Detection of microparticles from human red blood cells by multiparametric flow cytometry

Giulia Grisendi[1], Elena Finetti[2], Daniele Manganaro[3], Nicoletta Cordova[4], Giuliano Montagnani[4], Carlotta Spano[1], Malvina Prapa[1], Valentina Guarneri[5], Satoru Otsuru[6], Edwin M. Horwitz[6,7], Giorgio Mari[2], Massimo Dominici[1]

[1]Division of Oncology, Department of Medical and Surgical Sciences for Children & Adults, University-Hospital of Modena and Reggio Emilia, Modena, Italy; [2]R&D Filter Department, Fresenius HemoCare Italy, Cavezzo; [3]BD Biosciences Italy S.p.A, Buccinasco; [4]Division of Transfusion Medicine, University Hospital of Modena, Modena, Italy; [5]Department of Surgery, Oncology and Gastroenterology University of Padova, Istituto Oncologico Veneto IRCCS, Padua, Italy; [6]The Research Institute, [7]Division of Hematology/Oncology/BMT, Nationwide Children's Hospital, Columbus, Ohio, United States of America

Background. During storage, red blood cells (RBC) undergo chemical and biochemical changes referred to as "storage lesions". These events determine the loss of RBC integrity, resulting in lysis and release of microparticles. There is growing evidence of the clinical importance of microparticles and their role in blood transfusion-related side effects and pathogen transmission. Flow cytometry is currently one of the most common techniques used to quantify and characterise microparticles. Here we propose multiparametric staining to monitor and quantify the dynamic release of microparticles by stored human RBC.

Material and methods. RBC units (n=10) were stored under blood bank conditions for up to 42 days. Samples were tested at different time points to detect microparticles and determine the haemolysis rate (HR%). Microparticles were identified by flow cytometry combining carboxyfluorescein diacetate succinimidyl ester (CFSE) dye, annexin V and anti-glycophorin A antibody.

Results. We demonstrated that CFSE can be successfully used to label closed vesicles with an intact membrane. The combination of CFSE and glycophorin A antibody was effective for monitoring and quantifying the dynamic release of microparticles from RBC during storage. Double staining with CFSE/glycophorin A was a more precise approach, increasing vesicle detection up to 4.7-fold *vs* the use of glycophorin A/annexin V alone. Moreover, at all the time points tested, we found a robust correlation (R=0.625; p=0.0001) between HR% and number of microparticles detected.

Discussion. Multiparametric staining, based on a combination of CFSE, glycophorin A antibody and annexin V, was able to detect, characterise and monitor the release of microparticles from RBC units during storage, providing a sensitive approach to labelling and identifying microparticles for transfusion medicine and, more broadly, for cell-based therapies.

Keywords: microparticles, CFSE, red blood cells, flow cytometry.

Introduction

Blood transfusion from healthy donors is a very widely used medical therapy. It has been estimated that about 40% of critically ill adult patients receive at least one unit of packed red blood cells (RBC) while in an intensive care unit, with a mean of five units per patient[1,2]. Although RBC transfusion can be considered a life-saving therapy, this procedure is not without risk. In the last 10 years a growing body of evidence has indicated that RBC transfusion can be associated with adverse clinical outcomes including infections, multiple organ failure and death, particularly in compromised patients[3,4]. Recently, it was suggested that a large part of all these side effects could be associated with prolonged storage of the RBC prior to their transfusion[5,6].

Under standard blood banking conditions, RBC undergo progressive structural and biochemical changes commonly referred to as "storage lesions"[7]. This process includes alterations in pH, lactate, nitric oxide and ATP levels, as well as loss of biconcave shape and RBC lysis (haemolysis)[8]. Moreover, attention has been given to the spontaneous release of microparticles, a physiological step of erythrocyte senescence that may be enhanced by various stimuli occurring during storage[9,10]. Microparticles are small phospholipid vesicles of less than 1 μm in size, containing a subset of proteins derived from their parent cells[11,12]. These cellular elements have long been considered as cell debris with no biological functions[13]. Recently, however, several

studies have demonstrated that microparticles play roles in intercellular communication and are involved in a broad spectrum of pathological conditions such as thrombosis, cardiovascular disease, diabetes and infections[14-20]. It has also been reported that the number of microparticles in RBC units gradually increases with storage time, suggesting that these microparticles might contribute to adverse transfusion reactions. It is, therefore, important to establish novel approaches to careful monitoring and precise quantification of the release of microparticles in RBC units[9,21,22].

