 'intrinsic' adhesion properties, which have been already evidenced with platelet-derived MPs [16]. We observed heaps of erythrocyte MPs by electron microscopy (data not shown), thus erythrocyte MPs may also have adhesion properties. This observation is important for the quantitative results, because MPs counts are evaluated according to their sizes using flow cytometry. Thus, in samples with high concentrations of MPs, their number are probably underestimated due to the fact that MPs tend to form more heaps and flow cytometry does not distinguish between big MPs or aggregated MPs, even if the technique is the method of choice to study MPs [36]. So, the counting approach of MPs presented in this study (as well as in other published studies) should be considered as semiquantitative.

According to our proteomic and Western blot studies, MPs from stored RBCs appeared to be enriched with stomatin. Remarkably, the enrichment in stomatin, depletion in actin and stability of glycophorin A (as compared to erythrocyte membranes) were the same at day 7 and day 40. In this respect, MPs generated after a few days of storage or at the end of storage appear equivalent. Those results are well in line with previous reports [37,38]. Stomatin is a

membrane protein involved in regulation of monovalent cation transport through lipid membranes [39]. Interestingly, stomatin (which has a structure similar to caveolin) is a major lipid-raft component of erythrocytes [40]. Precise reasons of stomatin enrichment in MPs are not well known and are still subject of investigation, but may have a role in membrane microdo- mains modulation leading to membrane budding and MPs release [38]. The cell membrane plays a key role in the formation of MPs. Indeed, following a stimulus, increase in intracellular Ca++ occurs and activates proteases that cleave cytoskeleton proteins (actin and spectrin). Membrane is thus less rigid and can bud until formation of MPs. Furthermore, the asymmetry between the neutral phospholipids on the outer membrane and the negatively charged phospholipids on the inner membrane held by translocases is broken [37]. Consequently, phosphatidylserine, a negatively charged phospholipid, is also located on the outer side of MP membrane. Using annexin V, flow cytometry confirmed that phosphatidylserine was present on MPs derived from erythrocytes but essentially lacked from fresh RBCs and was externalized in only a small fraction of old RBCs. Finally, as shown by Western blots, actin was not a dominant protein of MPs when compared to RBC membranes. The precise reasons for the huge increase in MP counts during storage of EC observed in this study are unknown. Although controversial, it has been speculated that MPs could be a means for erythrocytes to prevent a premature removal from circulation when they are still functional or when lesions are reversible [41]. According to this hypothesis, MPs would allow erythrocytes to clear away non-functional molecules that would trigger an apoptosis-like pathway, or to get rid of autologous IgG binding senescent erythrocytes for removal by spleen macrophages [8,10,42]. Indeed, Willekens et al. have recently shown that MPs contain erythrocyte removal proteins such as bound IgG and altered Band 3, and thus concluded that microvesiculation serves as a removal pathway for damaged proteins [42]. Complementarily, a detailed proteomic investigation of RBCs and MPs generated during storage led Bosman et

al. to hypothesize that there are two possible mechanisms at work in MPs generation: first, immunoglobulins could bind to senescent surface proteins, thereby triggering microvesiculation. Alternatively, oxidatively damaged proteins could bind to or disrupt normal interactions within the cytoskeleton, thereby altering the tight balance between the cytoskeleton pressure and the membrane bending stress [10]. These two passive mechanisms could well be only one part of the picture, because activation of protein kinases has been demonstrated to trigger phosphatidylserine exposure in erythrocytes [43], as well as tight concomitant regulation of microvesiculation and Band 3 phosphorylation/ dephosphorylation [44]. Lastly, lysophosphatidic acid, an important lipid mediator, has been shown to be able to trigger phosphatidylserine exposure and microvesiculation in erythrocytes [45]. These studies show that MP generation can be triggered by various processes, including senescence or protein alteration, external or internal exposure to lipid mediators, and that phosphorylation plays a role in microvesiculation control.

In this study, we also showed evidence that Rhesus blood group antigens are located on erythrocyte MPs. The presence of these proteins was evidenced by determination of Rh peptides by MS and by flow cytometry, indicating that the antigenic parts of the Rh proteins are located outside MPs membranes. The presence of the Rh complex is also reinforced by the expression of CD47 (shown by flow cytometry), which is a member of the complex within the RBC membrane. Those blood group antigens present on MPs are likely immunogenic, and thus may play a potential role in RBC alloimmunization after transfusion.

As recently shown by Koch et al., there is a link between duration of RBC storage and complications after cardiac surgery [28]. According to the result of this research, trans- fusion of erythrocytes that have been stored for more than 14 days in patients undergoing cardiac surgery significantly increases the risk of postoperative complications and reduces survival time. Reasons for such complications remain unclear; however, storage lesions may be a

possible mechanism. Physicochemical changes occurring during storage of ECs are indeed known to affect RBCs function and viability. Our results confirm that important changes occur during storage of RBCs and that storage techniques allowing a better con- servation of the integrity of the membrane should be thus developed in the future.

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Review

Pre-analytical and methodological challenges in red blood cell microparticle proteomics

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Transfusion

ABSTRACT

Microparticles are phospholipid vesicles shed mostly in biological fluids, such as blood or urine, by various types of cells, such as red blood cells (RBCs), platelets, lymphocytes, endothelial cells. These microparticles contain a subset of the proteome of their parent cell, and their ready availability in biological fluid has raised strong interest in their study, as they might be markers of cell damage. However, their small size as well as their particular physico-chemical properties makes them hard to detect, size, count and study by proteome analysis. In this review, we report the pre-analytical and methodological caveats that we have faced in our own research about red blood cell microparticles in the context of transfusion science, as well as examples from the literature on the proteomics of various kinds of microparticles.

Abstract

Microparticles are phospholipid vesicles shed mostly in biological fluids, such as blood or urine, by various types of cells, such as red blood cells (RBCs), platelets, lymphocytes, endothelial cells. These microparticles contain a subset of the proteome of their parent cell, and their ready availability in biological fluid has raised strong interest in their study, as they might be markers of cell damage. However, their small size as well as their particular physico-chemical properties make them hard to detect, size, count and study by proteome analysis. In this review, we report the pre-analytical and methodological caveats that we have faced in our own research about red blood cell microparticles in the context of transfusion science, as well as examples from the literature on the proteomics of various kinds of microparticles.

Introduction

By its importance for living organisms, blood is often called “fluid of life”. Transfusion is indeed vital and indicated in numerous clinical situations such as severe haemorrhage, anaemia or hypovolemia, in which case red blood cell (RBC) concentrates are administered to sustain the oxygenation of tissues, haemostasis imbalance or disorders, in which case platelet concentrates are administered, or deficiency of coagulation factors (transfusion of fresh frozen plasma). Since the beginning of transfusion, numerous efforts have been made to secure blood products and gain knowledge about their molecular structures. With millions of blood product transfused worldwide every year, each incremental piece of progress has a potential broad effect on a great number of lives. Nevertheless, there is still a risk of side effects associated to transfusion such as fever, inflammation, and iron overload or autoantibody formation [1,2].

Erythrocyte concentrates (ECs), platelet concentrates (PCs) and fresh frozen plasma (FFP) are the three main labile blood products and have to be stored according to their particular components. Those components are subjected to modifications or degradations during storage, a process known as the “storage lesion” [3]. RBC ageing in blood banking conditions differs from physiological in vivo ageing. Indeed, conditions to which RBCs are exposed during storage, such as temperature and nature of the medium, are dramatically different from physiological conditions. Several preservative solutions such as citrate–phosphate–dextrose–adenine (CPDA), saline–adenine–glucose–mannitol (SAGM) or phosphate–adenine–glucose–guanosine–saline–mannitol (PAGGSM) are available in routine [4]. These additive solutions allow to store red blood cell concentrates from 35 days up to 49 days in accordance with the European transfusion standards [5], requiring that at least 75% of erythrocytes must survive in vivo 24-h after transfusion (see Table 1). During storage many physiological and biochemical alterations occur in the supernatant of ECs including an increase in the concentration of lipids,

MPs, free haemoglobin and a pH reduction. Red blood cells also undergo several changes such as loss of potassium, adenosine triphosphate or 2,3-diphosphoglycerate. Their membranes become more rigid, there is a disruption of phospholipid asymmetry, lipid raft rearrangement, loss of fragments or even release of MPs [6]. The effects of storage lesions, including the shedding of MPs, on transfusion efficiency and potential side effects are not clearly understood. Among other approaches, proteomics allows the investigation of important issues in blood research [7] and transfusion science [8] in order to gain better knowledge of the blood products delivered to patients as well as gain insight into the mechanisms at work in transfusion side effects.

This review focuses on methodological and analytical challenges in the proteomic analysis of red blood cell-derived microparticles. However, examples from other microparticle types (platelet, endothelial,. . .) are discussed as well to highlight some analytical or methodological aspects. Moreover, whereas flow cytometry is a central tool for the analysis of microparticles, it is beyond the scope of this review to discuss the various techniques used to analyze microparticles by flow cytometry. Excellent reviews have been published [9], and we discuss here only challenges and recent development in flow cytometry.

What are microparticles ?

MPs are plasma membrane vesicles shed in blood flow by various types of cells such as platelets, red and white blood cells, or endothelial cells. Those MPs, also known as microvesicles [10] or in some cases as ectosomes [11] have a size of less than 1µm [12] and contain a subset of proteins derived from their original cells as well as surface receptors allowing the identification their origin. Most studies generally agree that MPs are heterogeneous and vary in size, concentration, phospholipid composition, surface antigens and protein content. Release of MPs is thought to be a highly controlled process prompted by various stimuli such as shear stress, complement attack, pro-apoptotic stimulation or damage [13]. Although still subject to discussion, a model of vesiculation has been established. This model brings in translocases, lipid rafts, various protein modifications and irreversible membrane rearrangements. [14]. Recently, an association between erythrocytes aging processes and MPs formation has been proposed as a part of an apoptosis-like form in erythrocytes [15]. This "aging" process of red blood cells has also been observed during storage in blood bank condition [16].

MPs have long been considered as cell fragments or "dust" without any biological role. Although their functions are still largely unknown, there are more and more evidences that MPs are involved in a broad spectrum of biological activities [14] such as haemostasis [17], thrombosis [12], inflammation [17], transfer of surface proteins [18] or even angiogenesis [19].

In the case of haemostasis, MPs provide an additional negative phospholipid surface for the assembly of the tenase enzymatic complex involved in the coagulation cascade [20]. Moreover, a study demonstrated that platelets MPs have from 50 up to 100- times more procoagulant activity than platelets [21]. Nonetheless, not only platelet MPs are involved in

this process, indeed, erythrocyte MPs and other MPs have a procoagulant activity as well [22]. In addition, a recent paper by Furie and Furie [20] mentions a “microparticles accumulation pathway” as a part of the coagulation process. Briefly, according to this model, there are constitutive MPs at low concentration expressing inactive tissue factor (TF). These MPs are then captured in a developing thrombus and their accumulation in the injury site leads to activation of MPs TF and helps to amplify coagulation.

Another impelling example is the implication of MPs in erythrocyte ageing process [23]. Indeed, during their 120 days of lifespan, red blood cells lose between 15% and 20% of their volume and haemoglobin concentration increase by 14% [24]. Thus, microvesiculation would be amean for red blood cells to eliminate denatured haemoglobin which could be toxic [25], and besides, microparticles release would also be a mean for red blood cells to get rid of specific membrane proteins which could prevent or induce their removal from blood flow according to the situation. In a protective role, MPsmay help to clear away the C5–9 complement attack complex, band 3 neoantigen, IgG or other harmful agent from the membrane when the red blood cell is still viable [13,25–28], and thus prevent early removal from blood flow. In contrast, MPs could promote removal of erythrocytes: CD47 is an integral membrane protein present on erythrocytes surface, acting as a marker for self [23]. Thanks to CD47, normal red blood cells are recognized as self by the macrophages (through their signal regulatory protein alpha) and phagocytosis is inhibited. Senescent or damaged red blood cells whose CD47 expression is reduced by shedding of MPs enriched in CD47 would be no longer recognized as self and thus be eliminated by macrophages.

Although the presence of MPs in blood is common in healthy individual, an increase in the concentration of MPs in plasma has been demonstrated under various pathological conditions such as thrombocytopenic disorder [29], cardio vascular disease [30], diabetes [31] or sepsis

[32]. It is also important to refer that a fewpathologies such as Scott syndrome [33] are linked to decrease in MPs concentration in plasma.

In ECs and platelet concentrates, an increase in the number of MPs during storage has been evidenced [34,35]. Although MPs have been detected in FFP too, in contrast to ECs and PCs containing cells, there is no increase in MPs during storage. It has been proposed that MPs are implicated in different vascular pathologies. For example, a recent paper by Lawrie et al. [36] has demonstrated the effect of MPpresence on clotting time of FFP. Thus, as MPs affect the clotting time, they could alter the quality of the blood product. Whereas the overall impact of MP presence on the blood product value is still largely unknown, there is little chance that their presence be totally innocuous.

Table 1 The development of RBC storage solutions. ACD, acid citrate dextrose; AS-3, additive solution 3; CPD, citrate phosphate dextrose; CP2D, high dextrose CPD; CPDA-1, CPD plus adenine; CPDA-2, CPD with adenine and extra dextrose; ½ CPD, half volume CPD; PAGGSM, phosphate, adenine, glucose, guanosine, saline and mannitol; RAS-2, research additive solution 2; SAG, saline adenine glucose; SAGM, SAG plus mannitol. Adapted from Ref. [4].

	Typical recovery	Haemolysis	Vesicles
Three-week storage			
ACD	75%[63]	0.1%[63]	
CPD	79%[63]	0.1%[63]	
Five-week storage			
CPDA-1	72%[64]	0.5%[64]	70%[65]
CPD/SAG	83%[66]	0.6%[66]	
Six-week storage			
CPDA-2	80%[67]		
CPD/SAGM	78-84%[66]	0.4%[66]	25%[68]
CP 2D/AS-3	78-84%[66]	1.0%[66]	
Seven-week storage			
CPD/PAGGS-M	74%[69]	0.5%[69]	
1/2CPD/RAS2	78%[70]	0.5%[70]	
Eight-week storage			
CPD/EAS-81	85%[68]	0.4%[68]	10%[68]

Methods of microparticle analysis

The increasing interest in MPs lies in the fact that as they circulate in blood flow, they could constitute hallmarks of cellular activation or damage. Therefore new efficient methods have to be developed with the aim to obtain qualitative or/and quantitative data on MPs. It is important to add that there is no standardized method for MPs analysis, making any comparison difficult between different studies. In order to analyze MPs, several approaches are available in the literature such as electron microscopy, ELISA, proteomic methods and flow cytometry [9,37].

Isolation of MPs from biological medium

Prior to analyze MPs, isolation or concentration of MPs from samples (whole blood, platelet or red blood cells concentrate, fresh frozen plasma) could be needed according to the experiment. Indeed, in most studies, classical differential centrifugation (see Table 2) is principally employed. Notice that the references in Table 2 deal with platelet MPs, nevertheless it is a good illustration of the plentiful isolation methods available. For centrifugation, there are two main steps for MP isolation. First, low speed centrifugation (from 200 to 13,000×g) removes intact cells, which leaves a MP-rich supernatant that can be directly analyzed. Alternatively, a second high-speed centrifugation (18,000–100,000×g) can be used to pellet microparticles from the supernatant. Worth noticing is that most studies deal with platelet MPs, and that centrifugation conditions have to be adapted from one cell type to the other. Fig. 1 shows the optimisation of the centrifugation conditions to remove RBCs from ECs while keeping the MPs in the supernatant. In our experience, the best centrifugation conditions were determined to be two centrifugations at 1850×g, 4◦C, 20 min, to remove all

RBCs from the supernatant. In these conditions, there is a 15-fold loss of microparticles compared to untreated EC, but there are no detectable RBCs in the supernatant. In cases when concentrated microparticles are necessary (for example for proteomic studies), an additional ultracentrifugation is usually performed to pellet MPs from the supernatant; this is usually accomplished by centrifuging the MP-rich supernatant at 100,000×g to obtain a MP-free supernatant. The main drawback of this approach is that the MP pellet might also contain a lot of contaminants. It is our experience that the RBC supernatant after low speed centrifugation contains mainly MPs that can be detected and counted by flow cytometry with cell-specific markers (such as antibodies against CD47 or CD235a) or markers of phosphatidylserine externalization such as Annexin V, with very little background noise. On the contrary, when microvesiculation is induced in vitro by challenging RBCs with calcium ionophore or calcium alone, there is a huge increase in the number of small fragments of the same size as MPs (at least as can be estimated from flow cytometry, see below), but that are negative to any labeling with anti-CD47, anti-CD325a and Annexin V. When pelleting the MPs by ultracentrifugation, there is no doubt that a large part of these fragments are pelleted as well and may obscure the biologically relevant information about MPs.

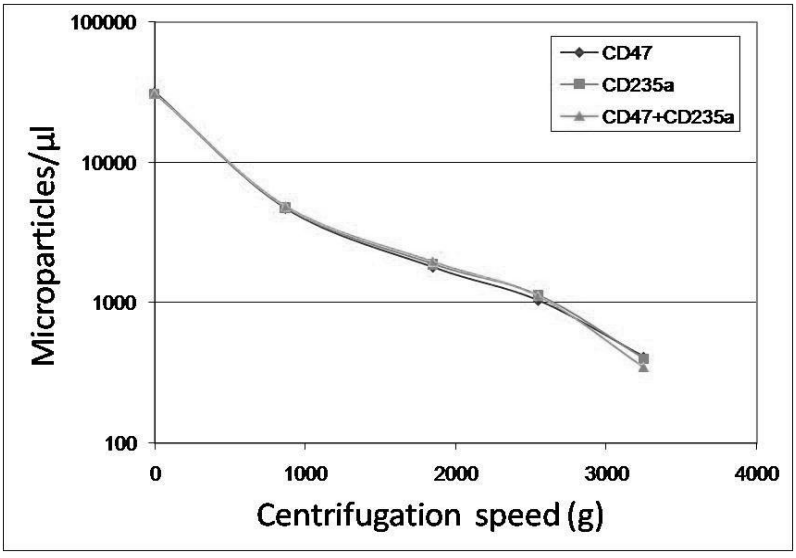


Fig. 1

Count of microparticles in supernatant of ECs. The number of microparticles measured decreased with increasing centrifugation speed (test on one ECs stored for 38 days). Note that the numbers measured were identical with the two antibodies used in this study (anti-human CD235a or CD47). Adapted from [34].

Table 2 Classical methods for MP isolation reported in the literature. Adapted from Ref. [37].

						Cell-specific identifications		
Main technique	**Quantitation**	**Anti-coagulant**	**Prepare PPP**	**MP pelleting**	**Generic MP detection**	**Platelet**	**Endothelial**	**Leukocytes**
Flow cytometry	Counts	Citrate	1550 g, 20 min	18000 g, 30 min	Annexin V	CD62P, CD61, CD63	CD31, CD62E, CD144	CD4, CD8…
Flow cytometry	Counts	Citrate	1550 g, 15 min 13000 g, 2 min		Annexin V	CD	CD51, CD144, CD146	CD45
Solid-phase capture	Prothrombinase capture	Citrate	1500 g, 15 min 13000 g, 2 min		Annexin V, tissue factor	CD62P, GPIba.	CD31, CD62E	CD45
Flow cytometry	Counts	Citrate	200 g, 10 min 1500 g, 7 min			CD41, CD42b, CD31	CD31+, CD42-, CD62E	CD45
ELISA	Standard PMP	EDTA	1500 g, 20 min			GP IX (capture), CD62P, CD40L		
Flow cytometry	Counts	Citrate	13000 g, 10 min	100000g, 60 min	Annexin V	CD41a	CD144	CD14

MP counting

As mentioned above, an increase in the number of MPs in blood is linked to various pathologies, hence the idea of using MPs as biomarker and thus the need to develop method allowing to quantify MPs. Flow cytometry is often considered as the method of choice to analyze MPs, indeed it allows analyzing thousands of MPs in one sample of whole blood or in a fraction with determination of many different markers at the same time. Additionally, flow cytometry enables not only qualitative but also semi-quantitative analysis. However, as MPs have a size smaller than 1µm, this requires working at the inferior limits of the instrument, which have for consequences a loss of precision and/or accuracy. In addition, flow cytometry is not able to distinguish MPs, small cell debris and aggregates of MPs. This observation is confirmed by electron microscopy of erythrocyte MPs from EC: their size is around 0.15µm

and they tend to form clumps consisting of 10–15 MPs with an aggregate size of 1µm (see Fig. 2). Note that whereas in Fig. 2, MPs are issued from stored erythrocytes, however similar observations have been made in sample from treated red blood cells [38].

In our hands, flow cytometry analysis was performed with TruCount tubes (with a precise number of fluorescent beads to determine the number of MPs in a sample) and fluorescent cell-specific antibodies (either anti-CD47 or anti-CD235a) or phosphatidylserine-specific Annexin V. It was found that during storage of ECs in blood bank conditions, the number of MPs increased from around 3300±1200 MPs/µl at day 5 of storage and it increases up to 64,000±37,000 MPs/µl after 50 days of storage (the storage limit being of 42 or 49 days depending on the additive solution used, see Table 1).

Interestingly, a wide variability between the different concentrates was observed (see Fig. 3). The variation is likely due to the physico-chemical changes that occur in blood units during storage. Those changes affecting erythrocyte's viability reinforce and accelerate haemolysis and the associated release of MPs. Changes take place at different storage time, more or less rapidly according to the blood donor; indeed this might be caused by intrinsic factor such as sex, age, health, genotype, or diet, due to the fact that each sample was identically processed.

Fig. 2

Pictures of erythrocyte microparticles taken by scanning electron microscopy. (A) Magnification of 4000×,MPs surrounding an erythrocyte. (B) Magnification of 37,000×, heap of MPs.

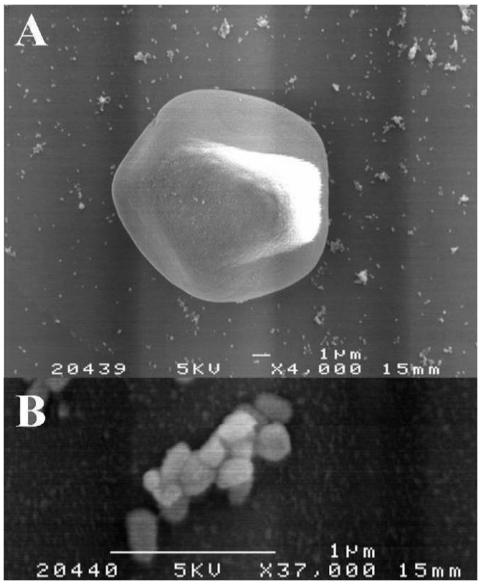
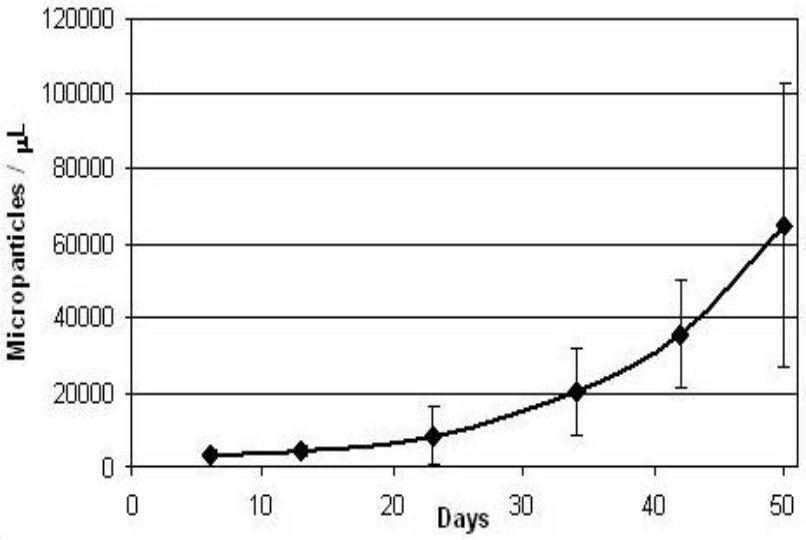


Fig. 3



Count of microparticles directly in erythrocyte concentrates during storage (without centrifugation). Data are expressed as the mean±SD experiment (N= 7).

At day 5, 3371±1188 microparticles/µl were counted, whereas at day 50, their concentration was 64’858±37’846 microparticles/µl. Anti-human CD47 was used to label MPs [34].

Pre-analytical factors

It has been observed that factors such as temperature, shaking, or the dilution buffer influence the number of MPs counted; it is not totally clear from the literature and our experience if these factors affect the MPs counts by favouring MP in vitro aggregation, for example, or if they indeed induce artifactual microvesiculation of RBCs.

The impact of sample manipulation out of the cold room was tested with a fresh EC split in 2 similar blood bags. One was simply stored at 4 ◦C for 40 days while the other one was put out of the cold room for 1 h twice a week (i.e. 10 times during 40 days). MPs were then counted after 40 days according to the methods described by Rubin et al. [34]. The experiment was performed on two different concentrates and in both, much more MPs were observed in bags that underwent 10 cycles of 4 ◦C–room temperature (see Fig. 4). Another experiment to test the impact of working temperature was done with two different ECs, one stored for 15 days and the other one for 38 days in standard blood banking conditions. Few millilitres of each EC

were incubated for 1 h at three different temperatures (4, 24 and 37 ◦C, respectively). Samples were then centrifuged and MPs were counted in the supernatant by flow cytometry. Fig. 5 shows the variation in MP concentration for the two different samples handled at 4, 24 and 37 ◦C. Not surprisingly, the EC stored for 38 days contains more MPs than the one stored for 15 days, but both samples show that increasing handling temperature induces a higher MP concentration.

Moreover, the solution used to dilute MP samples just before flow cytometry analysis also has an influence on MP counts. Here, three different ECs, stored for 2, 8 and 43 days in standard blood banking conditions, were diluted in different solutions: PBS, NaCl 0.9% and FACS Flow (FF) solution (BD Biosciences, Franklin Lakes, NJ). Flow cytometry analysis was performed as quickly as possible after the dilution of the sample. While the variation in the number of MPs was relatively small in concentrates stored for 1 and 8 days according to the buffer, a large increase of MPs was observed in the samples diluted in PBS compared to other buffers, as shown in Fig. 6.

An experiment to test if freezing/thawing sample of MPs free of cells affects MP counts has been conducted. Indeed, comparisons between MPs count in fresh supernatant and in supernatant frozen once have been done on ECs stored from 9 to 45 days (both supernatant were issued after two centrifugations of erythrocytes concentrates). Briefly, MPs were labeled and then counted by flow cytometry according to the protocol designed by Rubin et al. [34]. Notice that freezing sample could only be done after centrifugation, on cell-free samples. The difference between fresh and frozen supernatants does not exceed the standard variation coefficient of our standard experiment, which usually is from 3% to 13%. To give an example, in a 16 days stored sample, the difference between thawed and non-frozen sample was around 3%. So, MP counting, sample freezing did not affect the number of MPs detected.

Finally, the impact of shaking on MP count was checked: few millilitres of two different erythrocyte concentrates (the first one stored for 8 days and the other one stored for 42 days) were shaken using a Vortex mixer for different times (5, 10 and 20 s). Flow cytometry analysis was finally performed in the concentrate with TruCount tubes to count MPs beforehand stained with anti-CD47. Fig. 7 shows the increase in microparticle concentration after various vortexing times.

Fig. 4.

Effect of cold room/RT cycles on MP counts. White bars represent samples continuously kept at 4 ◦C, black bars represent samples exposed 10 times to room temperature during storage.

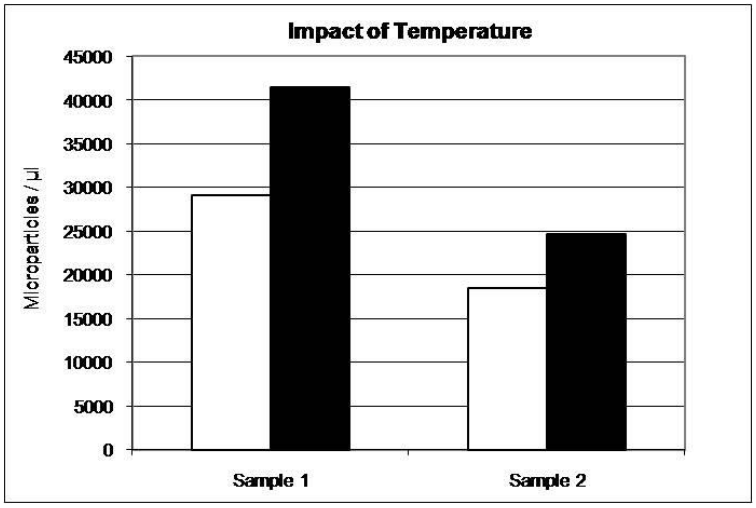


Fig. 5.

Effect of handling temperature on MP concentration (white bars represents samples handled at 4 ◦C, grey bars at 24 ◦C, and black bars at 37 ◦C).

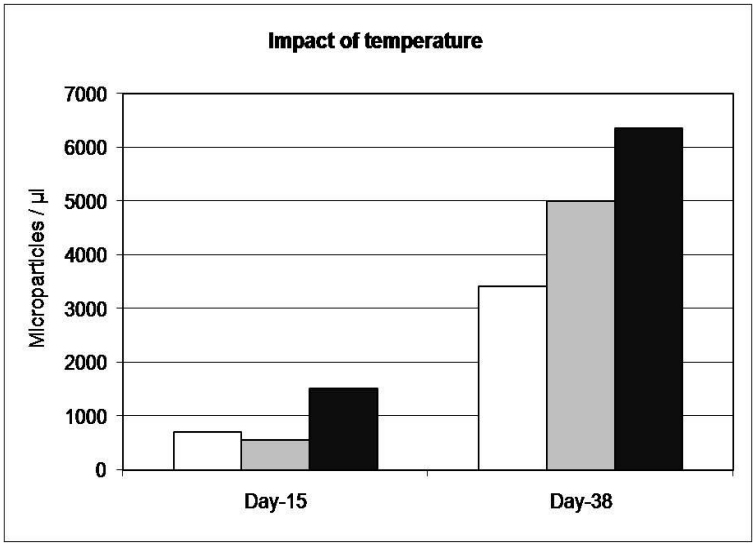


Fig. 6.

Effect of the dilution solution used for flow cytometry analysis on the MP count after 2, 8 or 43 days of storage in standard blood banking conditions. White bars correspond to PBS, grey bars to NaCl 0.9%, black bars to FACS Flow.

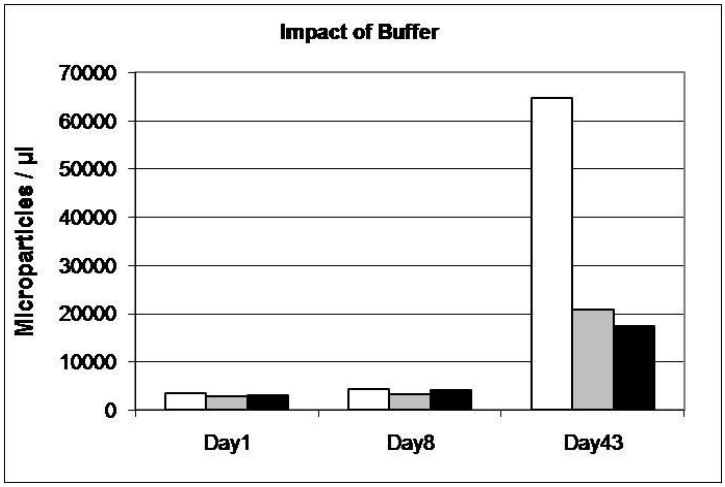
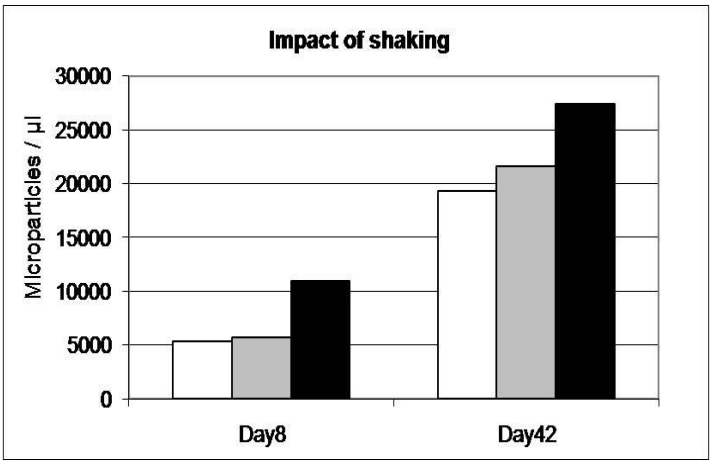


Fig. 7.

Effect of vortexing on theMPcount. White bars correspond to a 5 s vortexing, grey bars to a 10 s vortexing, and dark bars to a 10 s vortexing.



These four examples illustrate the impact of sample handling, dilution, and manipulation on the measured microparticle concentration. Whereas no clear rationale exists for the observed effects, some guidelines can be drawn from these experiments:

(1) The measured MP concentration does not depend on the detection system used (be it cell-specific antibodies or phosphatidylserine-specific Annexin V), as shown in Fig. 3.

(2) All other factors might have an influence on the measured MP concentration, and best efforts should be made to standardize sample handling and preparation as much as possible; this is especially true for the temperature to which sample are exposed, the solution used to dilute the sample before flow cytometry analysis, and the way solutions are mixed.

Microparticle counting and sizing

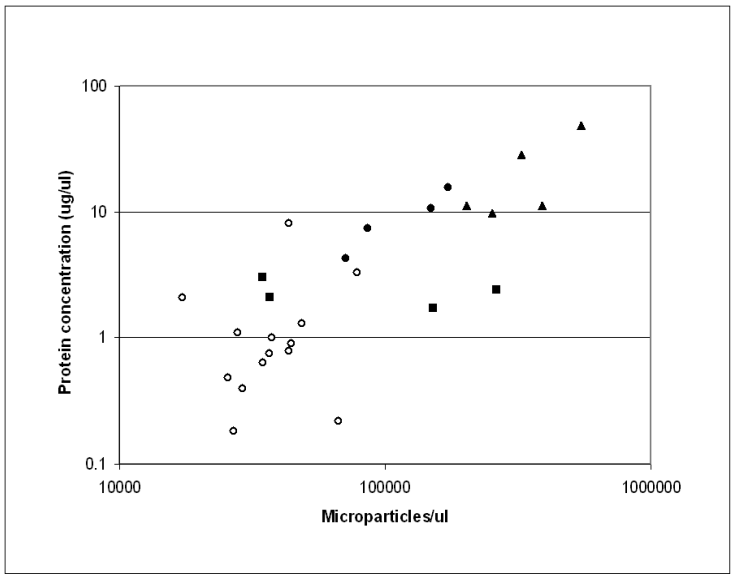
To return to MPs count, a solid-phase method was developed by Nomura and co-workers [39] allowing indirect quantification of MPs. Briefly, Annexin V coated plates are used to capture phosphatidylserine-exposing microparticles. After washing, the (indirect) procoagulant activity of the bounded MPs can be determined using a prothrombinase assay. The main drawback of this approach is that the results are expressed as an area of accessible phosphatidylserine and not as amicroparticle concentration, which makes the comparison with flow cytometry results hardly feasible [40,29]. Alternatively, a specific antibody can be added to quantify a cell-specific MP type. Although this assay is very sensitive to detect weakly expressed antigens, the relative non-specificity and non-sensitivity of Annexin V binding for MPs makes the technique difficult to use in routine.

Though standard flow cytometers have been extensively used to detect, count, and probe antigens at the surface of microparticles, it has to be clear that scattered light cannot be used to size microparticles. Most flow cytometers use light sources of wavelengths of 488nm for example, which places the diffraction limit at half amicron. In practice, it can be difficult on most instruments to discriminate platelets from background noise based solely on scattered light, and it is hardly possible to size even smaller objects such as microparticles. Flow cytometric analysis of microparticles has thus to rely on specific probes and labels to discriminate relevant objects from background noise, but very little can be said about the size of the detected objects. A definitive demonstration of this difficulty has been provided by Becker et al. who analyzed a calibration bead mixtures ranging from 3 to 8µm and showed that the scatter response of commercially available flow cytometers is not necessarily monotonous for smaller objects [41].

In this context, the laboratory of Bruce Furie modified a commercial flow cytometer that embedded a coulter counter, the Cell Lab Quanta SC from Beckman Coulter, to be able to measure objects of a few hundreds of nanometers. Fig. 8 shows the dot plot analysis of 780nm fluorescent beads, and authors claim they were able to accurately size particles down to half a micron. This modified flow cytometer had been applied to the study of tissue factorbearing microparticles in cancer [42,43]. Unfortunately, to the best of our knowledge, this instrument remains a unique prototype, whose technical specifications and modifications have not been disclosed, making the evaluation of its possibilities hard to truly evaluate. More recently, Lawrie et al. [44] tested two commercially available dynamic light scattering instruments (the Zetasizer Nano S from Malvern Instruments Ltd., and the N5 submicron particle Size Analyser from Beckman Coulter) to size microparticles from fresh frozen plasma. Whereas both instruments were able to correctly size calibration beads, they showed ambiguous results when applied to microparticles from fresh frozen plasma: it seems that these instruments are more adapted to study objects with sharp monodisperse size distributions than biological material with broad and overlapping size distributions. Nevertheless, the availability of such instruments as well as their lower costs compared to high-resolution flow cytometers might make them interesting complementary tools in microparticle research.

Fig. 8.

Correlation between protein titration and microparticle concentration. Different markers correspond to different samples, the coefficient of correlation according to Bravais–Pearson test is R = 0.85.



Microparticle proteomics

Recent advances in proteomics and new available techniques could play an important role in order to elucidate the exact roles of MPs studying their protein content. It is beyond the scope of this review to detail the particular biological results obtained by different studies, which are highly dependent on the cell type under study. In this section, we would rather insist on methodological aspects and difficulties that are shared by all investigators working on microparticle proteomics. Numerous publications describe microparticle proteomic studies [45–49]. To the best of our knowledge, most studies have adopted the same workflow for proteomic analysis of microparticles. First microparticles are pelleted by ultracentrifugation as discussed above, then diluted in a standard buffer (e.g. PBS) for protein titration by classical assays such as Bradford. In our hands, there appeared to be a correlation between protein concentration and microparticle concentration (coefficient of correlation (Bravais–Pearson): R = 0.85), as shown in Fig. 8, indicating that in terms of protein quantity, microparticles of various RBC origins (e.g. from RBCs stored in different additive solutions, or of different storage duration) are relatively homogeneous.

Because microparticles are not amenable to straightforward lyses such as hypotonic shocks or freeze/thaw cycles, most studies have adopted the same protein solubilization protocol: microparticles are directly solubilized in one-dimensional gel electrophoresis (1D-GE) loading solutions such as Laemmli, containing SDS and a reducer such as dithiothreitol, and proteins are separated by 1D-GE, protein bands are cut, and further digested by a proteolytic enzyme (usually trypsin) and identified by various mass spectrometry techniques. The clear-cut advantage of this technique compared to gel-free approaches is that to some extent 1D-GE is compatible with the analysis of hydrophobic proteins. This feature is important since microparticles, due to their formation mechanism and surface-to-volume ratio are expected to contain more membrane proteins with respect to their total proteome compared to their parent

cells. For example, using this approach, Miguet et al. found 34% of plasma membrane proteins in the microparticles derived from malignant lymphocytes, which is twice as much as in their parent cells [48]. Additionally, in our experience, 1D-GE provides another level of information that is complementary to the MS/MS identification of proteins based on a few peptides: the molecular weight of the identified protein can indeed be deduced from its position on the gel, which can sometime provide some indications about protein processing. For example, in a slice of a RBC microparticle separated by 1D-GE, we identified proteins such as Band 3 and RhD protein (see Fig. 9) that are much larger than the gel band in which they were found, suggesting that truncation or cleavage has occurred. Conversely, we also found proteins, such as haemoglobin subunits, that are much lighter than the gel band in which they were found, which is consistent with the fact that haemoglobin tends to be cross-linked to cytoskeleton proteins, especially under stress conditions [50]. The observation of such variance between observed and expected molecular weights has already been repeatedly reported in RBC proteomics: for example, Pasini et al. reported that such proteins originate either from organellar proteins, in which case they are most likely to originate from degradation products processed during cell maturation (case for proteins with lower molecular weight than expected), from ubiquitinated proteins targeted for degradation (case for proteins with higher molecular weight than expected), or from detergent- and reducing agent-resistant macromolecular assemblies, such as cytoskeletal assemblies [51].

Interestingly, following the same approach Bosman et al. differentially analyzed so-called microvesicles, isolated from the supernatant of RBCs at 40,000×g, and so-called nanovesicles, further isolated by centrifugation at 100,000×g [6]. Though this differential isolation relies on a somehow arbitrary criterion, the differential proteomic analysis performed by the authors showed marked differences between micro- and nanovesicles: main protein categories found in microvesicles are membrane and cytoskeletal proteins as well as

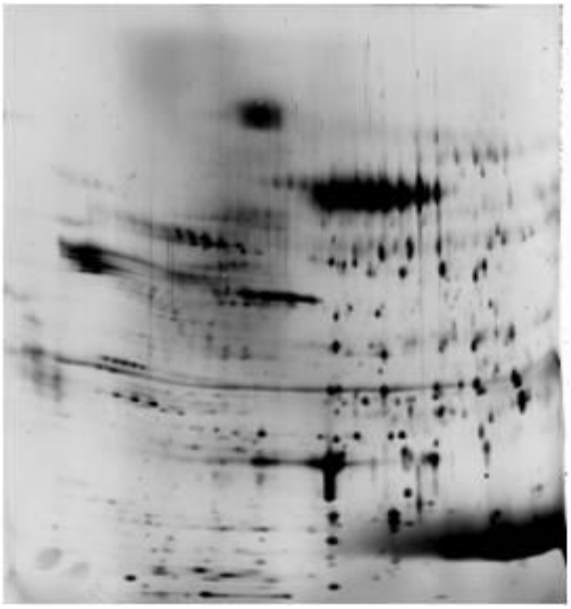
metabolic enzymes, whereas nanovesicles are particularly enriched in uncategorized proteins. On the contrary, whereas immune proteins such as immunoglobulins and complement proteins are almost absent from RBC membranes and microvesicles, they are clearly identified in nanovesicles. This differential protein sorting between micro- and nanovesicles might indicate that the two populations are generated by different mechanisms and play different roles in RBC ageing and stress response.

Following the observation that microparticle proteins are not necessarily found at the right position on a 1D gel, and thus that some important protein processing occurs at the microparticle level, we undertook a systematic effort to separate the microparticle proteome at the protein level by two dimensional gel electrophoresis (2D-GE) so as to be able to analyze differentially processed proteins. Unfortunately, 2D-GE is poorly amenable to the analysis of membrane and hydrophobic proteins. Hence following the work by Rabilloud et al. about membrane proteins solubilization for 2D-GE [52,53],we solubilized aMP pellet in urea, thiourea, DTE, pharmalyteTM and detergent (either CHAPS, ASB-14 or Brij 35). The protein mixture was then fractioned by OFFGEL electrophoresis and the fractions corresponding to pH 3–7 were pooled, so that haemoglobin was left in the most basic fractions. Following this haemoglobin depletion step, proteins were separated by 2D-GE with the detergent of choice. A typical 2D gel is shown in Fig. 10 (where Brij 35 was used to solubilizemembraneproteins). Whereas some haemoglobin can still be observed at the lower right corner of the gel, as pointed by several authors [54–57], haemoglobin depletion helps resolving more spots compared to the unfractioned sample. Moreover, the picture obtained by this differential solubilization strategy is dramatically different from the one obtained by using classical protocols (e.g. CHAPS solubilization).

Fig. 9 Proteins identified from a 25–35 kDa region of a 1D-GE of RBC membranes and RBC microparticles by MALDI-TOF/TOF mass spectrometry. Greyed proteins have a molecular weight that does not correspond to their position on the gel.

AC	Entry name	Protein name	MW (Da)	RBC (sequence coverage %)	RBC (identified peptide)	MPs (sequence coverage %)	MPs (identified peptide)
P31946	1433B_HUMAN	14-3-3 protein β/α	28 082	25·2	7	16·7	4
P62258	1433E_HUMAN	14-3-3 protein epsilon	29 174	34·9	11	NO	NO
P61981	1433G_HUMAN	14-3-3 protein γ	28 303	NO	NO	13·4	4
P27348	1433T_HUMAN	14-3-3 protein θ	27 764	14·3	5	NO	NO
P63104	1433Z_HUMAN	14-3-3 protein ζ/δ	27 745	44·5	11	8·2	2
P02730	B3AT_HUMAN	Band 3 anion transport protein	101 792	NO	NO	6·8	3
Q4TWB7	Q4TWB7_HUMAN	β-Globin chain (Fragment)	11 487	93·3	15	NO	NO
P07738	PMGE_HUMAN	Bisphosphoglycerate mutase	30 005	45·9	13	37·8	10
P00915	CAH1_HUMAN	Carbonic anhydrase 1	28 870	72·8	27	65·5	20
P00918	CAH2_HUMAN	Carbonic anhydrase 2	29 246	72·7	25	71·9	15
P07451	CAH3_HUMAN	Carbonic anhydrase 3	29 557	36·2	8	17·7	4
A0N071	A0N071_HUMAN	δ-Globin chain (haemoglobin δ)	16 055	55·1	10	55·1	9
P27105	STOM_HUMAN	Erythrocyte band 7 integral membrane protein	31 731	14·9	4	76·4	43
P17931	LEG3_HUMAN	Galectin-3	26 188	11·6	3	NO	NO
P78417	GSTO1_HUMAN	Glutathione transferase ω-1	27 566	33·2	10	7·9	2
P69905	HBA_HUMAN	Haemoglobin subunit α	15 258	71·1	9	71·1	9
P68871	HBB_HUMAN	Haemoglobin subunit β	15 998	83·0	16	93·9	15
Q16775	GLO2_HUMAN	Hydroxyacylglutathione hydrolase	28 860	8·8	2	NO	NO
P30041	PRDX6_HUMAN	Peroxiredoxin-6	25 035	25·9	6	16·1	4
P18669	PGAM1_HUMAN	Phosphoglycerate mutase 1	28 804	8·3	2	NO	NO
Q06323	PSME1_HUMAN	Proteasome activator complex subunit 1	28 723	16·5	4	NO	NO
P25788	PSA3_HUMAN	Proteasome subunit α type 3	28 433	6·7	2	NO	NO
P25789	PSA4_HUMAN	Proteasome subunit α type 4	29 484	12·3	4	NO	NO
P60900	PSA6_HUMAN	Proteasome subunit α type 6	27 399	9·3	2	NO	NO
O14818	PSA7_HUMAN	Proteasome subunit α type 7	27 887	21·4	4	NO	NO
P00491	PNPH_HUMAN	Purine nucleoside phosphorylase	32 118	59·5	16	17·6	4
Q0KG01	Q0KG01_HUMAN	RhD protein	45 052	NO	NO	4·1	2

Fig. 10. 2D-GE separation of 100µg erythrocyte microparticles proteins beforehand depleted in haemoglobin by OFFGEL electrophoresis. The first dimension is a 4–7 linear pH gradient, second dimension is a 4–12% gradient polyacylamide gel. In the present gel, Brij-35 detergent was used to solubilize membrane proteins.