Although flow cytometry procedures have been reported, currently there is no standard method to analyse microparticles in blood samples[23-25]. It has been established that RBC microparticles can be identified by flow cytometry thanks to their expression of glycophorin A, a protein uniquely expressed by erythrocytes, and phosphatidylserine[26-28]. Phosphatidylserine is normally exposed on the inner leaflet of the cell membrane; however, during apoptosis it is exposed on the outer side of the membrane, becoming detectable by annexin V binding[29]. Although widely used, this approach may generate some errors because of staining of cellular fragments and debris, in addition to microparticles. Moreover it has been recently demonstrated that not all microparticles express phosphatidylserine[22,26,28].

In this study, we investigated the dynamic release of microparticles by stored RBC using a new multiparametric approach based on carboxyfluorescein succinimidyl ester (CFSE) dye in combination with anti-glycophorin A antibody and annexin V. We demonstrate that the inclusion of CFSE dye in combination with anti-glycophorin A antibody and annexin V enables clear identification of closed vesicles, providing an effective strategy for visualising and quantifying microparticles in biological samples.

Materials and methods

Red blood cell preparation

Ten randomly selected RBC units were supplied by the Division of Transfusion Medicine at the University Hospital of Modena upon approved agreement n. 0030164/0003928. Only units that did not satisfy quality criteria for transfusion, because of elevated liver enzymes or leucocyte number, were used for this study. Whole blood (450 mL±10%) was collected from healthy volunteer donors into blood bags (Fresenius Kabi, Zverinek, Czech Republic) containing CPD anticoagulant (63 mL). After centrifugation in a Sorvall™ RC12BP (Thermo Scientific, Waltham, MA, USA) centrifuge at 4544g for 10 minutes at room temperature, plasma and buffy coat were separated and the RBC were suspended in SAG-M additive solution (100 mL) and leucodepleted by filtration. RBC units were stored under standard blood bank conditions (4±2 °C) up to day 42. Samples were collected by a sterile sampling device on days 0, 10, 20, 30 and 42 and were tested for the presence of microparticles and underwent standard RBC quality controls. RBC haematocrit and total haemoglobin were measured using a CeLL-DYN Ruby haematology analyser (Abbot Diagnostics, Illinois, IL, USA). Extracellular haemoglobin concentration was determined by a HemoCue Plasma/Low Hb System (HemoCue AB, Ängelholm, Sweden) after sample centrifugation at 3,000 rpm for 5 minutes. The percentage of haemolysis was calculated using the following formula:

$$\text{Haemolysis rate \%} = \frac{fHb_{mg/dL} \times (1\text{-}Hct/100)}{(Hb_{g/dL} \times 1{,}000) \times 100}$$ [30]

Flow cytometry

Ten microlitres of RBC concentrate were stained for 30 minutes on ice in 100 µL binding buffer (BD Pharmingen, San Diego, CA, USA) containing 5 µL phycoerythrin anti-human glycophorin A (CD235a) antibody (BD Pharmingen), 2 µL CFSE (Molecular Probe, Eugene, OR, USA) and 10 µL annexin V-allophycocyanin (BD Pharmingen). The optimal concentration was experimentally determined for each antibody or dye by titration experiments. After staining, samples were diluted with binding buffer to reach a final volume of 1 mL. Using a reverse pipetting technique, 50 µL of diluted stained sample were pipetted into a TruCount tube (BD Biosciences,San Jose, CA, USA) to which 300 µL of binding buffer were then added. TruCount tubes, containing a standardised number of fluorescent beads, were used to quantify microparticles. The microparticles were detected by a FACSAria III flow cytometer (BD Biosciences) equipped with two air-cooled lasers at 488 and 633 nm wavelengths. An unstained sample was acquired to detect the sample auto-fluorescence and set the photomultiplier for all the considered channels; fluorescent cross-talk was controlled by compensation adjustment. Compensation settings were established by acquiring single-colour stained tubes. Collected data were analysed by Diva software (BD Biosciences). Microparticles were identified by relative size; forward and side scatter channels (FSC and SSC) were used on a logarithmic scale. The setup of the FSC and SSC photomultiplier was calculated using background noise as the lower limit. The FSC photomultiplier was increased until the background noise filled the dots available of dot plots. The threshold, set on the FSC channel, was increased to reduce the noise progressively, in order to detect microparticles. To provide a reference size, Megamix beads (BioCytex, Marseille, France) were

acquired with the same settings used for microparticles. Sample acquisition was discontinued when the number of TruCount beads in the relative region reached 5,000 events. The number of microparticles per microlitre was calculated as follow:

$$\frac{\text{N. of events in gating containing microparticles}}{\text{N. of events in absolute count bead region}} \times \frac{\text{N. of bead counts per test}}{\text{Sample volume}(\mu L)} \times \text{Dilution factor}$$

Statistical analyses

Data are given as mean values and represented by box plots. The data were subjected to one-way analysis of variance (ANOVA) where ANOVA assumptions were not violated (residual diagnostics). In other cases a robust algorithm for variance stabilisation (Box-Cox power transformation) was applied and ANOVA was performed on transformed data. Statistical computations were carried out using Minitab®16. Two-tailed *t*-tests and Pearson's tests were used when applicable. A p-value<0.05 was considered statistically significant.

Results

Microparticles from red blood cells can be efficiently labelled by CFSE

We here targeted a common RBC marker by anti-human glycophorin A antibody along with annexin V in combination with CFSE, a fluorescent dye never used before for the detection of RBC microparticles (Figure 1). CFSE has been used for years in assessing the proliferation of nucleated cells and to specifically label RBC with no evidence of toxicity[31-33]. Given its chemical properties we hypothesised that CFSE could definitively distinguish microparticles with intact membrane structures from cellular debris. In particular, we wanted to take advantage of the fact that CFSE passively diffuses into microparticles and once within the microparticles can be hydrolysed by intracellular esterase, becoming fluorescent and easily distinguishing the microparticles from cellular fragments[34].

As shown in Figure 1, the population of microparticles was first identified by morphological gating according to their light scattering profile (Figure 1A). The identified vesicle population was then analysed for CFSE fluorescence (Figure 1B). Given the small size of microparticles and their reduced cytoplasmic content, the intensity of the CFSE signal released by microparticles was one logarithm lower than that observed for the RBC, allowing the microparticles to be clearly distinguished from their parental cells (Figure 1B). Subsequently, only CFSE-positive microparticles were investigated for glycophorin A expression in order to consider microparticles that had originated from erythrocytes (Figure 1C). In addition, a simultaneous comparative analysis of annexin V-positive elements in the CFSE/glycophorin A-positive population revealed that only a small fraction of microparticles was labelled by annexin V (Figure 1D). Collectively this multiparametric approach confirmed that the majority of RBC microparticles resulted negative for annexin V and revealed that CFSE can efficiently and selectively label microparticles, indicating that closed, intact vesicles containing cytoplasmic enzymes are able to metabolise CFSE.

CFSE/glycophorin A staining allows proper quantification of red blood cell-derived microparticles

Having established the protocol and the gating strategy, we then wanted to test them by quantifying microparticles in stored RBC. The microparticle measurements were initially performed considering all events included in the morphological gate according to a well-defined light scatter profile, illustrated in Figure 1A. The mean number of microparticles detected from day 0 (T0) to day 42 (T42) progressively increased from $1.17\times10^5\pm0.067\times10^5$ to $2.58\times10^5\pm0.34\times10^5$ (Figure 2A; $p<0.0001$ by ANOVA). Since the use of a morphological gate alone may result in enumeration of non-specific microparticles, including contaminating cellular fragments with similar FSC and SSC values as those of microparticles, we combined this gate with double CFSE/glycophorin A labelling (Figure 2B). As expected, the trend in microparticle numbers was comparable for both the described gating strategies, although the absolute number of microparticles counted in the CFSE/glycophorin A-positive gate was lower ($1.03\times10^5\pm0.072\times10^5$ to $2.09\times10^5\pm0.32\times10^5$, Figure 2B) than that in the morphological gate. The reason why the results from the morphological gate did not completely overlap with those from the CFSE/glycophorin A-positive gate was the exclusion of cellular fragments which were negative for CFSE staining; these fragments accounted from up to 19% of the events at day 42.