Conclusion

There has been increased interest in the recent years in microparticle proteomics, mainly because microparticles are shed in biological fluids (in blow flow or in urine) by their parent cells and are thus readily accessible for analysis. Furthermore, they are in many situations hallmarks of cell lesion, and their proteomic analysis promise to shed some light on the biology of their parent cell, which may ultimately provide some valuable biomedical information. Somewhat differently, in the field of transfusion medicine, RBC and platelet microparticles are inherent parts of the blood product delivered to patients, and there is little chance that their presence be totally innocuous. However, proteomic analysis of microparticles turned out to be much more difficult than expected, mainly because of pre analytical caveats. As we have attempted to demonstrate in this review, every single manipulation of the sample might have a direct influence on its microparticle content. Factors such as temperature, the dilution solution used, the way solutions are mixed, are crucial in the determination of microparticle concentration, and thus in the subsequent proteomic analysis. Additionally, the small size of microparticles makes their detection and sizing very difficult by standard techniques such as flow cytometry. The numerous caveats we tried to exemplify in this paper make the comparison between different studies almost impossible, and this is the reason why so much debate exists in the literature about the microparticle properties and functions. However, there is no doubt that proper control of the pre-analytical factors, as well as proper reporting of the methods used, will prompt sound and valuable insights into RBC biology through proteomic analysis.

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REVIEW

Microparticles in stored red blood cells: submicron clotting bombs?

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Introduction

More and more knowledge on blood microparticles (MPs) has been accumulated this last decade. However the role(s) of those small phospholipid vesicles still remain unclear. MPs, also named microvesicles or ectosomes, range from 0.1 to 1 μm in size, and are released in blood flow by various types of cells [1]. They are found in healthy individuals as well as in patients suffering from different diseases. The release of MPs is a highly controlled process prompted by various stimuli such as shear stress, complement attack, agonist (or pro-apoptotic) stimulation or damage [1,2]. If MPs were first described as cellular debris without any biological function [3], nowadays they are known as cellular effectors involved in numerous physiological processes such as haemostasis and inflammation [4], transfer of surface proteins [5], angiogenesis [6] or apoptosis [7]. Moreover, there is a link between MPs and several diseases such as thrombocytopenic disorder [8], thrombosis [1], cardiovascular diseases [9], diabetes [10], sickle cell disease [11], haemolytic anaemia [12] or sepsis [13], where an increase of the number of MPs in plasma has been demonstrated. Interestingly, a decrease in MPs numbers in plasma of patients presenting with the Scott syndrome, a rare bleeding disorder, has been reported [14].

MPs are also present, and accumulate, in blood products such as erythrocyte and platelet concentrates during storage [15-17]. Because the surface of MPs is made of negatively charged phospholipids, they may play a role in the coagulation cascade. Therefore, the purpose of this paper is to review the potential procoagulant effect of erythrocyte MPs and thus, their potential adverse effect after blood transfusion.

Microparticles and Coagulation

Blood coagulation is an essential mechanism that prevents bleeding; this highly controlled process is carefully regulated in order to maintain blood circulation in case of injury while preventing vessels obstruction by clot formation. Coagulation factors, calcium ions and procoagulant membrane surfaces are the primary components involved in coagulation activation. In eukaryotic cells, including blood cells, it is established that neutral phospholipids (such as phosphatidylcholine) are mainly present on the outer membrane, whereas negative phospholipids (such as phosphatidylserine) are at the inner side of the membrane. Concerning blood cells various translocases are activated upon stimulation and phospholipid asymmetry is disrupted. Phosphatidylserine (PhSer) is externalized, modifying the neutral membrane charge into negative [18]. Activated blood cells and their MPs expose negative membrane and are thus thought to be procoagulant. The presence of negatively charged phospholipid membranes and calcium ions is required for the assembly of coagulation complexes such as tenase or prothrombinase. The role of tenase complex is to convert factor X (FX) into activated factor X (FXa), in both extrinsic and intrinsic pathways. The extrinsic tenase complex is composed of tissue factor (TF) and FVIIa [18] whereas the intrinsic tenase is composed of co-FVIIIa and factor FIXa [19,20]. The function of prothrombinase, constituted of co-FVa and FXa, is to convert prothrombin into thrombin. FIXa and FXa can convert their substrate without their respective cofactors, however negatively charged membranes catalyses the reaction, for example $3x10^5$ fold faster in the case of prothrombinase [21,22]. Figure 1 presents the possible site of actions of MPs on the coagulation cascade. The importance of negative phospholipids is to facilitate complex association, speeding up their association by around 1000 fold for efficient haemostasis [23]. Because express PhSer on their membrane, they could contribute to coagulation by providing catalyzing negatively charged membranes. It has been demonstrated that platelets-derived

MPs have from 50- up to 100-times more procoagulant activity than platelets [24]. MPs membranes support more efficiently thrombin formation than platelet membrane when corrected for unit of surface, at least *in vitro* [4,25]. Not only platelet MPs are involved in this process, but erythrocyte MPs and other MPs have also a procoagulant activity through their negatively charged membrane as well [1,11,26].

Although controversial, some authors claim that MPs support clotting not only through their negatively charged membranes, but also by expressing an inactive form of tissue factor (TF) on their surfaces. The origin and mechanism of action of blood-born TF is still subject to debate, nevertheless authors have suggested that MPs may be a reservoir of blood-born TF [27]. Furie *et al* delineated a "*microparticles accumulation pathway"* as part of the coagulation cascade [25]. According to this model, some monocyte-derived MPs expressing TF in an "inactive" form bind to a forming clot in vessels and display active TF that helps to amplify coagulation. In an other hand, Connor *et al* proposed an activated factor X assay to measure clotting time of various samples containing MPs and their results show that there is a correlation between the number of MPs and clotting time; interestingly enough, the assay was insensitive to the presence or the absence of TF [28]. Other studies mention the presence of TF on endothelial-derived MPs [29], platelet-derived MPs [30,31]**,** or even on erythrocyte-derived MPs [32], while some others did not detect any MPs-exposing TF [11]. All these pieces of evidence are difficult to explain, in particular because results often depend on the study design, methods of MPs isolation, and various pre-analytical factors; therefore one cannot exclude that discrepancies might result from different experimental methodologies [33].

Figure 1

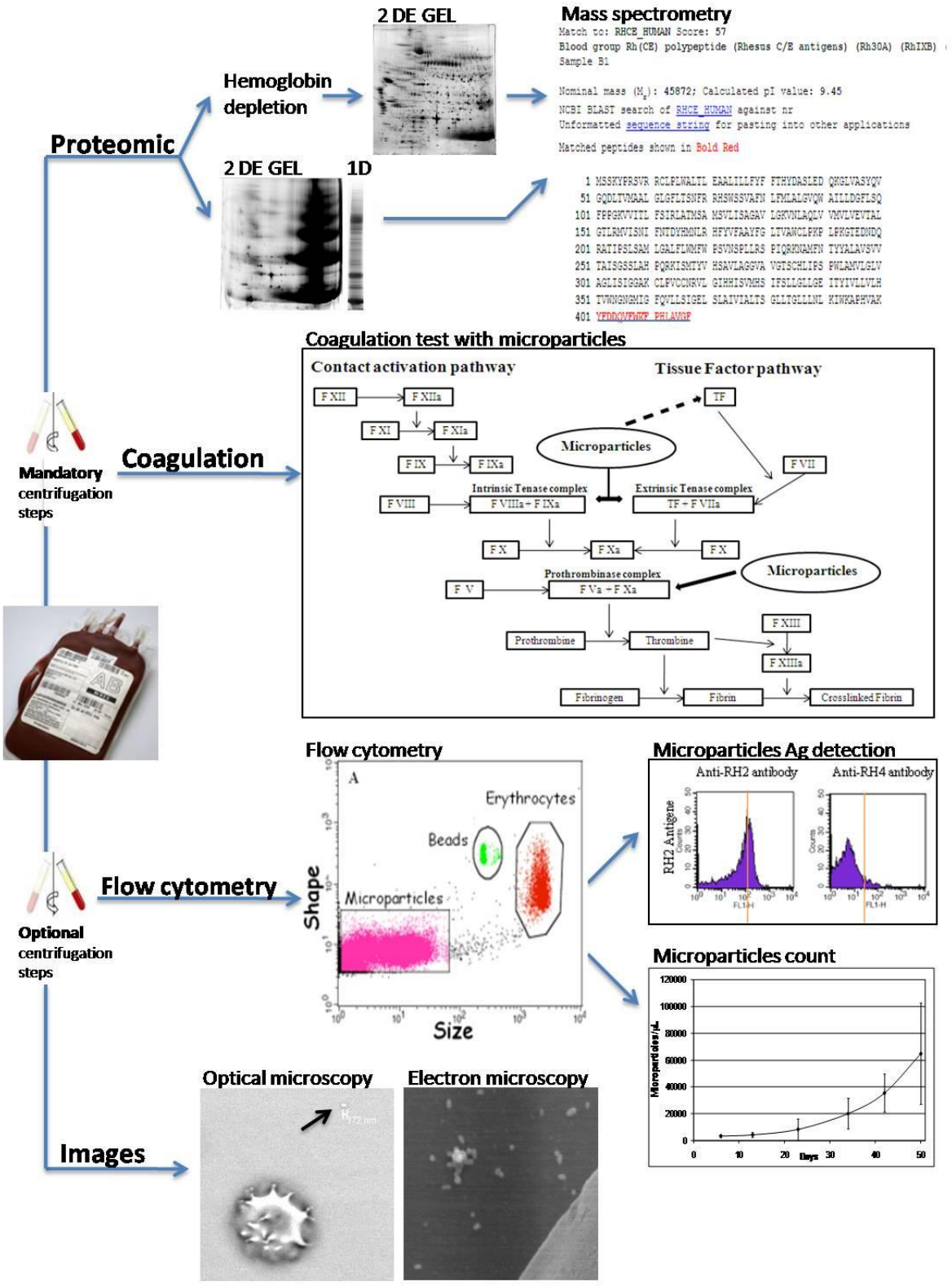
Proteomic
Hemoglobin depletion
2 DE GEL
Mass spectrometry
Match to: RHCE_HUMAN Score: 57
Blood group Rh(CE) polypeptide (Rhesus C/E antigens) (Rh30A) (RhIXB)
Sample B1
Nominal mass (Mr): 45872; Calculated pI value: 9.45
NCBI BLAST search of RHCE_HUMAN against nr
Unformatted sequence string for pasting into other applications
Matched peptides shown in Bold Red
2 DE GEL
1D
Coagulation test with microparticles
Contact activation pathway
Tissue Factor pathway
Mandatory centrifugation steps
Coagulation
Microparticles
Intrinsic Tenase complex
Extrinsic Tenase complex
Prothrombinase complex
Prothrombine
Thrombine
Fibrinogen
Fibrin
Crosslinked Fibrin
Flow cytometry
Microparticles Ag detection
Anti-RH2 antibody
Anti-RH4 antibody
RH2 Antigene
Optional centrifugation steps
Erythrocytes
Beads
Microparticles
Shape
Size
Microparticles count
Images
Optical microscopy
Electron microscopy

Figure 1: *General scheme of experiments on blood microparticles*.
Proteomics, coagulation tests, flow cytometry and imaging by microscopy are the most common approaches to investigate microparticles. It is possible to study microparticles from various origins (whole blood, plasma as well as platelet or red blood cells concentrates). Of note, agonists such as calcium ionophore or protaglandine E2 are frequently used to induce cells microparticles release. Various centrifugation steps may be used to further isolate microparticles depending on the experiment condition. Indeed, in proteomics or coagulation tests, it is important to work on "purified" microparticles.

- Proteomics approach: The characterisation of microparticle proteins is aimed to gain insights on the various biological processes involved in their production and function. Generally, after isolation of microparticles, they are directly solubilised in one-dimensional gel electrophoresis buffer. After migration and staining, protein bands are excised, digested by a proteolytic enzyme (usually trypsin), and identified by various mass spectrometry techniques. One-dimensional gel electrophoresis allows separating membrane as well as soluble proteins with a good compatibility to mass spectrometry analysis. Two-dimensional gel electrophoresis allows a better protein sorting, but this technique is poorly amenable to analysis of hydrophobic protein. In all technique, elimination of very abundant proteins, such as haemoglobin should be performed to detect the less abundant ones. This could be done by chromatography, "off gel electrophoresis" or by using "proteominer" techniques. As for one-dimensional gel electrophoresis, it is possible to excise spots of interest for identification by mass spectrometry. By using approaches described by Rabilloud *et al* [58] it is possible to improve the migration of hydrophobic proteins after two-dimensional gel electrophoresis, but the drawback of such approach is its lower compatibility with mass spectrometry. Therefore, most microparticle protein lists published were established using one-dimensional gel electrophoresis techniques followed by mass spectrometry. By this approach, we identified the presence of RHCE proteins in erythrocyte microparticle membranes, as showed on the mass spectrometry results presented in the scheme (a peptide corresponding to amino acids 401-417 from the RHCE protein was identified).

- Coagulation approach: There are more and more evidences that microparticles are involved in coagulation. Therefore, different assays could be done on microparticles. Standard clinical coagulation tests could be done such as determination of the thrombin time, the activated partial thromboplastin time or new available tests allowing determination of FXa. These approaches will allow determining at what level microparticles are involved in clotting.

- Flow cytometry approach: This is one of the most frequently used techniques to analyse microparticles. Indeed, it allows analyzing thousands of microparticles in one sample with determination of different markers and enables semi-quantitative analysis at the same time. For the semi-quantitative analysis, a precise number of fluorescent beads are used to determine the number of microparticles in a sample. Using the same number of beads, it is thus possible to compare the number of microparticles among different samples. Fluorescent cell-specific antibodies or annexin V (a phosphatidylserine marker) are used to tag microparticles and to differentiate them from the rest of the events. Using the antibody of choice and according to the type of microparticles, it is to detect and highlight surface antigens such as CD-235a, CD41, or even RH2 or RH4 (as presented on the scheme).

- Imaging approach: Observation of microparticles by microscopy give information about their size and shape. Though numerous steps of preparation, scanning electron microscopy allows observing if microparticles are homogeneous in size or not. By confocal differential interference contrast microscopy, it is possible to observe forming microparticles from echinocyte.

Microparticles and hypercoagulability

Hypercoagulability state is associated with an increased risk of fibrin deposition in blood vessels, which could result in thrombus formation [34]. Hypercoagulable state has been often observed in case of chronic haemolytic anaemia's (sickle cell disease, thalassemia, paroxysmal nocturnal haemoglobinuria, autoimmune anaemia) [35]. Frequently, there are erythrocyte membrane alterations with accumulation of negative procoagulant membranes in blood flow, platelet activation, and a decreased level of anticoagulant proteins, amplifying the risk of thrombosis [35]. In thalassemic patients, the main determinant of the thrombotic risk seems to be related to altered red blood cell membranes as well as to platelet activation, but not to plasma protein abnormalities [36]. Erythrocyte membrane alterations leads to the exposure of PhSer on the outer layer, cytoskeleton cleavage, membrane budding and release of MPs [8]. An elevated number of MPs in blood flow has been reported in patients suffering from different vascular diseases [1]. Consequently, MPs could have an essential causal role in hypercoagulable states, as reported by Van Beers *et al* in sickle cell disease [11]. The authors showed that, while the number of platelet-derived MPs was stable, there was an increase in erythrocyte-derived MPs during crisis compared to steady state.

Microparticles and red blood cells storage

Modification, alteration or even degradation of erythrocyte concentrates (ECs) occurs during storage (a phenomenon called the storage lesions) [7,37], and accumulating MPs have been considered as being one of the expression of storage lesions [15]. Red blood cells' ageing in blood bank conditions clearly differs from physiological *in vivo* ageing. Undeniably, conditions to which red blood cells are exposed during storage such as temperature and nature of the medium are dramatically different from *in vivo* conditions. Erythrocytes degrade and loose efficiency during storage. Indeed, erythrocytes change shape; their membranes become more rigid and there is a disruption of phospholipids asymmetry and release of fragments and MPs [37]. In addition, during the conservation of erythrocyte in plastic bags, not only physiological alterations occur, but also biochemical changes in the storage medium principally due to red cells metabolism. These modifications, physiological and biochemical are part of the storage lesions. There is an increase in the concentration of lipids, free haemoglobin, potassium, lactate and in contrast a reduction of pH, glucose, 2,3-diphosphoglycerate, sodium or adenosine triphosphate [38]. For example diminution of glucose and increase of lactate concentration reflects red blood cells glycolysis.

In blood flow, MPs are rapidly removed in the liver by Kupffer cells [39]. Release of MPs occurs throughout erythrocyte lifespan [39] and continues during storage, therefore they accumulate during storage [17]. Precise reasons of erythrocyte vesiculation are not elucidated, however MPs is a possible mechanism preventing red blood cell removal. MPs may help to clear away the C5-9 complement attack complex, band 3 neoantigen, IgG or other potential harmful agent from the membrane, when the erythrocyte is still viable [40-42]. In addition, MPs formation could be a mechanism involved to clear out methaemoglobin which is no more functional [37].

The precise consequences of storage, including storage lesions and MPs release, on transfusion efficiency are not clearly understood, and relatively little is known about their molecular bases and even less about their possible biomedical consequences on recipients. There is accumulating evidence that receiving "old" blood is not as advantageous as receiving fresher blood. In 2008, Koch *et al* showed a link between the age of the transfused ECs and post-transfusion complications. They claimed that life expectancy at 5 years was better for patients who received fresher blood (ECs stored for less than 14 days) as compared to older blood (stored for more than 14 days) in the course of cardiac surgery [43]. A recent study by Spinella *et al* concluded that ECs stored for more than 28 days was associated with an increased incidence of deep vein thrombosis and death from multi-organ failure [44]. Other studies on post-transfusion complications or mortality and storage time were also published and controversial [45-47]. However, although these studies raise important questions about transfusion safety and storage lesions, they are subjected to many discussions [48-51].

Due to the fact that transfused people are sick and polytransfused recipients are often in poor condition, it is difficult to attribute clearly an event to transfusion rather than on the clinical situation of the recipient. In a recent review, Zimrin and Hess analyzed several papers on clinical studies examining the effect of storage of red blood cells in transfusion and concluded that well designed epidemiologic studies are needed to demonstrate that a clear clinical effect may be due to ECs storage lesions. Despite the lack of conclusive evidence that storing blood longer is worse, it is current practice to transfuse fresher blood to patient considered at high risk (such as in neonatology for example or cardiac surgery).

As stated before, MPs may have procoagulant activities; therefore, we may reasonably suggest that transfusion of "older" ECs containing a high number of MPs could increase the risk of adverse reactions, by inducing a hypercoagulable state leading to thromboembolic

complications. Inversely, in many situations requiring blood transfusion, a hypercoagulable state may be useful to diminish or even helping to stop the bleeding.

Table 1: Overview of studies on proteins of microparticles reported in the literature

Type of MPs	Theme	Technique	Main Results	Ref.
Red blood cells	Red blood cell membranes and MPs proteome	Centrifugation, LC-MS/MS	Storage-dependent changes in the RBC membrane proteins. MPs display similar change in their protein composition during storage.	[53]
Red blood cells	MPs during storage of EC	Centrifugation, Flow cytometry, 1 D SDS PAGE, Western Blot, MALDI-MS	Number of MPs increase during storage. Proteomic analysis revealed changes of protein expression comparing MPs to erythrocyte membranes.	[17]
Plasma	Proteome of Plasma MPs	Centrifugation, Flow cytometry, 2 D-GE, MALDI-MS	Comparison between plasma and MPs 2D-GE and LC-MS/MS to list MPs proteins. From the spotted sample, 83 different proteins and their respective isoforms were identified in which 13 have never been reported in human plasma.	[55]
Platelet	Proteome of platelet MPs	Centrifugation, 1D SDS-PAGE, LC-MS/MS	The identification of 578 proteins of platelet MPs was accomplished among which 380 were not identified previously in platelet proteome	[52]
Endothelial	Proteome of endothelial procoagulant MPs	Centrifugation, 1D SDS-PAGE, LC-MS/MS	This study describes the protein composition of endothelial cell MPs. Among proteins, presence of metabolic enzymes and bioactive effectors has been evidenced.	[56]
Malignant lymphocyte	Characterization the T-lymphocytes MPs proteome	Centrifugation, 1D SDS-PAGE, LC-MS/MS, MALDI-MS	In total, 390 proteins were identified, 34% of those proteins were from plasma membrane among which proteins involved in hematopoietic clusters of differentiation.	[57]

Proteomics and microparticles analysis

Recent advances in proteomics provide new tools allowing studying protein content of MPs in order to elucidate their exact role. Several publications are focused on proteomics of MPs derived from various type of cells (see table 1). Results are different according to the MP cell types and the methodological aspects. To the best of our knowledge, most studies have adopted the same workflow for proteomic analysis of MPs as shown in the "proteomic" part of figure 1 (excepting for the initial sample that is not ECs in all cases). Briefly, once isolated by centrifugation, MPs are directly separated by one-dimensional gel electrophoresis (1D-GE) and finally protein bands are excised, and further digested by a proteolytic enzyme (usually trypsin) and identified by various mass spectrometry techniques. The main advantage of this technique is the compatibility with the analysis of hydrophobic proteins in contrast to two dimensional electrophoresis gel (2D-GE) approaches. Among different proteomic studies on MPs, various lists of MP proteins have been established. Mapping of MP proteins involved in coagulation is still lacking. In the study of platelet MPs proteome by Garcia *et al*[52] when the list of proteins is submitted to a QuickGO search with the GO reference "blood coagulation", 12 proteins out of 578 were found to be involved in blood coagulation. Recently, Bosman *et al* [53] established a reference proteome of red blood cells MPs. When the protein list was submitted to a QuickGO search with the same parameters as mentioned above, 5 out of 308 proteins were involved in coagulation, namely phospholipid scramblase 1, plasminogen precursor, fibrinogen beta chain precursor, complement component C9 precursor and beta-2-glycoprotein 1. Except for phospholipid sramblase 1, proteins were neither identified in red blood cells membrane proteome established by Bosman *et al* [53] nor by Pasini *et al* [54]. As procoagulant proteins of MPs were not found on red blood cells, we could hypothesize that those proteins were either of plasmatic origin with a non-specific binding to MPs or were proteins present on red blood cell membranes, but enriched on MPs microdomains.

Discussion

The important issue raised by the presence of MPs in blood products is to determine their exact involvement for the recipient. It has been demonstrated that MPs are present in blood products, and that they number increases during storage. Series of articles and reviews were published on the association between red cell storage duration and complications after transfusion. However few of them mentioned MPs as potential cause of these complications. Therefore, there is an urgent need of large clinical studies with the aim to determine precise effects (if any) of MPs in blood recipients. Furthermore, it is important to determine how MPs are involved in the coagulation cascade, by either passively acting by providing additional negatively charged membrane surface, or rather by allowing the expression of TF or other proteins implicated in the coagulation process. Furthermore, studies of MPs from platelets, endothelial cells or monocytes and comparison with EC-derived MPs are needed. Proteomic analysis, as well as quantitative proteomic evaluation of they protein content will open new avenues in haematology, because it will help to decipher the complexity of the hypercoagulable states and the effect of EC transfusion in routine clinical practice. Combining various approaches that are haemostatic investigations, MPs characterisation either by cytometry or by proteomics, clinical studies will certainly help to secure transfusion medicine.

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6

Thrombin generation by microparticles isolated in stored red cells

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Submitted

Summary

Microparticles are small phospholipid vesicles of less than 1 μm shed in blood flow by various types of including red blood cells. They are involved in many biological and physiological processes including haemostasis. Elevated number of microparticles in blood flow is often observed in various pathological situations.

Erythrocyte-derived microparticles accumulated in erythrocyte concentrate during their storage time. Their exact role being not elucidated, they are considered as storage lesion. The aim of this study was to evaluate the impact of erythrocyte derived microparticles in coagulation.

Using calibrated automated thrombogram, it has been demonstrated that erythrocyte derived microparticles isolated from red cells concentrates are capable not only to support thrombin generation, but also to trigger thrombin generation in assays. As the impact of those transfused microparticles is unknown on recipients, clinical study is needed to increase knowledge in the field of transfusion medicine.

Introduction

Microparticles (MPs) are small phospholipid vesicles of less than 1 µm circulating in blood flow, typically 0.15 µm for erythrocyte derived MPs (EMPs). They are heterogeneous in size, phospholipid content and surface proteins according to the cell they derived [14]. MPs are observed in healthy individuals and their increase has been observed in a variety of diseases [14, 23-27]. In contrast, a very low number of MPs has been observed in patients presenting with Scott syndrome, a rare bleeding disorder associated with apparent normal platelet function and coagulation factors level [28].

The release of MPs is a controlled process, triggered by various stimuli, including cell stimulation, shear stress or apoptosis [15]. If MPs have been first described as "cell dusts"[20], they are now recognized as being involved in a broad spectrum of biological activities [15, 22, 71, 72] including thrombosis and haemostasis [14, 21].

In contrast to their parental cells, the majority of MPs exposes negatively charged phospholipids on their outer membrane (mainly phosphatidylserine). As negatively charged phospholipids are required for the assembly of coagulation complex such as tenase or prothrombinase, MPs are thus thought to be procoagulant [16, 73, 74]. Indeed, it has been demonstrated that platelet derived MPs have from 50 up to 100 times more procoagulant activity than platelets [55]. As EMPs presents negatively charged membrane as well, those last could thus support coagulation [56].

During *ex vivo* storage of red blood cells (RBCs), numerous biochemical changes occur such as for example pH acidification and increase in free haemoglobin [41]. Those changes commonly referred as "storage lesions" [3] also include RBC modifications, such as increase in membrane rigidity, lipid rafts rearrangement, disruption of phospholipid asymmetry and MPs release. It has been demonstrated that EMPs accumulate during storage [29], with great variation of their number from an erythrocyte concentrate (EC) to another, most likely due to

the donor, conservative solution or even the preparation methods [62]. EMPs transfused within the EC could represent an additional source of negatively charged phospholipids and the impact on transfusion is still unknown [75].

The aim of our study is to evaluate the impact of EMPs isolated from ECs on transfusion studying their ability to support or trigger thrombin generation (TG) *in vitro.*

Materials and methods

Erythrocyte concentrates

ECs were provided and prepared at the Lausanne blood bank according to the standardized procedure. Briefly, 450 ± 50 ml of whole blood was drawn by venipuncture and collected in blood bags (Fenwal, Lake Zurich, IL, USA) containing citrate–phosphate–dextrose anticoagulant solution. RBCs were separated from platelet, anticoagulant solution and plasma by centrifugation and directly resuspended in 100 ml of preservative solution sodium–adenine–glucose–mannitol. Leucocytes were then removed by filtration. ECs were stored up to 42 days at 4°C. Only outdated ECs that were not transfused within 42 days of storage, but less than 45 days, were used for EMPs isolation.

Plasma

2.7 ml of venous blood were drawn with a 21-G needle directly into citrated tube (*Sarstedt,* Leicester, UK) containing 0.3 ml of sodium citrate and corn trypsin inhibitor (CTI) at a final concentration of 10.6 mM and 1.6 µM in whole blood, respectively. CTI inhibits intrinsic coagulation pathway at a concentration of at least 1µM in platelet-rich plasma (PRP) [76]. In addition, it allows reducing imprecision when measuring endogenous thrombin potential (ETP) by Calibrated Automated Thrombogram (CAT) in plasma samples at low TF concentration [77]. In order to avoid withdrawing TF and factor XII, light tourniquet and minimal suction was applied, and the first tube was not used for coagulation assay. Three sequential centrifugations (2 x 1500 g and 1 x 18’000 g) were proceeded in order to remove cells and the majority of MPs from plasma. For all experiments, only fresh plasma was used, and it remained at room temperature during sample preparation. As clotting factor concentration and thus haemostasis differ from one person to the other, in some experiment, pool of plasmas was used to avoid single person variability. For all CAT tests presented in

results, the same pool of fresh plasma from seven volunteers was employed. For western blotting, plasma samples were passed through ProteoMiner^{TM} columns (Biorad, Hercules, CA, USA) allowing to enrich low abundance proteins and compression of dynamic range of protein concentration in samples [78].

Microparticles Preparation

Isolation of EMPs

EMPs released during storage were separated from red blood cells by two successive centrifugations in 50 ml tubes of ECs at 1000 g, 10 minutes and 2000 g, 5 minutes at room temperature. Supernatant was collected and then another centrifugation at 18'000 g for 30 minutes at 4°C was performed. The supernatant was removed and the pellet was resuspended in filtered 0.9% NaCl solution. In order to avoid putative polymorphism effect, pools of EMPs issue from 4 different ECs were used. As EMPs also have blood group antigens on their surfaces [75], only group O concentrates were employed to avoid eventual agglutination reactions.

Tissue Factor coated EMPs

In order to obtain "artificial" tissue factor (TF) bearing EMPs (EMPs-TF) serving as a model, 200 µl of EMPs previously obtained were incubated with recombinant tissue factor (rTF) at a concentration of 100 nM at room temperature for 3 hours on constant agitation. EMPs were quantified by flow cytometry as described thereafter. Excess of rTF was removed by two washing steps consisting in centrifugation at 18'000 g, 30 min at 4°C and the pellet was resuspended in 200 µl of a 0.9% NaCl solution.

Flow cytometry

Samples of MPs were analyzed on a FACScalibur flow cytometer with CellQuest pro software (BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometer was calibrated with CaliBRIT™3 kit (BD Biosciences) containing different fluorescent beads. Size events were defined using flow cytometry size beads of 1–1.4 µm (Spherotech, Lake Forest, IL, USA). Each antibody for flow cytometry was purchased at BD Pharmingen (San Diego, CA, USA).

For MPs count, 5 µl of EMPs samples or fresh pooled plasma were incubated with 1 µl of Fluorescein isothiocyanate (FITC) anti-human CD235a (anti-human erythrocyte) and/or 1 µl of phycoerythrin (PE) PE anti-human CD142 (anti-human TF). After 20 minutes in the dark on constant shaking, 400 µl of PBS were added and flow cytometry analysis was carried out within 30 minutes in a Trucount™ tube (BD Biosciences). In order to avoid modifying the count, no centrifugation step to remove the excess of antibody was done.

Western blotting

Depending on the experimental conditions, from 5 to 40 µg of various proteins samples (EMPs, EMPs-TF or plasma) were loaded onto a 4–12% NuPAGE Novex Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, CA, USA) while from 0.0034 to 340 ng of pure rTF were load as positive control. After migration, carried out at constant voltage (150 V), proteins were transferred to Hybon-LFP polyvinylidene fluoride membranes (GE healthcare, Uppsala, Sweden) using a Novex blot module (Invitrogen) for 1 h 45 min at fixed voltage (30 V), according to the manufacturer's instruction. Membranes were then strained in Ponceau red to control if protein transfer occurred. After transfer, blotted membranes were soaked overnight in blocking solution with PBS, 0.1% Tween-20 (v/v), 5% milk and 1% bovine serum albumin (BSA) (w/v). Western blots were done with rabbit anti-human TF (American

Diagnostica, Stamford, CT, USA) at a dilution of 1:500. The goat anti-rabbit horseradish peroxidase conjugated secondary antibody (Dako, Baar, Switzerland) was used at a dilution of 1:10'000. Subsequent visualization was performed using enhanced chemiluminescence (GE Healthcare). The signal was finally captured using X-ray film.

Transmission Electron Microscopy and Immunogold Labelling

EMPs and EMP-TF samples were resuspended in PBS-BSA 1% (W:V) buffer and centrifuged at 18'000 g, 15 minutes. EMPs or EMPs-TF where then incubated for 1 h in PBS-BSA 1% with rabbit anti-human TF (American Diagnostica) at a dilution of 1:25. EMPs were washed twice in PBS-BSA 1%, and centrifuged at 18'000 g, 15 minutes. Then, samples were resuspended in PBS-BSA 1% with gold labelled secondary anti-rabbit antibody (Sigma, Saint Louis, MO, USA) for 30 minutes. Samples were washed twice in PBS-BSA 1%, at 18'000 g, 15 minutes and fixed in glutaraldehyde 2.5% (V:V) solution. Samples were washed twice in H_20. Sample of EMPs of EMPs-TF were absorbed onto grids and transferred onto drop of 2% uranyl acetat (W:V) for negative staining. Vesicles were finally visualized with a Philips CM100 transmission electron microscope (Philips, Amsterdam, Nederland).

Thrombin Generation with CAT

Measures of TG have been performed with a CAT following procedure developed by Hemker *et al* [79]. Assays were performed on a FluoroskanAscent plat reader (Thermo Labsystems, Helsinki, Finland) and Thrombinoscope software (Thrombinoscope BV, Maastricht, Netherlands) as described by the manufacturers.

For each well, 20 l of EMPs in 0.9% NaCl solution, containing different number of EMPs previously quantified by flow cytometry, were added to 60 µl of fresh pooled plasma. Two

different reagents were used to initiate TG in the test. PRP-regent (Thrombinoscope BV) containing TF and minimal amount of phospholipids was used to highlight the contribution in coagulation of additional negative phospholipid membrane provided by EMPs. Note that TG is phospholipid dependent [79]. MP-reagent (Thrombinoscope BV) did not contain any TF and made the assay specifically sensitive to TF present on the surface of MPs. Thrombin Calibrator (Thrombinoscope BV) was used to reduce donor or instrumental variability and allow comparing samples. CTI, a strong inhibitor of activated factor XII, was added in plasma in order to inhibit intrinsic coagulation pathway and to avoid the interference from *in vitro* contact factor activation. Indeed, *in vitro* contact activation occurs because factor XII binds to negatively charge surface and autoactivates [77]. Anti-human TF (American Diagnostica) was also employed as TF inhibitor at a dilution of 1:10 (and thus extrinsic pathway inhibitor) in EMPs samples when needed. Thus, 20 µl of PRP-reagent, MP-reagent or Thrombin Calibrator (Thrombinoscope BV) were added to 80 µl of each of sample into wells. The plate was then set in the fluorometer and after warming, thrombin rate measurement started with the addition of 20 µl of Fluo-Buffer (Thrombinoscope BV) containing 100 mM of $CaCl_2$ and 2.5 mM of fluorogenic substrat. During the measurement, the program compared the readings of sample with reagent and calibrator, calculated all parameters and displayed the concentration of thrombin in time. For each sample, 5 wells with the reagent and 2 wells with the calibrator were analysed. Data were validated and given as mean of the different wells for one sample by the Thrombinoscope software (Thrombinoscope BV).

Results

Evidencing TF

Various attempts to evidence TF on EMPs from ECs have been done. Neither flow cytometry, western blotting, nor immunogold allowed to evidenced trace of TF either on EMPs or in plasma even when plasma was threaded by ProteoMiner^{TM} technology (Fig 1). With pure rTF protein, threshold of detection was around 550 pM (corresponding to a load in gel of 0.34 ng of pure rTF). TF was clearly evidenced by all used methods on EMPs-TF (Fig 1).

Intrinsic pathway inhibitor

Plasma prepared for CAT experiments always contained 1.6 µM CTI, when there was no other indication. Flow cytometry also confirmed that there is no more platelet and almost no more MPs in three times centrifuged plasma (data not shown). In plasma sample without CTI, TG is sometimes observed, typically in one on three assays with a great variability in lag time. This is due to *in vitro* intrinsic coagulation pathway activation [77]. Without addition of EMPs in plasma containing 1.6 µM CTI, no TG was observed (Fig 2). No statistically differences in ETP were observed in sample with 10'000 EMPs/µl between plasma containing 1.6 µM and 3.2 µM CTI, (T-test; $P < 0.05$) (Table I).

Contribution of negative phospholipids from EMPs to support TG

Using PRP-reagent, contribution of EMPs phospholipids in TG was evaluated. Results demonstrate that more there were EMPs in plasma, the greater and faster was TG (Fig 2A). In addition, control with demonstrate that there was no significant TG in plasma without EMPs.

Contribution of EMPs to trigger TG

TG was observed when from 5000 EMPs/µl to 20'000 EMPs/µl were added to plasma, even if no TF was added (Fig 3). Note that in plasma with 1000 EMPs/µl, no TG was observed (Table I). Thus, without addition of exogenous TF, there is TG in plasma with more than 1000 EMPs/µl (Fig 3). As positive controls, from 250 to 10'000 EMPs-TF/µl were used in the assay. TG with EMPs-TF was always stronger comparing to EMPs. Additionally, the amount of EMPs-TF needed to generate thrombin is much smaller (from 250 EMPs-TF/µl) than with EMPs (Fig 3).

As intrinsic coagulation pathway is inhibited with CTI, anti-human TF was employed to inhibit putative plasma TF or EMPs-TF even if no TF was evidenced in those samples. In this way, both coagulation pathway triggers are inhibited. Interestingly, TG was not inhibited when EMPs were added to plasma containing anti-human TF at a concentration of 1:10 (Table I).

As positive control, plasma samples with thromboplastin, here Innovin (Siemens Healthcare Diagnostics, Marburg, Germany) or with EMPs-TF were tested. For both, rapid TG with a high ETP was observed (Fig 2B). Efficiency of anti-human TF was tested in plasma samples containing Innovin or EMPs-TF. When Anti-human TF was added to those samples, a partial inhibition of TG was observed in the sample with EMPs-TF whereas total inhibition was observed in the sample with Innovin (Table I and Fig 2B). These results also show that EMPs-TF are strong activator of TG and have a "thromboplastin-like effect".

Discussion

The exact role of EMPs remains unclear, and their function might be most likely multiple. This study demonstrated that EMPs are capable to support TG. This is particularly interesting in the field of transfusion due to the fact that EMPs are numerous in blood concentrate and they tend to increase during storage [29]. A recent study by Sweeny *et al* suggest that "something" in ECs supernatant facilitate TG [80]. We provide evidences that the element facilitating TG in supernatant are EMPs.

Results of CAT gave ETP reflecting a measure of global coagulation in a sample. This study demonstrates that in CAT test with TF, TG is observed in presence of EMPs in plasma. Thus, EMPs could support TG providing negative phospholipids membranes. Additionally, in CAT test without TF addition, TG is also observed when EMPs are added to plasma. When CTI and/or TF-antibody are added to plasma samples with EMPs, TG is still observed, while no TG was observed in plasma samples without EMPs. In positive control, EMPs-TF added to plasma, there is a strong and rapid TG comparing to EMPs alone. Indeed, while there is an input of TF leading TG in EMPs-TF samples, another mechanism is most likely involved to trigger TG with EMPs in plasma. Interestingly, the presence of inhibitors that triggers the two coagulation pathways does not affect TG if EMPs are added to plasma. So, as FXIIa or TF are not present or inhibited, it could be inferred that EMPs have the capacity to trigger TG by a mechanism that appears independent of both intrinsic and extrinsic coagulation pathways.

It is not easy to decipher find what trigger TG when EMPs are added to plasma. It might suggested that the "microparticles coagulation pathway" proposed by Furie *et al* triggers TG [52]. Another hypothesis would be that EMPs carry a cryptic form of TF or that EMPs are able to activate cryptic TF in plasma [57, 81]. However, neither in plasma nor EMPs samples

TF was evidenced or was below the detection threshold. Moreover, Bosman *et al* [3] did not highlight TF in an extensive study on EMPs proteome.

As time preceding the thrombin burst or lag-time variations are usually relatively wide when testing intrinsic coagulation pathway *in vitro* [77]. Therefore it could by hypothesized that EMPs might support or imitate intrinsic coagulation pathway. It has been demonstrated that factor XI could be activate by thrombin in a factor XII independent manner. Thus, EMPs could generate subnanomolar amount of thrombin sufficient to activate factor XI resulting in the observed thrombin explosion in CAT [77, 82].

According to literature, there is typically around 2000 platelet-derived MPs/µl and 60 EMPs/µl in PRP [15, 83]. It is considered that PRP reflects the number of MPs in blood flow. In ECs, a previous study using the same count method demonstrates that there are from around 3300 EMPs/µl ± 1200 at day six and this increase until around 35'000 EMPs/µl ± 15'000 after 42 day of storage [29]. Consequently, in case of massive transfusion, it is likely that the total number of EMPs in blood flow of the recipient rise up. As patients undergoing cardiac surgery are often massively transfused, an additional source of EMPs is provided by blood concentrates [29]. Moreover, it is known that such surgery produce a release of TF in patient's blood, even if techniques and procedures are developed to reduce this release [84, 85]. Taking together, it could be thus hypothesized that under certain circumstances, transfused EMPs could be involved in TG and could be linked to post transfusion complications or adverse clinical outcome.

Several studies have shown a link between storage time of blood products and post transfusion complications [4-6]. However, this is still controversial and some recent studies

limited this link only under specific circumstances [7-10]. Indeed, meta-analysis failed to point out a "cause" of complication after transfusion related to the storage time. As MPs are bioactive, it could be suggested that EMPs are mediators of these complications [11].

In summary, we provide evidence that i) EMPs in plasma are able to induce generation of thrombin, ii) this generation is not abolished using anti-TF, iii) thrombin generation is enhanced by adding EMPs previously incubated with TF, with an activity similar to that observed with thromboplastin.

Acknowledgements

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Table I : Endogenous Thrombin Potential (nM.min) are given for the various samples tested in calibrated automated thrombogram with MP-reagent. ETP is given in mean ± SD of five independent measures (N=5). For each tested plasma sample, table give also concentration of Corn Trypsin inhibitor, number of erythrocyte-derived microparticles (EMPs) or tissue factor (TF) EMPs (EMPs-TF), addition of Innovin (1:250) or Anti-human TF (1:10).

CTI [µM]	EMPs/µl	EMPs-TF/µl	Innovin	Anti-TF	ETP ± SD
1.6	-	-	-	-	0
1.6	1000	-	-	-	0
1.6	5000	-	-	-	962 ± 99
1.6	10'000	-	-	-	986 ± 101
3.2	10'000	-	-	-	1063 ± 45
1.6	20'000	-	-	-	1120 ± 42
1.6	-	250	-	-	1255 ± 41
1.6	-	1000	-	-	1477 ± 91
1.6	-	10'000	-	-	1516 ± 23
1.6		10'000	-	+	1304 ± 42
1.6	-	-	+	-	1355 ± 46
1.6	-	-	+	+	0

Legend to figures

Figure 1: Attempts to evidence tissue factor (representative experiments)

Western blotting

Using anti-human tissue Factor (TF), detection threshold of recombinant tissue factor (rTF) by western blot was around 550 pM (0.34 ng). In plasma samples (treated with Proteominer™ or not), as well as in erythrocyte derived microparticle samples, no TF was detected. In positive controls, samples of erythrocyte derived microparticles coated with tissue factor, tissue factor was clearly detected.

Flow cytometry

(A) Shows event sorted according to their size and shape. Two different regions are determined, Region 1 (R1) for Truecount beads and Region 2 (R2) for microparticles count.

(B) and (C) show event from R2 according to their fluorescence due to the labelled antibodies. (B) Shows erythrocyte derived microparticles (EMPs), events are positive for anti-human CD235a (erythrocyte marker) and negative for anti-human CD142 (tissue factor marker). (C) TF coated EMPs (EMPs-TF); events were positive for human CD235a and 5 to 15 % were positive for anti-human CD142.

Electron microscopy

Transmission electron microscopy of erythrocyte derived microparticles (EMPs) and coated tissue factor erythrocyte derived microparticles (EMPs-TF); both samples were stained with anti-TF followed by immunogold staining. EMPs-TF are labelled (arrow) whereas no staining is observed in EMPs.

Figure 2: Measurement of the activity of erythrocyte-derived microparticles (EMPs) using thrombin generation assays. In the presented thrombograms, thrombin generation curves are the mean of five independent measures, in a representative experiment with pooled plasma and pooled EMPs.

Effect of negatively charged membranes of EMPs on thrombin generation in plasma. (Test with PRP reagent, tissue factor (TF) is added to trigger reaction). Thrombin generation was observed when EMPs where added to plasma at a final concentration of 1000 EMPs/µl (▲), 10'000 EMPs/µl (■) and 20'000 EMPs/µl (◆). The more there are EMPs, the stronger and faster is the TG. In the absence of EMPs in plasma, no significant TG was observed, 0 EMPs/µl (✕).

(B): Effect of EMPs on thrombin generation in plasma (Test with MP-reagent, no addition of tissue factor (TF)) No TG was observed in plasma alone, without EMPs (l). EMPs or coated TF EMPs (EMPs-TF) are added to plasma at a final concentration of 10'000 per microliter. Plasma with EMPs-TF (■) or Innovin in the absence of EMPs (◆) are strong activator of thrombin generation in the test. Thrombin generation was also observed in plasma sample with EMPs (✕). A partial inhibition of thrombin generation was observed when anti-TF is added to EMPs-TF sample (▲), whereas inhibition was complete when anti-TF was added to plasma with Innovin in the absence of EMPs (●) .

Figure 3 Endogenous Thrombin Potential of erythrocyte-derived microparticles (EMPs) and coated tissue factor EMPs (EMPs-TF) at various concentrations.

Endogenous Thrombin Potential (nM.min) measurements of plasma samples containing 1.6 µM of corn trypsin inhibitor with the addition of 0 to 20'000 EMPs/µl (◆) or 0 to 10'000 EMPs-TF/µl (●) in thrombin generation assays with MP-reagent. Each dots represent the mean of five independent measures ± SD, N=5. ETP values for each sample are presented in Table I.