In addition, the analysis of microparticle number revealed a 2-fold increase in particles in stored RBC units from T0 to T42 ($p<0.005$ by ANOVA). Interestingly, a more in depth study of the microparticle population revealed that annexin V was able to detect only 27.56±3.47% of the entire population of CFSE/glycophorin A-positive microparticles at T0, while this percentage increased at T42, reaching 34.07±1.75%. Although the mean number of annexin V-positive vesicles increased during RBC storage, this value was always significantly lower (by as much as 4.7-fold) than the mean number of microparticles detected by the CFSE/glycophorin A gating strategy ($p<0.0002$ by the *t*-test) (Figure 2C).

Microparticle detection by CFSE correlates with red blood cell lysis during storage

RBC lysis is one of the main events occurring during storage and potentially gives rise to complications associated with blood transfusion[35]. We, therefore, determined the level of RBC lysis and correlated this with microparticle release (Figure 3). As already described by others, we found a significant increase in haemolysis rate (HR%) during storage (Figure 3A; $p<0.001$ by ANOVA)[30,36]. Moreover, we demonstrated a significant relationship (Figure 3B) ($R=0.625$; $p=0.0001$ by Pearson's test) between HR% and microparticle number, as detected by the combination of CSFE/glycophorin A, indicating that levels of haemolysis correlated with the number of microparticles detected, at all the time points tested. These data further underline the importance of precise detection of microparticles during storage as an indirect parameter of RBC preservation.

Discussion

Microparticles released by RBC are usually identified through the use of antibodies that bind to glycophorin A, a protein widely expressed on the erythrocyte membrane, and annexin V, which binds to the phosphatidylserine expressed on the outer membrane of the microparticles[26,28,37]. Several groups have recently reported that phosphatidylserine is not expressed by all microparticles and its use to identify microparticles could largely underestimate the total number of vesicles[38-40].

The clinical importance of microparticles has been progressively recognised, highlighting the need to better characterise their biological properties and their influence in pathological conditions[41]. Although flow cytometry is widely used to detect microparticles, there is still no consensus on the protocol for sample processing and microparticle detection in human blood[26]. In this study, we investigated a multiparametric approach that could provide a more sensitive tool for the detection of microparticles in stored RBC samples.

Alongside the dual labelling with glycophorin A and annexin V, we introduced CFSE as an unexplored RBC microparticle marker. CFSE was developed as a fluorescent dye that could be used to monitor cell proliferation both *in vitro* and *in vivo*[31,32]. The CFSE precursor, carboxyfluorescein diacetate succinimidyl