Figure 1

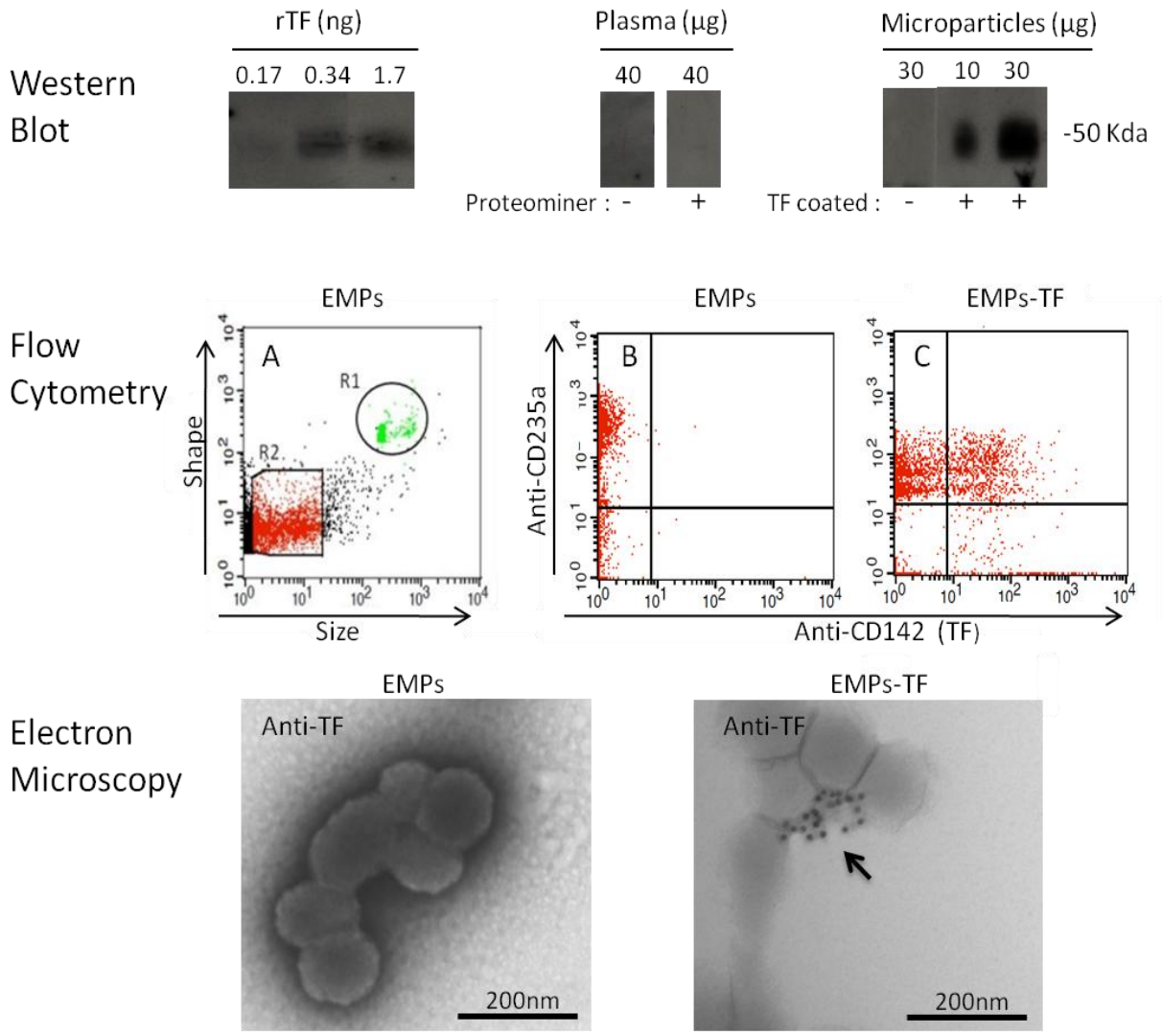

Western Blot
rTF (ng)
0.17 0.34 1.7
Plasma (µg)
40 40
Proteominer : - +
Microparticles (µg)
30 10 30
TF coated : - + +
-50 Kda
Flow Cytometry
EMPs
A
R1
R2
Shape
Size
EMPs
B
Anti-CD235a
EMPs-TF
C
Anti-CD142 (TF)
Electron Microscopy
EMPs
Anti-TF
200nm
EMPs-TF
Anti-TF
200nm

Figure 2

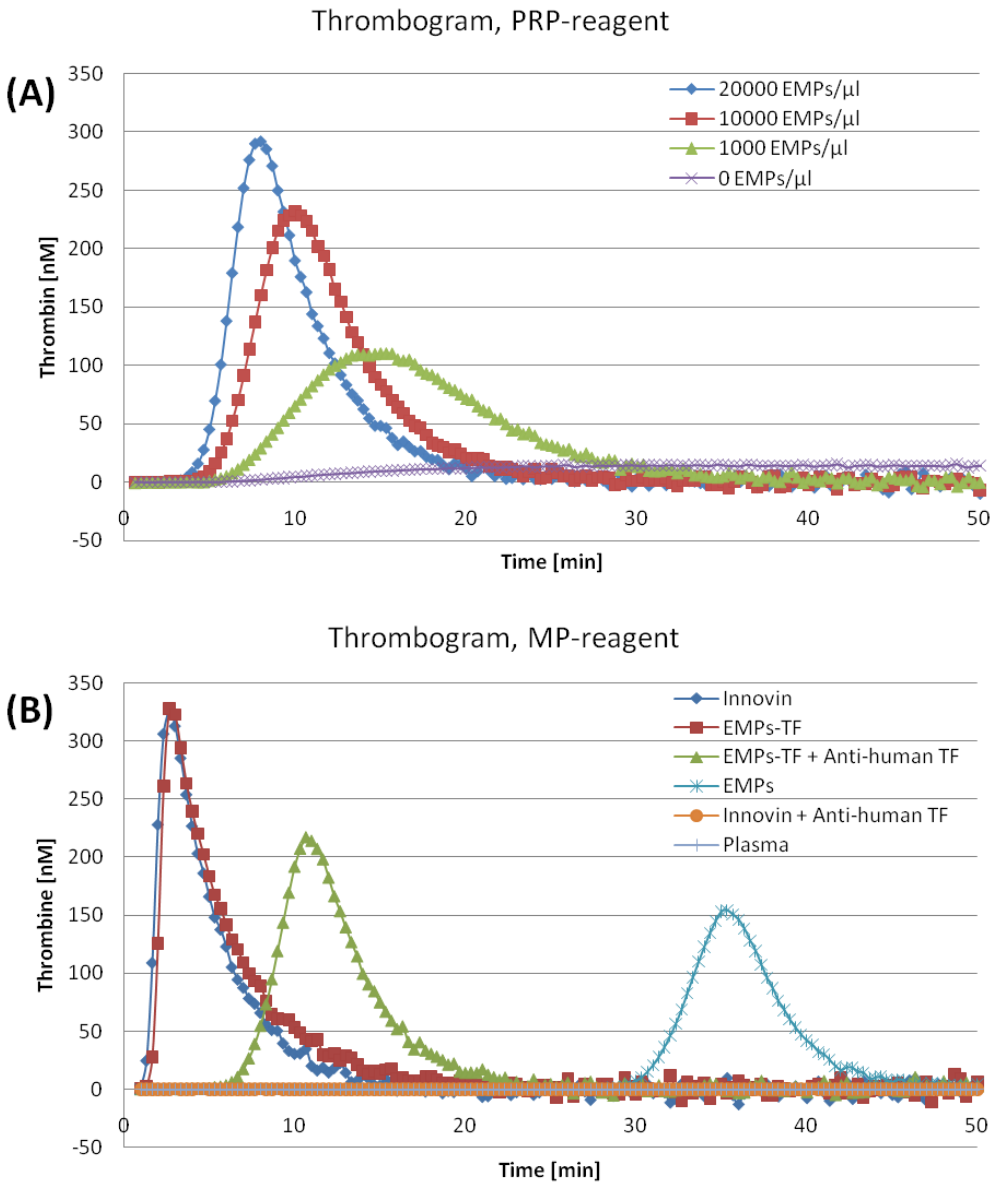
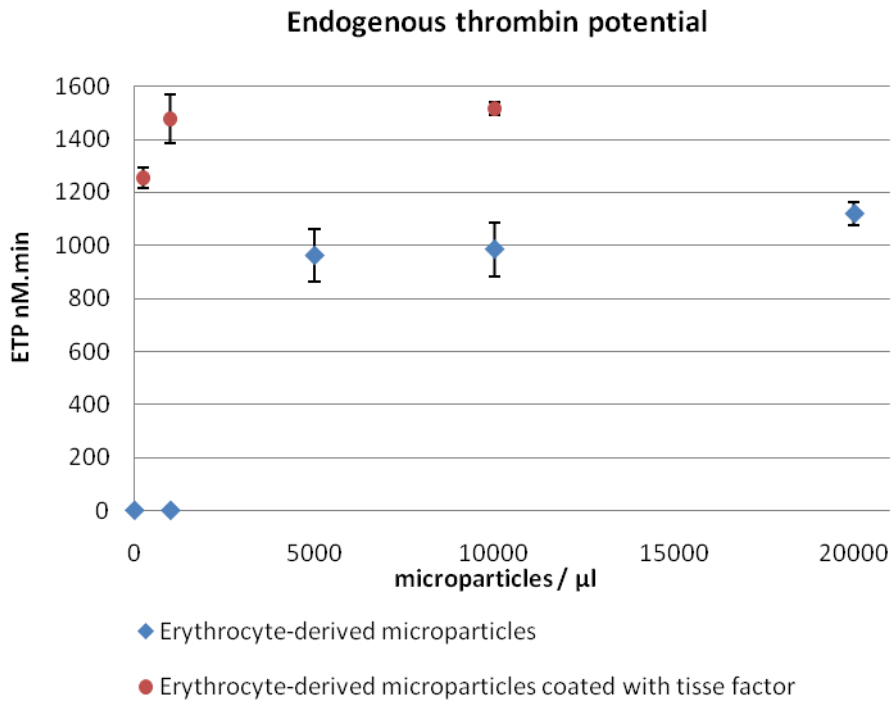

(A)
Thrombogram, PRP-reagent
Thrombin [nM]
Time [min]
20000 EMPs/µl
10000 EMPs/µl
1000 EMPs/µl
0 EMPs/µl
(B)
Thrombogram, MP-reagent
Thrombine [nM]
Time [min]
Innovin
EMPs-TF
EMPs-TF + Anti-human TF
EMPs
Innovin + Anti-human TF
Plasma

Figure 3


Endogenous thrombin potential
ETP nM.min
1600
1400
1200
1000
800
600
400
200
0
0
5000
10000
15000
20000
microparticles / µl
Erythrocyte-derived microparticles
Erythrocyte-derived microparticles coated with tisse factor

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7

TRANSFUSION
CLINIQUE ET BIOLOGIQUE

Le don du sang, entre enjeux éthiques et financiers

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Accepté sept. 2011

Avant-propos

Ce chapitre est consacré à des réflexions moins scientifiques, mais plutôt humanistes. Loin de moi la prétention d'être un philosophe ou écrivain, cependant le (jeune) chercheur que je suis aimerait partager ses préoccupations et interrogations sur les enjeux et problèmes éthiques que l'on peut rencontrer en transfusion.

J'aimerais dédier ce chapitre aux personnes qui nous ont quittées pendant mon travail de thèse.

A mon père, Willy Rubin, décédé en 2009

A Florence Rappaz-Rosset, partie trop tôt en 2010

Résumé

Le don de sang représente un acte solidaire. Il est le plus souvent bénévole et, selon les pays et les circonstances, non rémunéré. Face à l'augmentation des besoins, face aux critères d'exclusion de plus en plus nombreux, face à la dérive sécuritaire et tenant compte des modifications des structures de nos sociétés, de nombreux acteurs de la chaîne transfusionnelle s'interrogent sur la valeur du don, sur le sens du bénévolat et finalement sur l'opportunité de rémunérer l'acte de livrer une partie de soi, non plus comme un don, non plus comme l'expression de l'altruisme et de la solidarité, mais comme une prestation commerciale définie par des règles économiques pouvant inclure ou non des aspects éthiques d'un commerce dit équitable ou non.

Abstract

Ethical aspects of blood donation are apparently well defined, and in most countries, donors are not remunerated. However, in more particularly in countries in which plasma fractionation is particularly well developed, the question of a finical compensation is either in discussion or is already implemented. What is the truth? What are the ethical considerations that must be taken into account when discussing the organization of the blood supply? Health systems have developed ethics, known as medical or biomedical ethics. However, economy has also its own ethics. How to deal with these realities? Is remuneration of blood an interdict or a modern response of the society faced to the necessity of organizing blood transfusion for all people needing blood products?

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Introduction

Le sang est une ressource précieuse, sa symbolique, son mode de prélèvement et de distribution en font un élément unique dans l'histoire de la médecine et de l'homme. La demande des hôpitaux en produits sanguins labiles a tendance à augmenter d'années en années tandis que le nombre de dons, lui, diminue.

Lorsqu'on parle de don du sang, on regroupe deux mots portant des symboliques fortes, « don » et « sang ». Selon le Petit Robert « Donner, c'est abandonner à quelqu'un, dans une intention libérale ou sans rien recevoir en retour, une chose que l'on possède ou dont on jouit ». On trouve également comme première définition dans le dictionnaire Larousse « Céder, offrir gratuitement à quelqu'un quelque chose qu'on possédait ou qu'on a soi-même achetée à cet effet ». C'est en général, cette définition qui vient à l'esprit de la plupart des gens. La seconde définition donnée par le Larousse est « Remettre, attribuer quelque chose (de l'argent souvent) à quelqu'un comme récompense ou comme paiement en échange de quelque chose ». Dans ces deux définitions, on observe deux concepts très différents : dans le premier on parle d'offrir, l'action est dirigée dans un sens unique, dans le second on parle d'échange, l'action s'accomplit dans les deux sens. Selon Marcel Mauss, sociologue et philosophe français du 20ème siècle, il n'y aurait pas de don pur ou don totalement altruiste, dénudé d'intérêt. En quelque sorte, lorsque l'on fait un don, il y aurait toujours un intérêt et donc un contre-don effectué sous des formes les plus diverses. Le don est alors assimilé à une forme d'échange basé sur une exigence tacite de réciprocité. C'est la réciprocité qui est sous-entendue par l'idée de « contre-don » : cela peut inclure la satisfaction personnelle d'avoir donné, la gratitude, le renforcement de liens, ou encore se donner «bonne conscience». Cette vision est alors assez réductrice de l'acte de donner, puisqu'accomplie par intérêt et jamais par altruisme. Et malgré le sens premier de donner auquel on pense, on observe de nombreuses

expressions qui sous-entendent le contre-don, par exemple « c'est donnant-donnant » ou « il faut donner pour recevoir ».

Sans vouloir diminuer la portée du don du sang, il est intéressant de comprendre ce qui motive une personne à donner son sang. Selon un sondage effectué en Suisse en 2004, à la question « pour quelle raison donnez-vous votre sang », plus de 75% des personnes interrogées ont répondu « parce qu'un jour, je pourrais en avoir besoin ». On est donc bien dans la logique du contre-don, ou plutôt contre-don « anticipé ». Ceci convient au principe de solidarité, aider les autres et être aidé en cas de besoin. Bien-entendu, parmi les réponses des donneurs, il y a aussi des réponses de type : « Pour sauver des vies » ou « Parce qu'un proche a été transfusé ». Celles-ci peuvent être considérées comme étant davantage altruistes.

Dans le monde de la transfusion, on oppose souvent « don-gratuit » (céder) à « don-rémunéré » (échanger). Un don devrait être forcément gratuit, selon son sens premier ; et donc un don rémunéré est en quelque sorte un oxymore. On devrait parler plutôt de vente de sang dans ce dernier cas. Peut-être est-il plus acceptable « éthiquement », dans nos sociétés, de parler de don rémunéré plutôt que de vente de sang. Par la suite, quand il sera mentionné don rémunéré, il sera sous-entendu vente de sang comme il est d'usage en transfusion.

Afin de rendre hommage aux donneurs qui, par leur geste, permettent de sauver de nombreuses vies, nous nous permettrons de citer Khalil Gibran. Dans le contexte du don du sang, on peut détourner - avec une petite pointe d'humour - le sens premier de sa phrase avec une interprétation au sens propre qui n'était probablement pas l'idée de l'auteur : « Vous ne donnez que peu lorsque vous donner vos biens, c'est lorsque vous donnez de vous-même que vous donnez réellement. »

L’éthique

On parle souvent de problèmes et enjeux éthiques. Selon Wikipédia : l’éthique (du grec êthikon, « la science morale ») est une discipline philosophique pratique (action) et normative (règles) dans un milieu naturel et humain. Elle se donne pour but d'indiquer comment les êtres humains doivent se comporter, agir et être, entre eux et envers ce qui les entoure. La morale et l’éthique sont donc relativement proches comme termes et le langage courant les emploie souvent comme synonymes : cependant en voici les principale différences ; la morale a souvent une connotation religieuse, se porte sur le bien et le mal et impose des obligations de l’extérieur. L’éthique est plutôt de nature laïque, se porte sur le positif et le négatif et nous fait réfléchir, nous responsabilise. L’éthique délimite des principes qui permettent de faire des choix libres et responsables face à une situation dans un cadre donné. En fonction de la culture, de la société, du temps, de l’évolution des mœurs, les principes éthiques peuvent varier. Avec l’essor des nouvelles technologies et de la science, les limites étant repoussées sans cesse, les principes éthiques doivent s’adapter face à des problématiques nouvelles. C’est donc pour cela que bien souvent l’éthique se retrouve à l’interface entre progrès médicaux, nécessité économique et protection de la dignité humaine. En Europe occidentale, les principes éthiques du don de sang reposent généralement sur quatre piliers :

Le volontariat : le don du sang doit relever de la seule volonté du donneur, et ne doit en aucun cas être lié à une quelconque obligation.

La gratuité : le don du sang ne doit pas donner droit à des contreparties financières et les produits sanguins labiles ne doivent pas faire l’objet d’un commerce.

L’anonymat : l’identité du donneur ne doit pas être connue du receveur et vice-versa.

La responsabilité : le donneur s’engage à ne dissimuler aucun élément susceptible de nuire à la santé du receveur lors de l’entretien médical.

L'organisation mondiale de la santé (OMS), la Croix-Rouge ainsi que d'autres organisations tentent de faire adopter ces principes éthiques dans un maximum de pays à travers le monde.

Les motivations

Le nombre de produits sanguins disponibles pour les hôpitaux est directement dépendant de la volonté des donneurs de sang. On peut se demander quelle est la motivation de ces personnes. En effet, il n'y a pas de bénéfice direct, c'est donc un comportement qui peut être considéré comme altruiste et pro social, relativement rare dans notre société de consommation moderne individualiste. Plusieurs théories tentent d'expliquer un tel comportement qui fait passer le bénéfice des autres avant le sien.

Pour tenter d'expliquer cette forte motivation à donner son sang, revenons brièvement à l'histoire de la transfusion. La première transfusion « moderne » réussie fut effectuée en 1818. Avec l'amélioration des techniques et de meilleures connaissances, c'est dans les années 1930, plus d'un siècle plus tard, que les premiers services de transfusion furent mis en place. L'essor de la transfusion eut lieu pendant la seconde guerre mondiale. Durant cette période, le principe de solidarité sera mis en avant. En effet, en donnant leur sang, les citoyens pouvaient « soutenir » l'effort de guerre et « sauver » indirectement la vie de soldats blessés sur les champs de bataille. De ce fait, dès le début de la transfusion moderne, le don du sang (sous-entendu sans contrepartie) fut perçu par la population comme un acte positif, voire même un acte de résistance et de nationalisme. L'urgence de la guerre a probablement permis à la transfusion d'échapper non seulement aux réticences de certains médecins (la transfusion comporte des effets indésirables et des risques) mais aussi à la méfiance de la population vis-à-vis d'une technique nouvelle. A la fin de la guerre et, en conséquence des avancées de la chirurgie - grande consommatrice de sang -, il fallut de nouveaux moyens pour motiver les

gens à donner leur sang afin de subvenir aux besoins des hôpitaux. Dès le début des années 50, aux Etats-Unis notamment, le principe de don rémunéré s'imposa tandis qu'en Europe, d'une manière générale, c'est le don gratuit qui fut valorisé en tant qu'acte de solidarité citoyen, rassembleur et fédérateur, symbole de l'unité d'un peuple en bonne santé. Aujourd'hui encore le don du sang continue de bénéficier d'une image très positive, et cela malgré les scandales des affaires du sang contaminé des années 80. Actuellement, dans notre société, l'action de donner son sang semble encore et toujours perçue comme un acte considéré « moralement » bon par essence.

L'image du don du sang

L'image positive du don du sang, action solidaire envers son prochain ou signe de bonne santé, permet le postulat collectif suivant dans notre société : « il est normal de donner son sang ». Malencontreusement, l'exclusion d'une personne pour une raison ou une autre fait que cette dernière n'est plus dans la « normalité » et sous-entendrait même qu'elle serait malade. De ce fait, par l'exclusion du don, c'est comme si le donneur potentiel ne faisait plus partie du système de solidarité, comme une « mise au ban » de la société. Les critères d'exclusion sont devenus, au fil des ans, de plus en plus drastiques et de plus en plus nombreux - afin de garantir la sécurité des produits sanguins -. Cette dérive sécuritaire a eu pour conséquence que les personnes exclues sont devenues de plus en plus nombreuses. A titre indicatif, au service de transfusion de Lausanne, près du tiers des personnes qui se présentent pour la première fois sont exclues du don. L'exclusion est parfois très mal vécue, et les services de transfusion se retrouvent dans une position délicate ; en effet, ils doivent promouvoir le don du sang, mais en même temps refuser de nombreuses personnes. Le message contradictoire qui peut en ressortir est « donner votre sang, mais pas vous ! ». En voici quelques exemples :

- Certaines personnes âgées (mais que veut dire l'âge ?), habituées à donner leur sang depuis de nombreuses années, se voient refusées sous prétexte que le don du sang pourrait représenter un danger pour leur santé. Elles se sentent alors rejetées.
- Les personnes ayant été transfusées ne peuvent plus donner leur sang depuis la fin des années 90 car le prion (responsable de la maladie de Creutzfeldt-Jacob) pourrait être transmis du donneur au receveur qui à son tour, s'il donne son sang, propagerait la maladie. De même, la chaîne de transmission des maladies inconnues, par le sang, serait maintenue. De nombreuses personnes ayant été transfusées (11% de la population des donneurs de sang du Canton de Vaud) donnaient leur sang depuis des années, représentant ainsi une opportunité de rendre le geste que d'autres personnes avaient eues pour elles. C'est pourquoi cette mesure de précaution fut particulièrement mal comprise et mal acceptée par les personnes concernées.
- Les hommes ayant des relations sexuelles avec des hommes sont également exclus du don[1] en raison de la prévalence élevée du VIH dans ce groupe. Ils ressentent l'exclusion du don du sang comme particulièrement discriminatoire et injuste, compte tenu de leur volonté de faire partie de la « normalité », d'une recherche de reconnaissance sociale et de solidarité associée au don du sang. De plus, cela renvoie directement au lien simplificateur, encore souvent fait entre SIDA et homosexuels, dont ces derniers tentent de se défaire.