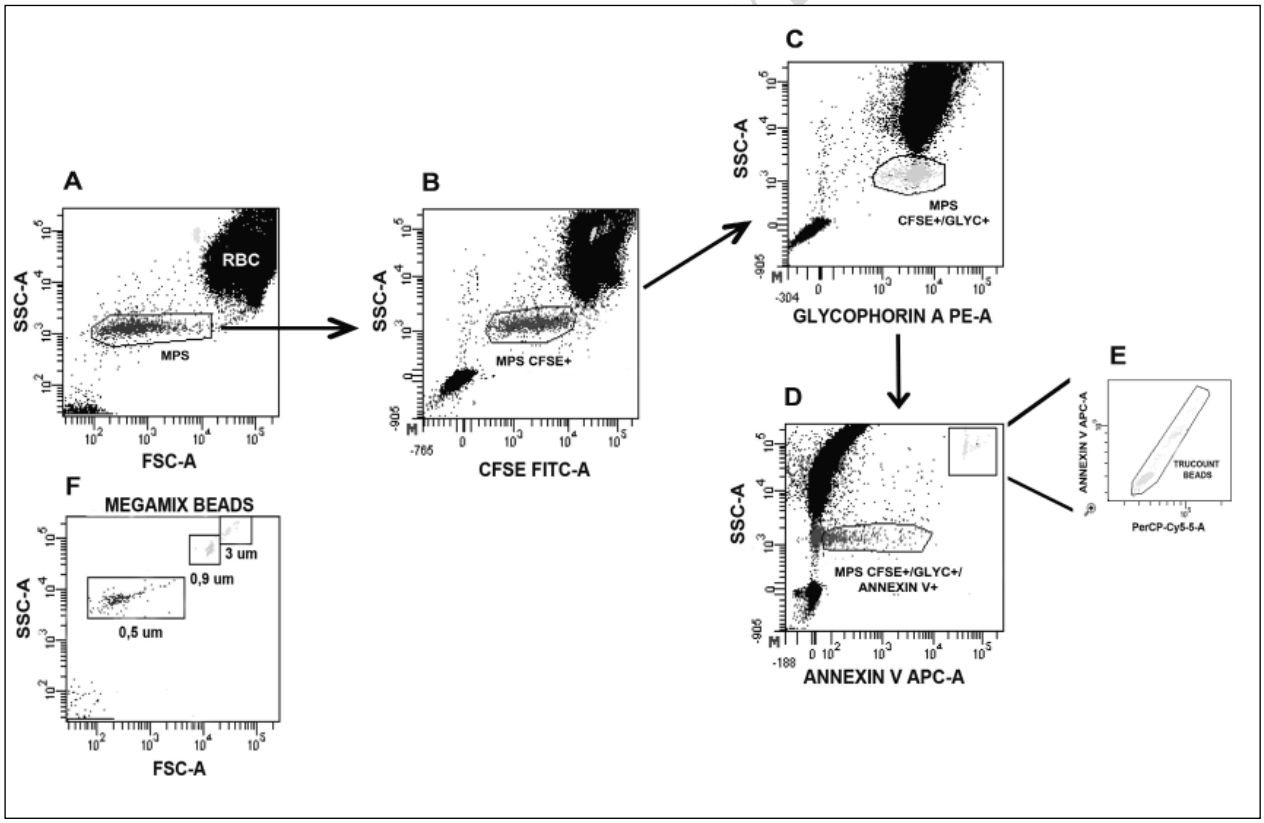

Figure 1 - Gating strategy for the identification of RBC microparticles.

A - Side scatter (SSC) and forward scatter (FSC) parameters of RBC microparticles (MPS). The MPS region lies between background noise (lower limit) and RBC (upper limit). B,C - The MPS detected in the morphological gating were also double-positive for CFSE and glycophorin A-PE. D - In contrast, only a small fraction of CFSE+/glycophorin A+ MPS were also positive for annexin V. E - TruCount fluorescent beads, as internal reference, were used to quantify the absolute number of MPS. F - Megamix beads were acquired with the same settings applied for MPS detection. As shown MPS are included in the 0.5 μm bead FSC size range.

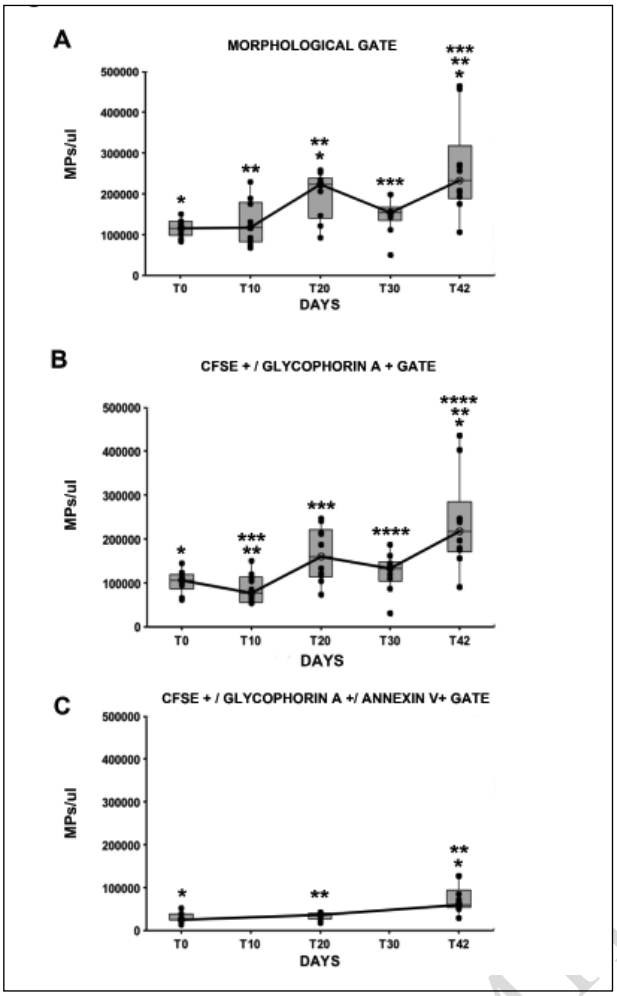

Figure 2 - Quantification and characterization of microparticles released by RBC during storage.
Graphs A and B show the number of microparticles (MPS)/µL evaluated according to light scattered profile or CFSE/glycophorin A gating strategy, respectively. Both analyses revealed the same trend characterized by a statistically significant increase of MPS number during storage (*,**,***,****p<0.0001 by ANOVA). At T42 the MPS number was 2-fold higher than at T0. C - CFSE/glycophorin A+/annexin V+ MPS.