Rémunération

Dans la plupart des pays européens, le don du sang fait partie des mœurs et jouit d'une image très positive. Ce n'est pas le cas dans les pays dans lesquels le don du sang a été mis en place

[1] Les mots « Etre exclu du don » portent toute l'ambigüité de la réalité du quotidien des médecins du don.

après la seconde guerre mondiale. La rémunération peut être un moyen incitatif, bien paradoxal dans les pays pauvres. La rémunération du sang pose aussi des problèmes éthiques car une personne dans le besoin peut « mentir » et ne pas répondre correctement au questionnaire d'évaluation et mettre sa propre santé en danger, par exemple en donnant son sang plus souvent que les prescriptions ne l'autorisent. Bien souvent également, dans les systèmes de santé qui poussent les individus à donner pour des raisons financières, ces donneurs risquent eux-mêmes de ne pas avoir accès aux transfusions thérapeutiques en cas de nécessité, par manque de moyens ou par absence d'assurance sociale. En quelque sorte, on prélèverait le sang des plus pauvres au profit des plus riches. C'est entre autre pour ces raisons que l'OMS a décidé de s'engager en faveur du don « véritable » du sang, sans rémunération.

Don volontaire et gratuit

Selon l'article 21 de la directive du conseil de l'Europe 2002/98/CE du 27 janvier 2003, également ratifiée par la Suisse, « Les États membres prennent les mesures nécessaires pour encourager les dons volontaires et non rémunérés en vue de garantir que, dans toute la mesure du possible, le sang et les composants sanguins proviennent de ces dons». Les pays européens sont « encouragés » à promouvoir « dans la mesure du possible » les dons volontaires et non rémunérés. Cette directive non contraignante laisse beaucoup de liberté à chaque état. Dans la plupart des pays, le don est « non rémunéré » alors que dans certain pays comme la Hongrie ou l'Autriche, les deux systèmes, rémunéré et non rémunéré se côtoient. Enfin, dans des pays comme la Lituanie, le don est rémunéré.

Indemnité, reconnaissance ou rémunération ?

Bien que le don volontaire et non rémunéré soit reconnu dans la plupart des états européens, son interprétation varie d'un pays à l'autre. L'absence de rémunération n'exclut pas que les donneurs puissent recevoir une « indemnisation » qui doit, en théorie, se limiter à compenser les dépenses occasionnées pour effectuer le don. En plus d'une petite collation, dans la plupart des pays, il est d'usage courant d'offrir un petit cadeau pour remercier le donneur allant du porte-clés aux billets de concert… Cependant, l'indemnisation ou un petit cadeau de remerciement ne doivent en aucun cas être la source de motivation des donneurs. Dans certains pays comme la Suisse, des cérémonies avec remise de broches, diplômes ou médailles sont organisées pour remercier les personnes qui ont effectué un certain nombre de dons. Seuls cinq pays en Europe appliquent strictement le principe de volontariat et non rémunération et n'offrent rien pour remercier ou inciter les donneurs à revenir.

La France fut l'un des premiers pays à inscrire le principe de gratuité du don du sang dans la loi, en 1952. En Allemagne, la loi sur la transfusion interdisant aussi la rémunération (mais autorisant le versement d'une indemnité) a été adoptée en 1998. En Allemagne, deux systèmes de collecte se côtoient, l'un public organisé par la Croix-Rouge et l'autre privé. Contrairement aux collectes organisées par la Croix-Rouge, les centres de collecte privés prévoient une « indemnisation » forfaitaire d'une vingtaine d'euros pour couvrir les frais de déplacement ou de dérangement pour un don de sang complet, l'indemnisation ne devant pas constituer la source de motivation. Cependant, un industriel du fractionnement comme Heama, lié à l'industrie pharmaceutique, a développé un site internet bilingue, en allemand et en polonais (http://www.haema.de/?L=1), ce qui incite ces derniers où le niveau de vie est inférieur à venir donner leur sang en Allemagne. Comme le don de sang n'est pas rémunéré, ni « indemnisé » en Pologne, cela peux créer des problèmes d'approvisionnement dans les

régions frontalières. L'Allemagne tolère cette pratique qui pourtant est discutable. Tout est donc une question d'interprétation. Si la plupart des citoyens européens soutiennent le don « bénévole », la perception du bénévolat et de ce qui est éthiquement acceptable varie d'un pays à l'autre, la frontière entre rémunération, reconnaissance ou indemnité n'est pas toujours aussi claire qu'elle n'y paraît au premier abord. Dans notre société de consommation, le don du sang fait partie des dernières actions altruistes avec un principe de solidarité qui perdure. Pourtant, dans cette société dites de « services », il est considéré comme normal d'être payé pour un service rendu. Dès lors, pourquoi ne pas appliquer ce principe au don du sang ? Cela pourrait se révéler efficace pour inciter plus de personnes à donner leur sang. Néanmoins, il ne faut pas négliger la symbolique du sang, le fait de savoir qu'un don de sang permet de sauver des vies et cela est jusqu'à présent une des sources de motivation suffisante pour justifier la gratuité de l'acte dans une perspective de solidarité. Au delà de la valeur de l'action, donner son sang est aussi une manière unique de relier les citoyens les uns aux autres. La rémunération pourrait changer la perception et la symbolique forte de l'acte, le sang deviendrait un bien de consommation comme un autre.

Le paradoxe du plasma

Par le don de sang, on sous-entend ce qu'on appelle un don de sang complet, soit le sang comprenant tous ses éléments, c'est à dire, le plasma, les globules rouges, les globules blancs et les plaquettes. Cependant, il existe également d'autres types de don de sang, à savoir le don de plaquettes ou de plasma, qui sont prélevés par une technique que l'on appelle « aphérèse ». Grâce à cette technique, on peut prélever un élément particulier du sang, et restituer le reste au donneur. Si le principe de don du sang « complet » bénévole est généralement le plus répandu en Europe, le don de plaquettes dans une moindre mesure, mais surtout le « don » de plasma,

sont rémunérés dans plusieurs pays. Le plasma sert à la fabrication de médicaments, comme des facteurs de coagulation ou des immunoglobulines. La demande en médicaments à partir de plasma augmentant, l'industrie pharmaceutique s'intéresse beaucoup à ce marché très lucratif. Le prix des médicaments à base de plasma humain augmente d'années en années. Dans un nombre non négligeable de pays, le don de sang complet bénévole coexiste avec le « don » de plasma rémunéré dans des centres de plasmaphérèse, bien souvent des succursales de compagnies pharmaceutiques.

Comme le plasma sert à la fabrication de médicaments, la symbolique autour de cet acte n'est pas la même que celui du sang complet. De ce fait, il est mieux accepté que le plasma soit rémunéré contrairement au sang complet. La vente du plasma relève d'ailleurs d'un autre cadre législatif que le don de sang. Dans ce contexte, la parole est donnée à un industriel du plasma dans la section suivante

Visions d'un industriel du fractionnement du plasma

Le Dr Ruedi E. Wäger, scientifique ayant dirigé des grandes entreprises de fractionnement de plasma, dans un chapitre récemment rédigé et consacré à l'industrie du plasma apporte un éclairage personnel sur cette éthique commerciale de la vente du sang[2].

Il note que « l'augmentation explosive de la demande en albumine, en immunoglobulines et en Facteur VIII a fait apparaître que le plasma, issu des dons de sang collectés par les services de transfusion, ne peut plus couvrir les besoins en produits dérivés et que, de ce fait, il n'est pas surprenant que l'industrie de fractionnement de plasma se soit intéressée à des solutions alternatives passant par le développement de machines automatisées pour prélever

[2] Ruedi E. Wäger : L'industrie mondiale du plasma sanguin. Dans : Sang : arts, sciences et vies (Ph. Schneider, J.-D. Tissot, M. Benzoni et O. Ribes, éditeurs). Editions Favre, Lausanne, Paris, sous presse.

sélectivement le plasma en restituant les autres composants du sang aux donneurs. Ainsi, des entrepreneurs et des investisseurs ont construit des centres de collecte de plasma aux Etats-Unis, permettant à la plupart des gros producteurs d'être pratiquement indépendants dans leur approvisionnement de plasma ». C'est dans ce contexte que les donneurs de plasma sont rémunérés et cette situation est justifiée par une personnalité comme Wäger qui indique « Personnellement, je pense qu'il est plus éthique d'indemniser des donneurs de plasma soigneusement sélectionnés et fiables que de priver les patients de traitement par des produits dérivés de plasma ». Le débat est ouvert, le système est culpabilisé. Nous sommes défenseurs d'une éthique du bénévolat, mis face à nos responsabilités. Alors, Wäger ajoute « S'il est vrai que les donneurs américains sont rémunérés pour leurs dons de plasma (entre 20 et 30 dollars par don en fonction du besoin en plasma, il ne faut pas oublier que ces donneurs doivent se déplacer jusqu'au centre de collecte et que la séance de plasmaphérèse (y compris le temps d'attente et l'examen du donneur) prend jusqu'à deux heures ». Dans son argumentaire, Wäger affirme ; « Lors de centaines d'entretiens avec des donneurs rémunérés aux Etats-Unis, j'ai appris que leur principale motivation est d'aider les patients qui ont besoin de produits dérivés de plasma. Elle n'est pas moins valable que celle des donneurs de sang bénévoles. Par ailleurs, je constate que dans de nombreux pays (France, Allemagne, Japon, etc.), les donneurs de sang et de plasma du secteur non commercial reçoivent fréquemment des compensations ou des incitations au don » et de poursuivre « Le débat sur la valeur et l'éthique respectives des dons de sang entier ou de plasma sur une base bénévole non rémunérée et des dons de plasma rémunérés représente le vestige d'une sorte de guerre de religion, qui est loin de la réalité. Comment le secteur à but non lucratif pourrait-il remplacer les 20 millions de litres de plasma qui lui manquent pour satisfaire les besoins des patients en produits dérivés de plasma, alors que ce secteur est juste en mesure de couvrir les besoins locaux/nationaux en globules rouges, plaquettes et plasma frais congelé par les services de transfusion sanguine ? Pourquoi les

patients, qui souffrent de maladies mettant leur vie en danger et qui ont besoin de produits contenant des protéines plasmatiques, ne seraient-ils pas traités de manière adéquate, sous prétexte que certains estiment que les dons de plasma rémunérés ne sont pas éthiques ? Les secteurs commerciaux et non commerciaux ont des logiques et des justifications fortes à continuer de servir la communauté et les patients, malgré des approches et des philosophies différentes, en étant fiers de leur tâche, en se concentrant sur leurs points forts et en respectant les principes éthiques établis ».

Le don dirigé, une alternative ou un interdit

Le don dirigé est mal aimé dans nos pays ; il est considéré comme peu sécuritaire, comme trahissant l'éthique établie par les pays occidentaux, comme un tabou, comme une dérive des pays pauvres inaptes à développer un système de transfusion à l'européenne ou à l'américaine. Il force des individus ne désirant pas donner pour un proche à dévoiler leurs secrets, à se mettre à nu au service d'un frère, d'un ami, d'un père. Mais, à l'image du commerce du sang, la transfusion dirigée, ou intrafamiliale est-elle un interdit définitif ?

La parole est donnée à Jean-Pierre Allain et à Dora Mbanya. Dans leur chapitre intitulé « La Transfusion en Afrique[3]), ces auteurs apportent un éclairage qu'il ne faut pas négliger, lorsque, de notre hauteur d'européens civilisés, nous examinons la transfusion sanguine dans une vision de mondialisation égoïste.

Le sang des pays pauvres

Allain et Mbanya rappellent que dans nos pays, les produits sanguins sont transfusés le plus souvent à des patients présentant un déficit de production (d'origine médicamenteuse

[3] Jean-Pierre Allain & Dora Mbanya : La transfusion en Afrique. Dans : Sang : arts, sciences et vies (Ph. Schneider, J.-D. Tissot, M. Benzoni et O. Ribes, éditeurs). Editions Favre, Lausanne, Paris, sous presse.

thérapeutique, suite à des transplantations d'organes, de moelle ou de chimiothérapie pour cancer, ou lié à l'âge des receveurs – ayant soit plus de 70 ans ou moins de 3 ans) et que les transfusions d'urgence représentent une part moins importante de l'acte transfusionnel. En Afrique, la transfusion reste une thérapeutique d'urgence pour les anémies aiguës (paludisme chez les enfants de 0 à 5 ans, hémorragies aigues péri- ou post-natales, patients subissant une intervention chirurgicale). Les cas de transfusion « médicale » sont une minorité, même en comptant les anémies congénitales comme la drépanocytose ou la thalassémie majeure. Dans toutes ces indications, le sang total (qui est quasi introuvable dans les pays développés) est le produit sanguin de base. En Europe, la « publicité » pour le don de sang dit que donner son sang sauve des vies. En réalité, ce n'est pas toujours le cas, les transfusions sont prescrites pour le confort du malade, pour des anémies modérées liées à des interventions ou pour maintenir en vie des patients. En Afrique, c'est l'inverse. Les dons de sang sauvent des vies. Les dons sont bénévoles et reposent en majeure partie sur les étudiants âgés de 16 à 19 ans, qui constituent 50 à 70% des donneurs de sang. Ceux-ci sont réputés sûrs parce que l'OMS et les instances transfusionnelles internationales ont estimé que le risque d'infection était moindre avec seul le sang de donneurs volontaires non-rémunérés. Néanmoins, la pénurie de sang est chronique. Dans beaucoup d'hôpitaux, la règle est : « pas de don familial, pas de transfusion ». Malgré les recommandations de toutes les organisations internationales, les donneurs familiaux continuent de fournir la majorité des dons de sang, même si les donneurs familiaux sont dangereux, car on ne peut les distinguer des donneurs « payés » par les familles incapables de fournir un donneur. Dans leur analyse, Allain et Mbanya examinent en détail les données épidémiologiques afin d'évaluer scientifiquement si, selon le credo officiel et occidental, les donneurs familiaux sont plus dangereux et doivent être écartés du don de sang. De leurs travaux, une conclusion essentielle s'impose : **seule la répétition du don de sang procure un avantage en termes de sécurité, pas le type de donneur**. Toujours selon Allain

et Mbanya, si les donneurs familiaux étaient intégrés dans les collectes de sang, moins de malades mourraient par manque de sang et la sécurité transfusionnelle n'en serait pas amoindrie. Ces observations sont importantes pour dégager objectivement une éthique globale du don de sang.

Le sang des Suisses

Le sang des Suisses a été vendu et a été versé durant des siècles. Des régiments suisses se battaient parfois entre eux, l'un défendant les intérêts d'un roi, d'un prince ou d'un Pape. Manière d'affaiblir la terre et les fermiers en les privant de bras, en les écrasant de labeur, en les privant des âmes de la révolte. Le sang des Suisses était exploité déjà par les propriétaires terriens, les nobles pour maintenir leur pouvoir et/ou leurs droits féodaux. Cette exploitation du sang a perduré, sous d'autres formes. Les anciens qui ont connu le Laboratoire Central de la Croix-Rouge suisse (ZLB) parlent encore de collectes massives de sang, avec comme objectif principal l'accumulation du plasma pour la préparation de produits sanguins dérivés stables, notamment pour produire des immunoglobulines intraveineuses. Ils se souviennent, sans dire précisément s'il s'agit d'une légende ou de la réalité, que l'excès de globules rouges collecté était déversé dans l'Aare, rivière bien connue des bernois. Légende ou réalité ? Toujours est-il que l'excès de globules rouges a bel est bien été une réalité et qu'il a fallu l'exploiter. Des milliers de concentrés érythrocytaires ont été vendus hors de Suisse. Des accords ont été passé entre le ZLB et le New York Blood Center d'une part, et avec le gouvernement grec d'autre part. Le risque de transmission de prions, les restructurations internes, puis la vente du ZLB, par la Croix-Rouge suisse, à des industriels du fractionnement du plasma ont totalement modifié la situation au XXI^{ème} siècle. Des concentrés érythrocytaires sont toujours vendus en Grèce dans le cadre d'une collaboration entre le Service suisse de

transfusion sanguine (Transfusion CRS Suisse) et le gouvernement Grec (http://www.blutspende.ch/fr/qui-sommes-nous/organisation/organisation-nationale/programme-dexportation-vers-la-grece/), en particulier pour soutenir les programmes de prise en charge de patients thalassémiques.

Vente, commerce, don bénévole, exportation…. ; tous ces mots sont porteurs de sens très contradictoires. Cette situation pour le moins paradoxale a fait l'objet de nombreux débats et questionnements dans la presse helvétique ces dernières années. La question de fond reste entière ; commerce de sang ou exploitation optimale des ressources ? La transfusion sanguine, en Suisse dépend de la Croix-Rouge suisse dans une organisation appelée en français « Transfusion CRS Suisse » et « Blutspende SRK Schweiz » en allemand, ce qui se traduit en français par « Don du sang CRS Suisse ». Premier paradoxe, le champ linguistique diffère et couvre des notions différentes. La transfusion est une activité clinique globale, qui a ses règles économiques, son marché, ses normes. Le don de sang représente un seul de ces aspects. Le don du sang en Suisse est bénévole et volontaire. La transfusion sanguine est payante. Comme le répètent à l'envi les responsables hospitaliers et les assureurs, le sang est cher, d'autant qu'il s'agit d'une matière périssable et rare. Second paradoxe, les hôpitaux ont des budgets importants pour acheter ce sang donné, ce sang gratuit, ce sang fourni bénévolement. Troisième paradoxe, voire scandale pour les médias, c'est la vente aux industries de fractionnement du plasma dérivé du sang complet ou collecté par aphérèse chez ces donneurs bénévoles. Scandale pour certains journalistes, qui ont dénoncé, dans une émission de la Télévision Suisse Romande, en 2007, le fait que du plasma collecté chez des bénévoles était vendu par les services suisses de transfusion sans que les donneurs puissent décider, en toute connaissance de cause, du devenir de leur don. En effet, l'information fournie aux donneurs était insuffisante, laconique souvent, inexistante parfois. Les donneurs ne pouvaient ni

comprendre les enjeux financiers liés à leurs dons ni les implications éthiques y relatives. Le principe du consentement éclairé était clairement bafoué.

Commerce du sang ; scandale du commerce du sang suisse ! En Suisse ce n'est pas le sang ou ses dérivés, comme les plaquettes ou le plasma destiné à la transfusion, qui sont vendus ; c'est le travail et les services nécessaires à sa préparation et à son conditionnement qui sont l'objet d'un commerce répondant aux règles économiques du marché de la santé. Mais le plasma destiné au fractionnement, dans ce contexte, pose un problème. En effet, sa vente en tant que matière première nécessaire à la fabrication de médicaments trahit, pervertit la notion de gratuité du don. Même si les revenus dégagés de cette vente permettent de diminuer les charges relatives au fonctionnement des systèmes de prélèvement, de conditionnement, de sécurité, d'analyses, de stockage, la vente du plasma représente un vrai commerce qui a ses règles et éventuellement son éthique propre.

En Suisse, les produits sanguins sont, sur le plan législatif, considérés comme des médicaments (Loi fédérale sur les médicaments et les dispositifs médicaux : http://www.admin.ch/ch/f/rs/8/812.21.fr.pdf). Il est dès lors peu étonnant que les industriels puissent considérer le plasma comme une matière première, qui a un prix et un marché. Les exigences des « GMPs » qui sont appliquées à la lettre pour la préparation du plasma, sont assez éloignées des considérations éthiques biomédicales et humanistes qui font les valeurs implicites de la médecine du don. Ces mondes divergents doivent se trouver, se rencontrer, éventuellement se confronter, afin d'éviter une schizophrénie du raisonnement et de la pensée qui nous guette et qui place les donneurs en otage de nos errances décisionnelles. En somme, en Suisse, ce n'est pas le donneur qui est rémunéré ; ce sont les services de transfusion, qui, par la vente du plasma, « corrompent » le sens du don, dans un souci de restreindre les coûts globaux. L'éthique commerciale domine l'éthique biomédicale. Les deux ont leur valeur et leur sens. Ceci dit, le message délivré aux différents partenaires de la chaîne transfusionnelle

est confus ; il nécessite urgemment un effort de clarification et d'information permettant aux donneurs de faire leur choix de manière éclairée.

Vers une psychothérapie de la médecine du don ?

Comme le dit si bien le philosophe allemand Andris Breitling « Il existe des actes de générosité qui échappent à toute logique économique ». Le don de sang est comme un acte d'amour, un geste altruiste vers son prochain. La générosité est une vertu sociale par laquelle on gagne son honneur. Et si, comme le pense Breitling « Le don du sang était un exemple de surabondance, voire de débordement de bonté ». Même si le don du sang est bénévole et gratuit, même si le donneur ne semble percevoir aucun bénéfice ou avantage matériel, il pourrait être malgré tout récompensé en se procurant une bonne conscience, et comme le dit Friedrich Nietzsche « Une bonne conscience permet un sommeil de qualité ». A la lecture de ces lignes, comment se positionner, quelle liberté prendre, comment reparler du sens du don ?

L'éthique doit nous conduire dans nos réflexions, elle n'est en aucun cas la solution. Revendiquer l'éthique est la placer dans un champ qui emprisonne la pensée. Utiliser les principes éthiques permet de penser la liberté, y compris dans les valeurs du don de sang. Ouvrons donc la porte de la psychanalyse de café du commerce : et si la « sexualité » - dans sa représentation symbolique - était une piste de réflexion nous renvoyant à – ou nous éclairant sur – nos convictions souvent dogmatiques touchant le don bénévole ou la rémunération du sang collecté ?

Eléments de réflexion :

- *La vie est donnée, transmise par l'acte sexuel.*
- *La vie est donnée, portée par le sang, transmise par le sang. La vie est offerte par l'acte de donner son sang.*

- *La mort est portée par le sexe – contaminé –.*
- *La mort est portée par le sang – contaminé –.*
- *Donner la vie, par le don du sang, est de fait une représentation symbolique de l'acte sexuel, porteur de vie et de mort.*
- *L'acte sexuel est acte d'amour : il est libre, il est partage.*
- *L'acte sexuel tarifé résulte d'une transaction commerciale ; il est considéré comme impur et est souvent tabou dans nos sociétés – prostitution –.*

Perspectives :

- *La vente du sang pourrait représenter, tout au moins sur le plan symbolique et dans l'inconscient collectif, un acte sexuel tarifé et de ce fait, être l'expression d'une relation impure, d'une relation porteuse d'interdit, d'une relation dénuée de la notion d'amour du prochain.*
- *Le don dirigé porte en lui une image incestueuse, est un symbole du mariage arrangé – forcé –, est une de perte de liberté.*
- *L'exclusion d'un donneur est l'expression cachée d'une impuissance sexuelle imposée au donneur, est une représentation allégorique de castration.*
- *La vente du plasma aux industriels, ou l'exportation du sang collecté « en trop » (ou désigné plus diplomatiquement comme « réserve nationale » en Suisse), raisonnent dans notre inconscient comme une sorte de proxénétisme, comme une parabole moderne et détestable de la traite des blanches.*

Sexe et sang ; un binôme indissociable comme la vie et la mort. L'argent comme vecteur de contrôle, de puissance. La transfusion sanguine est au carrefour des valeurs humaines. Ce sont ces valeurs qui nous confrontent dans nos réalités et rendent la réflexion si complexe.