ester (CFDA-SE) is a non-fluorescent molecule, which can passively diffuse through cellular membranes with no reported toxic effects for labelled cells[31,34]. Once inside the cells, the acetate groups are removed by intracellular esterases resulting in a less permeable fluorescent molecule, which is retained within the cytoplasm for long periods of time. Since it has been established that microparticles are encircled vesicles, containing part of the cytoplasmic content derived from parental cells, including esterase enzymes, we hypothesized that CFSE could be used to label and detect microparticles.

Our data demonstrate that CFSE can be successfully employed to label closed vesicles with an intact membrane, allowing exclusion of cellular fragments that may interfere with the detection and quantification of microparticles. At the same time, the use of glycophorin A was fundamental for determining the cellular origin of the microparticles. The inclusion of this antibody in our multiparametric approach revealed the presence of microparticles not expressing glycophorin A. The mean amount of CFSE+/glycophorin A− vesicles was 11.2% and decreased during storage, reaching 6% by day 42. These data suggest the presence of contaminating microparticles derived from other cellular components such as leucocytes, platelets or endothelial cells at the time of blood collection and that this population is lost progressively during storage. In brief, the combination of CFSE and glycophorin A antibody was particularly effective for careful monitoring and quantification of the dynamic release of microparticles from RBC during storage. Moreover, analysis of microparticles using annexin V showed that only a small fraction of the microparticles expressed phosphatidylserine on the outer membrane. Thus, the use of annexin V as a specific marker for RBC microparticles could lead to a significant underestimate of the number of particles in blood samples. Double staining with CFSE/glycophorin A increases vesicle detection by up to 4.7-fold *vs* the use of glycophorin A/annexin V alone and offers a more sensitive approach to the identification of microparticles in biological samples.

While this finding requires further investigation, we can speculate that during early stages of RBC storage only a small fraction of phosphatidylserine-expressing microparticles is released. The sensitivity of microparticle detection by CFSE/glycophorin A staining is, therefore, significantly higher than that achieved by glycophorin A/annexin V. On the other hand, after longer storage, cellular ageing generates an increased amount of damaged RBC, with a consequent rise in phosphatidylserine-expressing microparticles, which enhances annexin V sensitivity. RBC were not substantially affected by the CFSE dye used in the current setting, as demonstrated by an average 7.4% annexin V-positive signal in our samples. Although some studies indicated an increase of cell death and a block of proliferation using 10 µL of CFSE[42], others have introduced up to 50 µL with no evident toxicity[43]. The difference could be due to the variety of cell types considered and/or to the staining protocols. The effect of CFSE on cellular behaviour and on emerging biological features does, therefore, require further investigation.

The importance of quantifying microparticles correctly is becoming clearer. We wanted to correlate microparticle levels with haemolysis induced by storage as a possible novel step in the quality control of preserved, blood-derived products. We found that the amount of microparticles correlates with haemolysis rate. While the explanation of this correlation requires further investigation, we suspect that it is related to the capacity of microparticles to enclose