Quoiqu'il en soit, ne mettons pas l'éthique du don bénévole en otage d'une psychologie bon marché, par un examen, soit trop superficiel, soit inversement par une analyse exagérément poussée des données et des symboles de notre réalité de médecins impliqués dans la chaîne transfusionnelle. Les organisations transfusionnelles doivent expliquer, clarifier, définir des positions explicites, afin que les donneurs puissent décider librement, en conscience. Expliquons, clarifions, communiquons ; soyons vecteurs de la liberté du don ! Nous devons dépasser notre vision habituelle et simplificatrice des choses.

Ceci dit et pour paraphraser Breitling « **Les donneurs ne devraient pas trop s'attarder sur les symboles mais simplement faire ce qui est juste. Donner son sang est vraiment facile. Il est sans doute très intéressant de penser aux implications éthiques et philosophiques d'un tel geste mais, finalement, on devrait simplement le faire** ». Et nous, dans nos organismes de transfusion, soyons honnêtes, transparents et au service de cette chaîne d'humanisme qui lie le donneur et le receveur.

Conclusions

Ces quelques paragraphes ont été l'opportunité de pouvoir partager des préoccupations et des réflexions de personnes travaillant dans le domaine de la transfusion sanguine. Ces visions sont biaisées, volontairement peut-être. Des éclairages arbitraires ont été choisis. La sécurité transfusionnelle, le rationnement des ressources, l'adéquation avec les coûts de la santé et une vie humaine sauvée renvoient directement à des choix existentiels de société, et comme on peut le voir, ces questions dépassent largement le cadre de la transfusion.

Pour le moment, en Suisse et malgré les perversions énoncées plus haut, le système de don non rémunéré perdure grâce à de nombreuses personnes qui participent à la solidarité. En tant que trait d'union entre les donneurs et les receveurs, **les services de transfusion ont un**

engagement tacite et une responsabilité afin de fournir des produits sanguins sûrs et qu'il en soit fait bon usage. Il ne faut pas condamner d'autres approches, il faut les comprendre, les placer dans leur contexte et la réalité qui diffère si souvent d'un endroit à l'autre, d'une culture à l'autre.

8

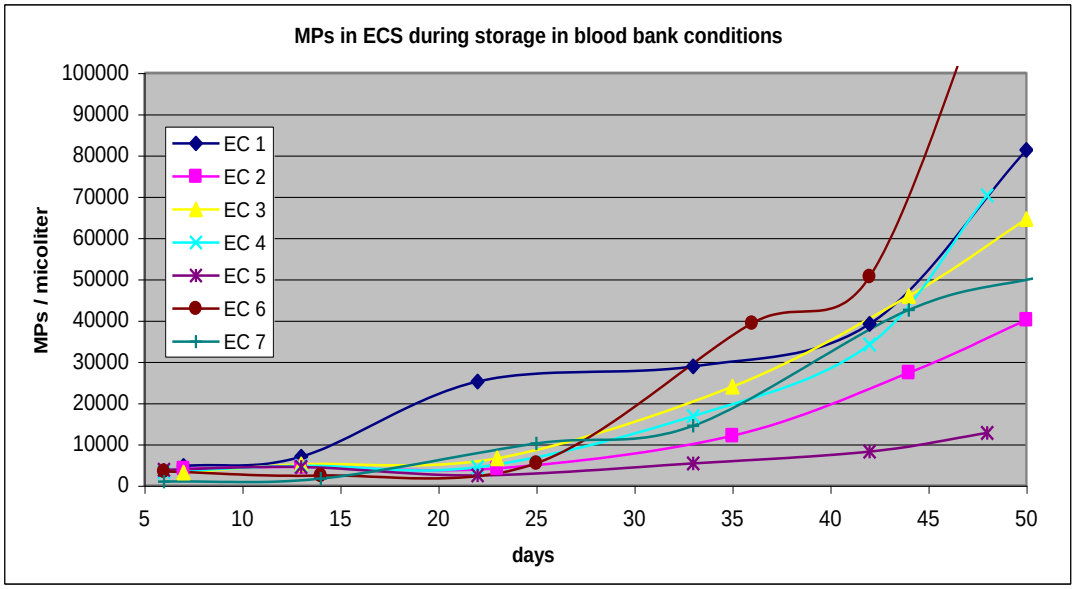
Main results, discussion and perspectives

Main results

Counting Methods

A counting method was developed using flow cytometry. The size of erythrocyte-derived microparticles (EMPs) and heaps of EMPs were defined using beads of 1 µm to set up the gate of EMPs. A precise number of fluorescent "trucount" beads allows obtaining a concentration of EMPs per µl. As it appears that EMPs tend to aggregate and form heaps, it is considered that this method is semi-quantitative. With double labeling and size it is possible to isolate MPs from background noise. In erythrocyte concentrates (EC) as well as in their supernatant, an increase in number of MPs according to storage time has been observed. Analysing directly in the EC, 3300 ± 1100 EMPs/ µl were counted after 5 days of storage. This number slowly increases until around 25 days of storage. After 35 days of storage, the increase is more pronounced and sharp and variability among samples goes wideer. After 42 days of storage corresponding to the usual shelf life of EC, the count reaches 35'400 ± 14'000 EMPs/ µl. This number almost double between 42 and 50 days of storage (Fig 1). This is most likely due to physicochemical changes that affect erythrocyte viability. As a result, the release of EMPs is amplified.

Figure 1: Count of microparticles in seven erythrocyte concentrates during storage



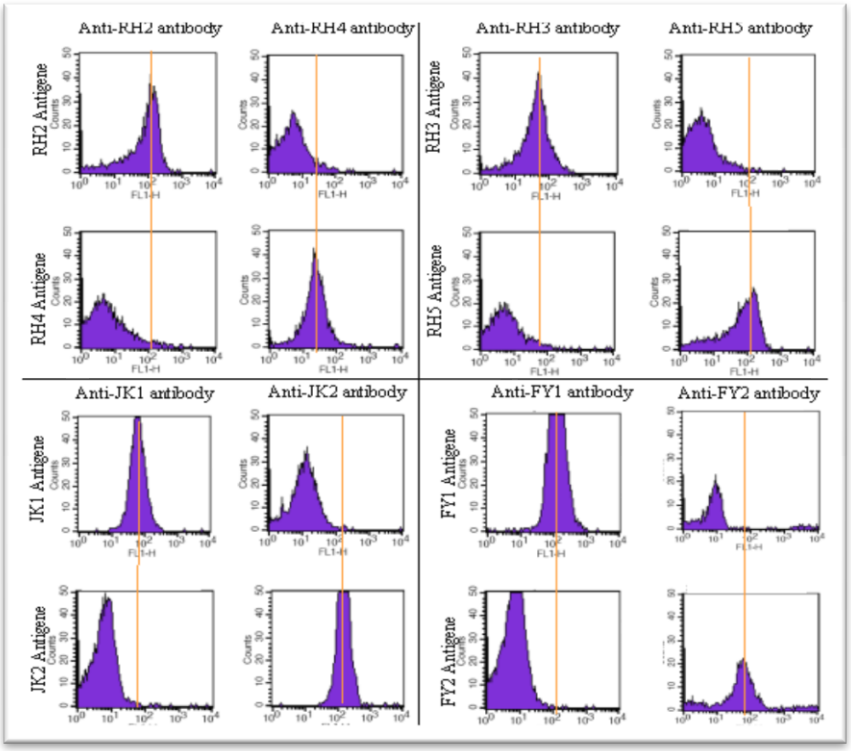
Surface Antigens

More than the half of RBC transmembrane proteins carries various group antigens. Those proteins play crucial role for the immune system and allow recognition of "self" antigens from "non-self" ones. Those antigens were shown to be present on EMPs too.

By proteomic study (1DE-LCMS/MS), Rh peptides were identified on EMPs. Rh group antigens also play a role in membrane stabilization suggesting that they may take part to the process of EMPs formation and release.

By flow cytometry, several blood group antigens were shown to be present on EMPs. Those groups of importance for transfusion are RH1, RH2, RH3, RH4, RH5, FY1, FY2, JK1, JK2 and KEL 1 (Fig 2).

Figure 2: Flow cytometry results showing the presence of various blood group antigens on EMPs.



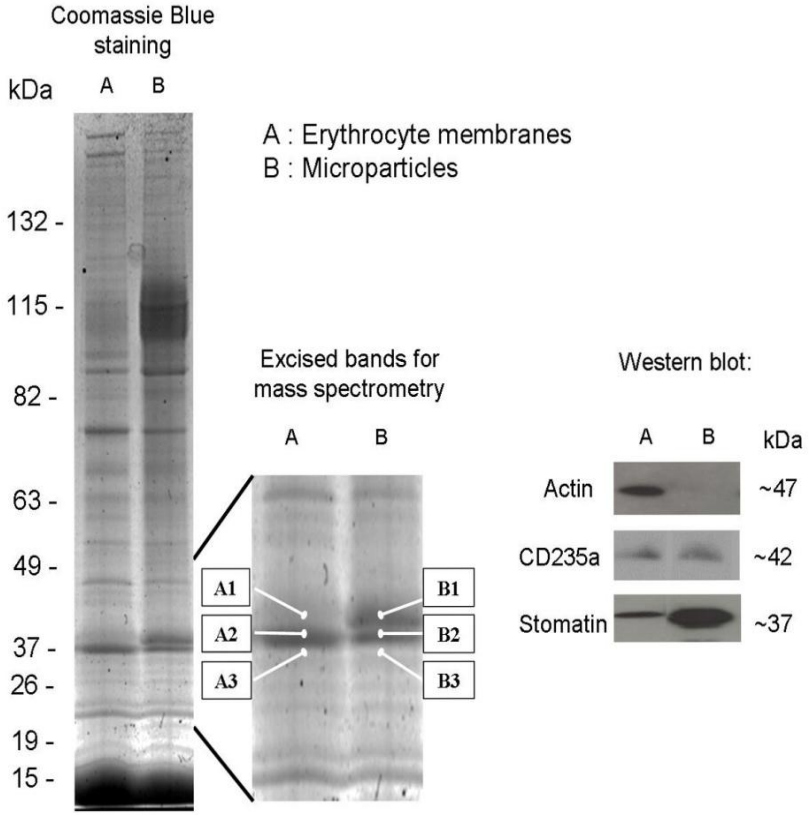
For each antigen, specific antibody was employed and as negative control an antibody against another antigen was employed. A line through the mean peak show labeled microparticles. In the histogram corresponding to the negative control the peak of microparticles appears on the left of the line, indicating non labeled events.

Erythrocyte-derived microparticle proteins

In order to elucidate the roles of EMPs, analysis of their protein content by one dimensional gel electrophoresis (1D-GE) was performed. EMPs were directly solubilized in 1D-GE loading solutions such as Laemmli, containing SDS and reducer. Proteins were then separated by 1D-GE. The protein bands of interest were cut, and further digested by trypsin and identified by MALDI-TOF/TOF. The clear-cut advantage of this technique, compared to gel-free approaches, is that to some extent 1D-GE is compatible with the analysis of hydrophobic proteins, due to the fact that EMPs contain more membrane proteins. Erythrocyte membrane and erythrocyte MP samples were first compared by 1D-GE (Fig 3). Both sample were obtained from a 42 days stored EC. The gel was stained with Coomasie blue for better compatibility with mass spectrometry. Slight differences of pattern were observed. A major difference was observed in the 25–35 kDa region; thus, in order to perform protein identification, bands of interest stained with Coomassie blue were excised and proteins were identified by MS (Fig 3). Western blot analysis allowed to thoroughly quantify the differences in specific protein content in both sample. Thus, by comparing erythrocyte membranes and EMPs, a clear reduction of actin and an accumulation of stomatin were observed on EMPs. The immunodetection of CD235a (glycophorin A) was similar in both gels (Fig3). Those results were well in line with previous report [86]. Stomatin is a membrane protein involved in regulation of monovalent cation transport through lipid membranes. Interestingly, stomatin (which has a structure similar to caveolin) is a major lipid-raft component of erythrocytes [87]. Precise reasons of stomatin enrichment in EMPs are not well known and are still subject of investigation, but may have a role in membrane microdomains modulation leading to membrane budding and EMPs release. The cell membrane plays a key role in the formation of EMPs. Indeed, following a stimulus, increase in intracellular calcium occurs and activates

proteases that cleave cytoskeleton proteins (actin and spectrin). Membrane is thus less rigid and can bud until formation of EMPs.

Figure 3: Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)of erythrocyte membranes and microparticles



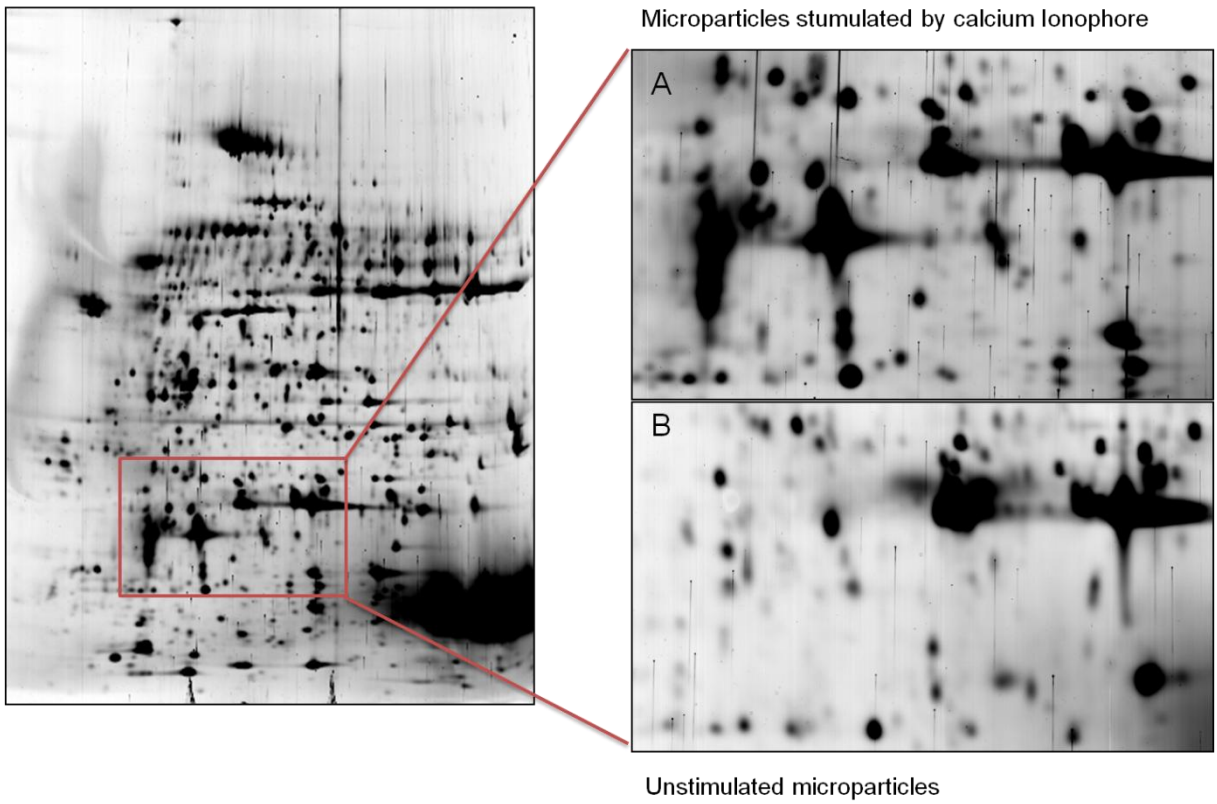
Both samples (A and B) were from a 42-day stored erythrocyte concentrate. The gel was stained with Coomassie blue (for better compatibility with MS). Inset shows enlargement of Coomassie blue-stained gel used for the preparation of the bands submitted to mass spectrometry analysis. Western blot analysis points out the variation of protein expression. Thus, by comparing erythrocyte membranes and microparticles, a clear reduction of actin and an accumulation of stomatin were observed on microparticles. The staining of CD235a (glycophorin A) was similar in both gels.

1D-GE provided another level of information that is complementary to the MS/MS identification of proteins based on a few peptides: the molecular weight of the identified protein can indeed be deduced from its position on the gel, which can sometime provide some indications about protein processing. For example, in a slice of EMPs separated by 1DGE, proteins such as Band 3 and RhD protein have a much heavier molecular weight than the corresponding gel band in which they were identified, indicating that some truncation has occurred.

Conversely, proteins, such as hemoglobin subunits have a lighter molecular weight than the corresponding gel band in which they were identified. It is consistent with the fact that hemoglobin tends to be crosslinked to cytoskeleton proteins, especially under stress conditions. Following the observation that EMPs proteins were not necessarily found at the right position on a 1D gel, and thus that some important protein processing occurred at the EMPs level, separation of the EMPs proteome was undertook by two-dimensional gel electrophoresis (2D-GE) so as to be able to analyze differentially processed proteins. Unfortunately, 2D-GE is poorly amenable to the analysis of membrane and hydrophobic proteins. Hence following the work about membrane proteins solubilization for 2D-GE by Rabilloud *et al*, EMP pellets were solubilized in urea, thiourea, DTE, pharmalyte and detergent (either CHAPS, ASB-14 or Brij 35). The most abundant protein of EMPs is hemoglobin as in RBCs. The presence of this highly abundant protein poses a dynamic range problem for proteomic analysis. Consequently, OFFGEL fractionator was used for hemoglobin depletion allowing resolving more sport on gel compared to unfractionned sample.

Among the numerous comparisons of EMP protein samples, one of the most relevant results was between samples of EMPs stimulated by calcium Ionophore and EMPs that are naturally released during ECs storage. Indeed, a certain number of proteins were over expressed in calcium Ionophore stimulated EMPs (Fig 4). Spots of interest were excised for subsequent protein identification by LC-MS/MS. All identified proteins were involved in calcium metabolism, such as for example Sorcin, Alpha-synuclein, Annexin V or Grancalcin. Those presented results are preliminary. Data analysis of those results and complementary experiments are still in process.

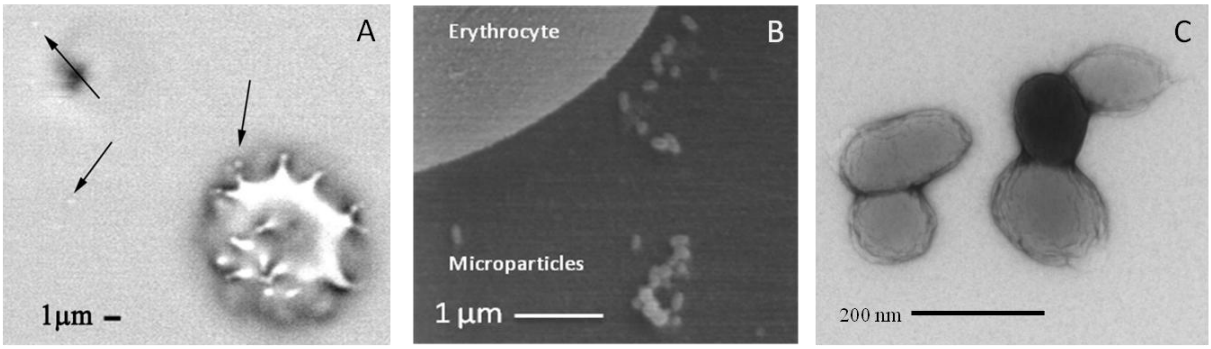
Figure 4: Comparison by 2 D-GE between two samples of EMPs, (A) stimulated by calcium ionophore and (B) unstiumated (”naturally” released during storage)



Microscopy

Observation of EMPs by microscopy gave information about their size and shape. By confocale differential interference microscopy, it is possible to observe forming EMPs from spicules of spheroechinocyte (Fig 5A). Electron microscopy allows observing that EMPs tends to aggregate and that they are homogeneous in size (Fig 5B and 5C).

Figure 5: Various picture of EMPs using different microscopy technique



(A) Confocal microscopy, magnification of 1500 x. EMPs formation from spicules. (B) Scanning electron microscopy, magnification of 4000 x, EMPs surrounding an erythrocyte (C) Transmission electron microscopy, magnification 37'000x. Membrane bilayer of EMPs is visible.

Coagulation

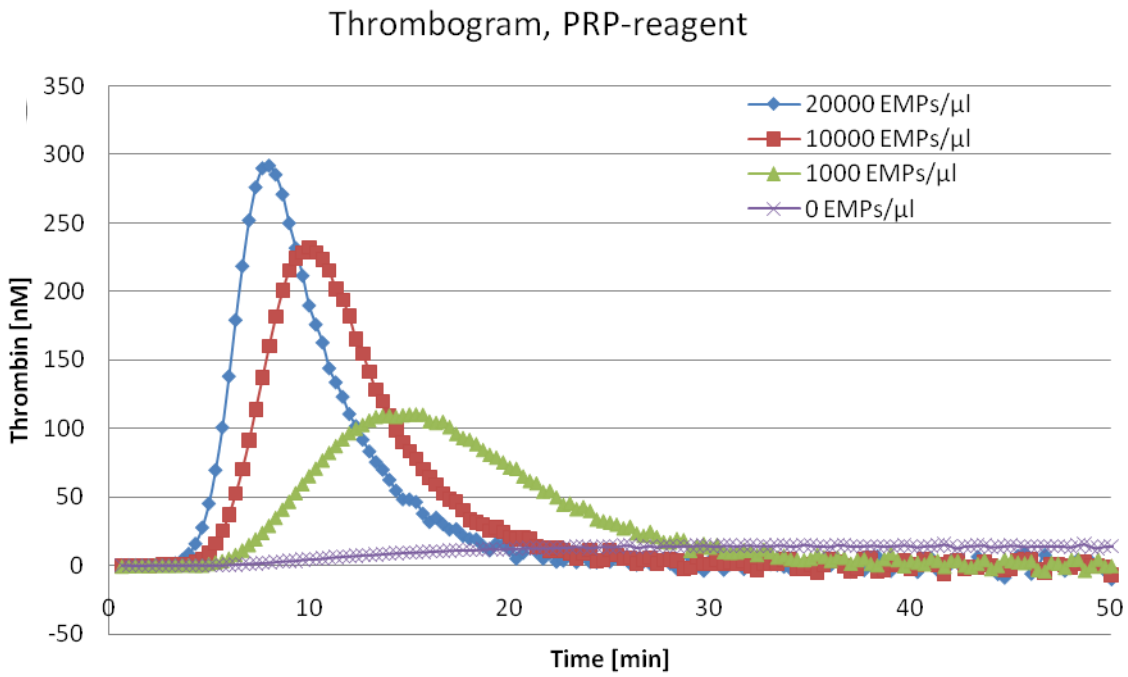
In order to verify whether EMPs play a role in transfusion medicine, it has been decided to examine if they could take part in coagulation process. As EMPs present negatively charged phospholipids on their membranes, they could indeed provide supplementary membranes required for the assembly of coagulation complexes such as tenase or prothrombinase.

Flow cytometry also confirmed that there is no more platelet and almost no more MPs in three times centrifuged plasma (data not shown).Plasma prepared for calibrated automated thrombogram (CAT) experiment always contained 1.6 µM corn trypsin inhibitor (CTI), an inhibitor of intrinsic coagulation pathway. In plasma sample without CTI, thrombin generation (TG) is sometimes observed, typically in one on three assays with a great variability in lag time. This is due to *in vitro* intrinsic coagulation pathway activation [77]. In plasma alone containing 1.6 µM CTI, no TG was observed.

Contribution of EMP phospholipids to support TG

Contribution of EMP phospholipids in TG was evaluated in a CAT, using PRP-reagent containing TF to trigger TG, but few phospholipids. EMPs at different concentrations (from 0 to 20’000 EMPs / µl) were added to pooled plasma to test if EMP membranes could amplify or generate thrombin in the assay. Results demonstrate that the more EMPs are added to plasma, the greater and faster was TG. In addition, there was no significant TG in plasma without EMPs (Fig 6).

Figure 6: Effect of negatively charged membranes of EMPs on TG



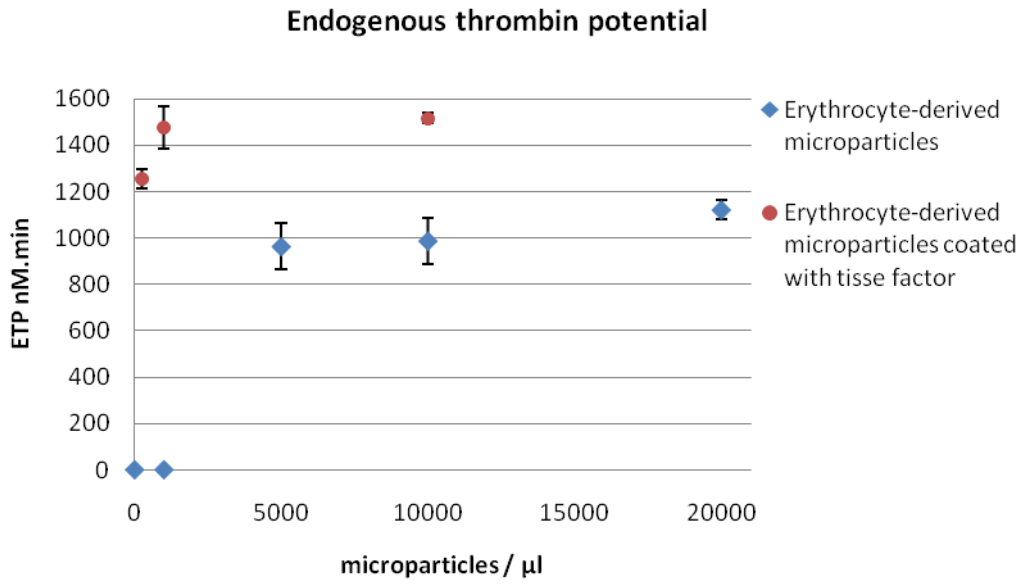
Measurement of the procoagulant activity of EMPs using thrombin generation assays (Test with PRP reagent, TF is added to trigger reaction). In the presented thrombogram, thrombin generation curves are the mean of five independent measures (N=5).
TG was observed when EMPs where added to plasma at a final concentration of 1000 EMPs/µl, 10'000 EMPs/µl and 20'000 EMPs/µl. The more there are EMPs, the stronger and faster is the TG. In the absence of EMPs in plasma, no significant TG was observed, 0 EMPs/µl.

Contribution of EMPs to trigger TG

In another assay with MP-reagent, it has been tested if EMPs not only support coagulation exposing their negative phospholipids, but also launch coagulation. MP-reagent do not contain any TF, no exogenous is added. TG was observed when from 5000 EMPs/µl to 20'000 EMPs/µl were added to plasma (Fig 7). It is of importance to note that in plasma with 1000 EMPs/µl, no TG was observed (Fig 7). Thus, without addition of exogenous TF, there is TG in plasma with more than 1000 EMPs/µl EMPs. As positive control, EMPs-coated with TF (EMPs-TF) were used in the assay. TG with EMPs-TF was always stronger comparing to EMPs. Additionally, the amount of EMPs-TF needed to generate thrombin is logically much

smaller than with only EMPs. Indeed, while TF leads TG in EMPs-TF sample, another mechanism is involved to trigger TG with EMPs in plasma.

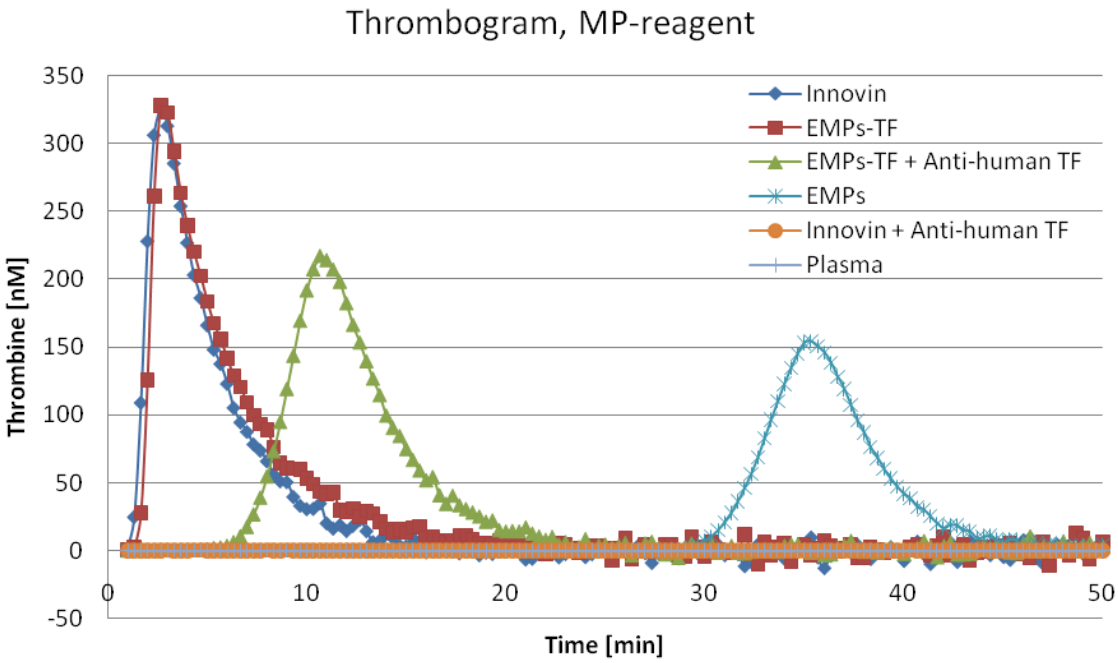
Figure 7: Endogenous Thrombin Potential of EMPs and EMPs-TF at various concentrations.



ETP (nM.min) measurements of plasma samples containing 1.6 µM of corn trypsin inhibitor with the addition of 0 to 20'000 EMPs/µl or 0 to 10'000 EMPs-TF/µl in thrombin generation assays with MP-reagent. Each dots represent the mean of five independent measures ± SD, N=5.

As positive control, plasma samples with thromboplastin, here Innovin (Siemens Healthcare Diagnostics, Marburg, Germany) or with EMPs-TF were tested. For both, rapid TG with a high ETP was observed (Fig 8). Efficiency of anti-human TF was tested in plasma samples containing Innovin or EMPs-TF. When Anti-human TF antibody was added to those samples, a partial inhibition of TG was observed in the sample with EMPs-TF whereas total inhibition was observed in the sample with Innovin (Fig 8). These results also show that EMPs-TF are strong activator of TG and have a "thromboplastin-like effect".

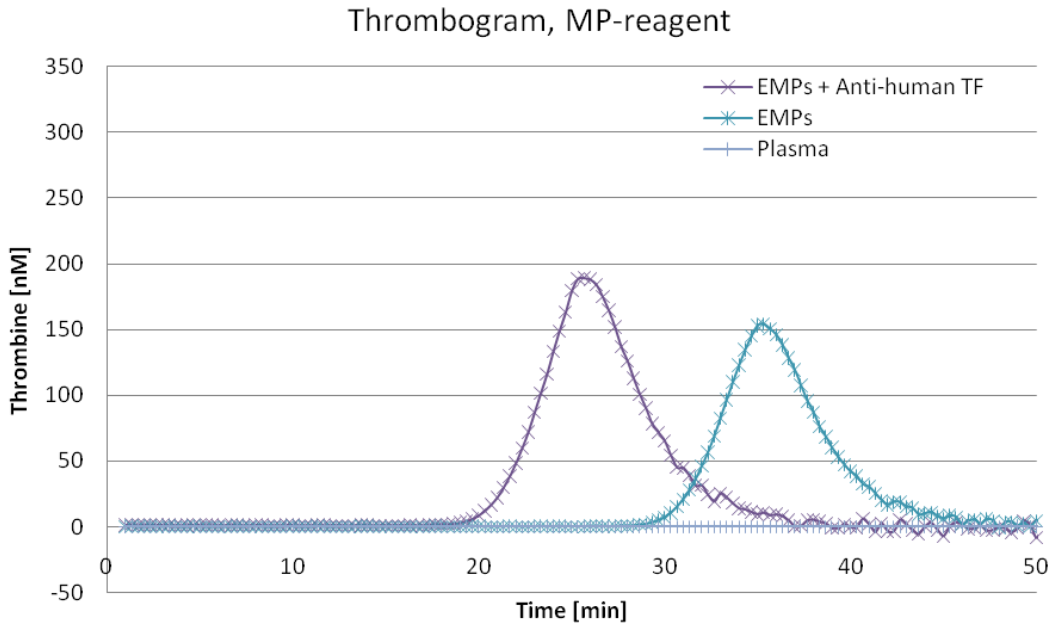
Figure 8: Contribution of EMPs to trigger TG



Measurement of the procoagulant activity of various samples using thrombin generation assays (Test with MP-reagent, no addition of TF). In the presented thrombogram, thrombin generation curves are the mean of five independent measures (N=5). No TG was observed in plasma alone. EMPs or EMPs-TF are added to plasma at a final concentration of 10'000 per microliter. Plasma with EMPs-TF or Innovin in the absence of EMPs are strong activator of thrombin generation in the test. Thrombin generation was also observed in plasma sample with EMPs A partial inhibition of thrombin generation was observed when anti-TF is added to EMPs-TF sample, whereas inhibition was complete when anti-TF was added to plasma with Innovin in the absence of EMPs.

Intrinsic coagulation pathway is inhibited by CTI, anti-human TF was employed to inhibit putative plasma TF or EMPs bearing TF even if no TF was evidenced in those samples. Additionally, no TF was identified in an extensive proteome study of EMPs [3]. In this way, both coagulation pathway triggers are inhibited. Interestingly, TG was not inhibited when EMPs were added to plasma containing anti-human TF at a concentration of 1:10 (Fig 9).

Figure 9 : Contribution of EMPs to trigger TG



Measurement of the procoagulant activity EMPs in plasma, with and without the addition of anti-human TF. (Test with MP-reagent, no addition of TF). In the presented thrombogram, thrombin generation curves are the mean of five independent measures (N=5). Final concentration of EMPs is 10’000 per microliter. No TG was observed in plasma alone. In both sample, TG was observed. Surprisingly, the generation was stronger and the lag time shorter in the presence of anti-human TF.

EMPs were not only capable to support coagulation providing negatively charged phospholipids but also are capable to trigger thrombin generation in the assays. In summary, we provide evidence that EMPs in plasma are able to induce generation of thrombin, and that this generation is not abolished using anti-TF. Thrombin generation is enhanced by adding EMPs previously incubated with TF, with an activity similar to that observed with thromboplastin.

Discussion and perspectives

The increasing interest in MPs lies in the fact that, as they circulate in blood flow, they could constitute hallmarks of cellular activation or damage. Consequently, there are always more studies on MPs, mainly dealing with platelets or white blood cells derived MPs, erythrocyte derived MPs remaining less studied. Therefore new efficient methods have to be developed with the aim to obtain qualitative or/and quantitative data on MPs.

The present thesis gave an extensive work to characterize those EMPs using various approaches, and demonstrate that those bioactive EMPs not only accumulate in red cells concentrate during storage, but they also could be implicated in coagulation processes in an active way. EMPs also bear blood group antigens on their surfaces. However, it is not known whether they are able to elicit an immune response. In addition, EMP protein contents change according to the stimulus, suggesting that there are potentially different kinds of EMPs with different function. Finally, microscopy gave information about formation and shapes of EMPs.

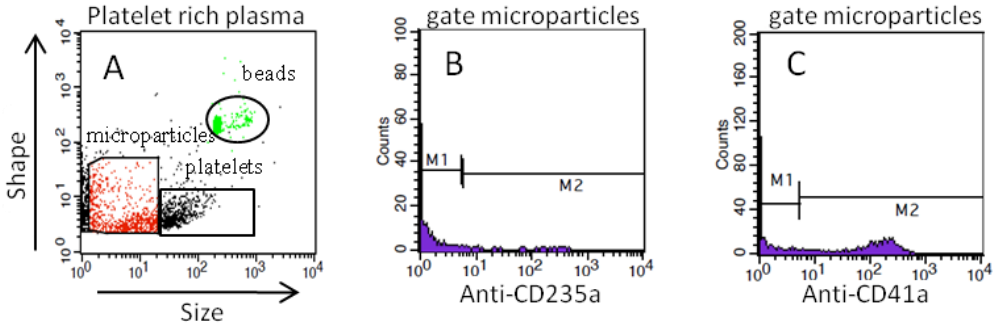
Standardization methods

In this work, we provide a semi-quantitative counting method of EMPs using flow cytometry. It is important to note that there is no standardized method to analyze or to count MPs [61]. For that reason, comparison among different studies is particularly difficult. Yet, since 2005, the vascular biology group within the Scientific Subcommittees of the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) has proposed to create an international workshop by using calibrated microbeads to clarify confusing data regarding MPs numeration, and the first step will be to establish a normal range for MPs. This comity also proposed to standardize analysis methods of MPs lipids or protein. To our knowledge, until now no consensus has been established to provide a standardized method of to count MPs

Counting Methods

Our developed counting method is also adapted to quantify MPs from various origins, using corresponding antibody. For example, in platelet rich plasma (PRP), we observed typically around 2000 platelet- derived MPs /µl and 100 EMPs /µl (Fig 10). Those results correspond to those founded in scientific literature [15, 83].

Figure 10 : Flow cytometry analysis of platelet riche plasma



A.General flow cytometry windows of a platelet rich plasma sample. Events are sorted according to their size and shape. Three different regions are delimited namely microparticles, platelets and counting beads. B. Events of MPs region according to their fluorescence with an anti-human erythrocyte antibody. M2 area shows the number of positively labeled MPs. C. Event of MPs region according to their fluorescence with an anti-human platelet antibody. M2 area shows the number of positively labeled MPs.

Considering that MPs in PRP reflects the number of MPs in blood flow, it could be of great interest to count MPs in plasma of patient suffering of various diseases comparing to a healthy individual baseline. Another perspective would be to count EMPs from patients (in cardiac surgery) before and after surgery/transfusion in order to observe whether an increase in number of EMPs is apparent or not. It would be also very interesting to verify if the number of EMPs or MPs rise in blood passed through a "cell Saver" machine. Those proposed experiments could help to clarify the process of formation and the role of EMPs in transfusion medicine.

Proteins

Interesting results were obtained comparing protein profile of erythrocyte membranes and EMPs. As 1 D-GE profiles were slightly different, it indicated some proteins are enriched in a sample comparing to another. Western blot confirmed those result, demonstrating that protein sorting occurs in process of EMPs formation and release.

2 D-GE analyses was proceeded to compare various storage conditions or different samples of EMPs. As shown in figure 5, it has been observed that some proteins were overexpressed in EMPs stimulated by calcium ionophore comparing to EMPs naturally released during storage. Subsequent mass spectrometry analysis identified overexpressed proteins involved in calcium metabolism.

This observation is of particular importance, it indicates that protein sorting occurs during the formation and release process of EMPs and thus that they are potentially different kinds of EMPs. According to the stimulus, those EMPs might be involved in different biological processes.

Coagulation

ETP reflects a measure of global coagulation in a sample. It has been demonstrated that EMPs could support coagulation, and trigger thrombin generation in plasma when they reach a concentration between 1000 and 5000 EMPs/µl, in the absence of triggers of coagulation. So, as FXIIa or TF are not present or inhibited, it could be inferred that EMPs have the capacity to trigger TG by a mechanism that appears independent of both intrinsic and extrinsic coagulation pathways.

It might be suggested that the "microparticles coagulation pathway" proposed by Furie et al trigger TG [52]. Another hypothesis would be that EMPs carry a cryptic form of TF or that EMPs are able to activate cryptic TF in plasma [57, 81]. As time preceding the thrombin burst or lag-time variations are usually relatively wide when testing intrinsic coagulation pathway *in vitro* [77]. Therefore it could by hypothesized that EMPs might support or imitate intrinsic coagulation pathway. It has been demonstrated that factor XI could be activate by thrombin in a factor XII independent manner. Thus, EMPs could generate subnanomolar amount of thrombin sufficient to activate factor XI resulting in the observed thrombin explosion in CAT [77, 82] (This process is also called "Josso loop" in coagulation). In future experiment, the use of a factor XI inhibitor would be interesting to determine if it is involved in TG with EMPs.

An unexplained observation have been done comparing sample of EMPs with and without TF inhibitor. Expected results were either no significant difference or (partial) inhibition of TG in the case of putative presence of TF on EMPs. Curiously, it has been observed that in sample with TF inhibitor, lag time was reduced and ETP was higher (Fig 9). Even if TF inhibitor does not fully inhibit TF, it has been demonstrated that this last was very efficient to inhibit TF of thromboplastin and quite efficient on EMPs bearing TF. It might be also supposed that this

observation is due to an *in vitro* artefact (but reproducible). In order to understand this unexpected result, it might be of great interest to test plasma and EMPs sample with FVIIa inhibitors instead TF inhibitor, however this last is not commercially available.

Numerous factors are involved in hemostasis, all having multiple roles in this carefully regulated process. Coagulation pathways are thus interdependent. In this field, EMPs are also a player whose role is not yet clearly define. Due to the fact that in case of injury, thrombin is also linked to inflammation [88], the TG observed by EMPs might support inflammation instead of coagulation as observed, this relevant hypothesis has also to be taken into account.

Final remarks

This thesis provides evidences that storage of RBCs is associated with the generation of EMPs. Those EMPs bear blood group antigen and are different according to the stimulus. In addition, coagulation assays demonstrate that EMPs support and could trigger TG under certain circumstances. This supports the hypothesis that among MPs, EMPs are also bioactive. Even if no clinical study on the involvement of EMPs in coagulation has been conducted yet, their presence in ECs is totally innocuous. As numerous studies suggest that there is a link between transfusion complication and storage time of ECs, we suggest that EMPs could be mediators of those complications under certain circumstances.

The role and implication of EMPs in biological processes are far from being entirely understood. There are numerous scopes on EMPs research, as mentioned. Therefore the need of investigations in various fields has to be done on MPs and specifically EMPs in order to allow increasing global knowledge on blood and blood products.

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Curriculum Vitae

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Formations

• **Doctorat ès sciences de la vie** 2011

Université de Lausanne, faculté de Biologie et de Médecine, unité de recherche et développement du service régional vaudois de transfusion sanguine, sous la supervision du Dr N. Lion et du Prof. J.-D. Tissot

• **Master en protéomique et bioinformatique** 2007

Université de Genève, faculté des Sciences et de Médecine

• **Bachelor en biologie** 2005

Université de Genève, faculté des Sciences

• **Maturité fédérale langues modernes** (anglais, allemand, italien) 2000

Collège Sismondi, Genève

Expériences professionnelles

• **Doctorant ès sciences de la vie** 2007-2011

Service régional vaudois de transfusion sanguine, Lausanne

- A l'unité de recherche et développement :
Gestion du projet de recherche sur les microparticules d'érythrocytes et leurs implications en médecine transfusionnelle. Ces expériences ont pour de mieux comprendre les processus de vieillissement des produits sanguins et à terme d'en améliorer la qualité si possible.

- Au laboratoire de contrôle qualité et cellules souches (à temps partiel) :
Contrôle qualité des produits sanguins, cryopréservation des cellules souches hématopoïétiques, de la congélation à l'infusion, mise en culture de produits sanguins lors de réactions transfusionnelles.

• **Stage de master** 2006-2007

Laboratoire de recherche et développement du service régional vaudois de transfusion sanguine à Lausanne, sous la supervision du Prof. J.-D. Tissot

• **Aide d'éducateurs spécialisés** 2002-2006

Centre de rééducation et d'enseignement de la Roseraie (Institution accueillant des jeunes de 4 à 18 ans souffrant d'un handicap moteur et troubles associés), Genève

• **Nombreux remplacements à l'école primaire et secondaire** 2002-2006

Département de l'instruction publique, Genève

Compétences

• Techniques

Electrophorèse sur gel 1 et 2 dimensions, western blot, dosage de protéines, chromatographie liquide, ELISA, cytométrie de flux, microscopie électronique, bonne connaissances en spectrométrie de mass, diverses techniques d'hémostase comme le coagulomètre ou le Calibrated Automated Thrombogram, cryoprésevation de cellules souches hématopoïétiques.

• Générales

Gestion d'un projet de recherche.

Très bonne connaissance du fonctionnement d'un service de transfusion : collecte, production, contrôles qualités et stockage des produits sanguins. Connaissances générales en hématologie et hémostase.

Participation à de nombreuses conférences et séminaires, présentations orales et de posters dans les domaines de la transfusion, de l'hématologie et de la protéomique. Intérêt pour l'éthique biomédicale.

• Personnelles

Persévérant, ambitieux, curieux, ouvert, créatif et jovial.

Langues

Français : Langue maternelle
Anglais : Bon niveau (écrit et parlé, cours de master suivis en anglais)
Allemand : Bonnes connaissances orales
Italien : Bonnes connaissances orales
Hébreu : Bonnes connaissances orales

Informatique

Systèmes d'exploitation : Windows, Linux
Applications : MS office & Open office
Utilisation de divers programmes de bioinformatique

Centres d'intérêts

Vélo, ski, voyages

Publications

Thrombin generation by microparticles isolated from stored red cells
Rubin O; Delobel J; Prudent M; Lion N; Tissot J-D; Angelillo Sherrer A — Submitted

Le don de sang : entre enjeux éthiques et financiers
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Plasma/serum proteomics: pre-analytical issues
Barelli Stefano; Crettaz David; Thadikkaran Lynne; **Rubin Olivier;** et al.
EXPERT REVIEW OF PROTEOMICS — JUN 2007

Conférences

- Swiss proteomics society congress 2007, Lausanne (CH), 08.2007
  Présentation de poster

- Journée de la recherche du CHUV Regenerative Medicine, Lausanne (CH), 17.01.2008
  Présentation de poster

- D. Day, *Université de Lausanne,* 14.02.2008,
  Présentation de poster

- Journée éducationnelle organisée par l'Association suisse de médecine transfusionnelle et les ETS Rhône-Alpes et Bourgogne Franche-Comté, Evian (FR), 14.03.2008
  Présentation orale

- D. Day, *Université de Lausanne,* 05.02.2009
  Présentation de poster

- 1ère journée scientifique de la plateforme CLIPP "Du nanomonde au patient", Besançon (FR), 26.05.2009
  Présentation orale

- Blood and proteomics in Viterbo, Viterbo (IT), 12-14.10.2009
  Présentation de poster

- Swisstransfusion joint congress, Interlaken (CH), 09.2010
  Présentation de poster et orale

- Swiss Proteomics Society PhD students' symposium, Basel (CH),12.2010
  Présentation orale