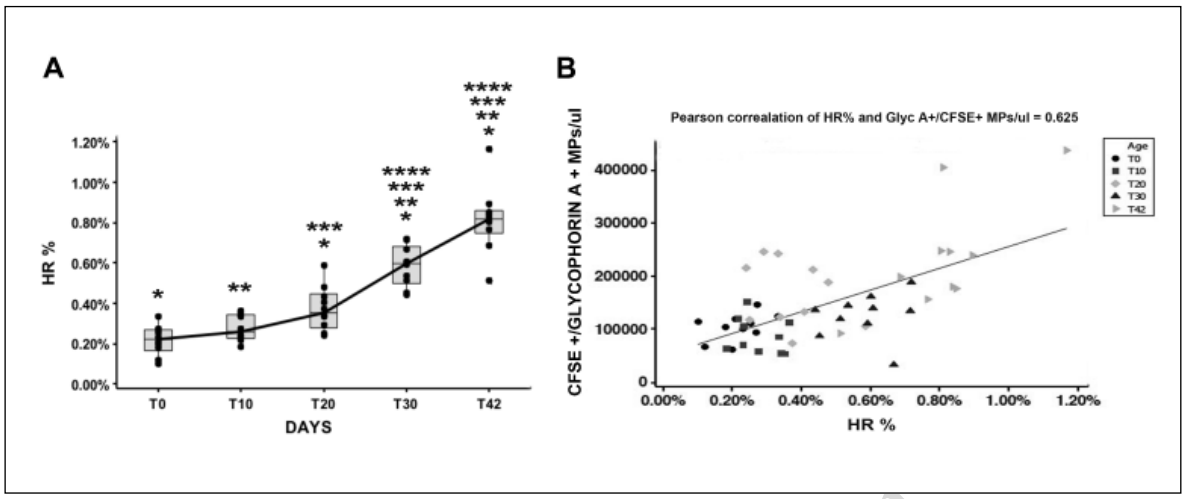

Figure 3 - Assessment of RBC lysis and correlation between number of microparticles and haemolysis rate of stored RBC. A - Haemolysis rate (HR%) at different time points. A significant increase of haemolysis was observed during storage, as expected (*.**.***.****p<0.001 by ANOVA). B - Correlation between the number of microparticles (MPS) and HR%.

haemoglobin, which is also detected as free haemoglobin during the analysis, as suggested by others[44].

The detection of microparticles in blood-derived products and their subsequent analysis may be relevant in clinical practice to provide further insights into transfusion-related side effects. Moreover, fluorescent labelling would allow *in vitro* and *in vivo* tracking of these elements, which could then be detected after injection into animal models. In conclusion, the use of a

CFSE-based approach could couple microparticle detection and sorting, ideally enabling isolation and further characterisation of the biology and clinical implications of these still poorly explored cellular particles.

Conclusions

In this study, we took advantage of the dynamic release of MPs by stored RBC to propose a multi-parametric approach based on CFSE labelling in combination with anti-Glycophorin A antibody and Annexin V aiming for an improved MPs detection. Our data demonstrate that CFSE can be successfully employed to identify closed vesicles characterized by intact membrane. The combination of CSFE/Glycophorin/Annexin V allows to determine the cellular origin of these elements resulting in a careful monitoring and quantification of their release during RBC storage time. The detection of MPs in blood derived products and their subsequent analyses may be relevant for clinical practice to further provide insights on transfusion related side effects. In addition, this multi-parametric staining may provide a sensitive approach to detect and study MPs in transfusion medicine and, more broadly, in cell based therapies.

Acknowledgements

This work was supported in parts by R&D Filter, Fresenius HemoCare Italia S.r.l. We thank BD Biosciences Italy S.p.A for technical support during the development of the study.

Authorship contributions

GG designed, performed, and analysed research, and assisted with preparation of the manuscript; EF designed and analysed research, and assisted with preparation of the manuscript; DM designed research, contributed with essential tools and assisted with preparation of the manuscript; NC, GM, CS, MP, VG, and SO performed research; EMH analysed research; GM designed and analysed research; MD oversaw the project, designed and analysed research, and prepared the manuscript.

Conflict of interest disclosure

EF and GM are employees of Fresenius HemoCare Italia S.r.l and DM is an employee of BD Biosciences Italy S.p.A. The other authors declare that they have no competing financial interests.

Reference

1) Vincent JL, Baron JF, Reinhart K, et al. Anemia and blood transfusion in critically ill patients. JAMA 2002; **288**: 1499-507.
2) Corwin HL, Gettinger A, Pearl RG, et al. The CRIT study: anemia and blood transfusion in the critically ill: current clinical practice in the United States. Crit Care Med 2004; **32**: 39-52.
3) Napolitano LM, Kurek S, Luchette FA, et al. American College of Critical Care Medicine of the Society of Critical Care Medicine, Eastern Association for the Surgery of Trauma Practice Management Workgroup. Clinical practice guideline: red blood cell transfusion in adult trauma and critical care. Crit Care Med 2009; **37**: 3124-57.

4) Spiess BD. Risks of transfusion: outcome focus. Transfusion 2004; **44**(12 Suppl): 4S-14S.
5) Edgren G, Kamper-Jørgensen M, Eloranta S, et al. Duration of red blood cell storage and survival of transfused patients. Transfusion 2010; **50**: 1185-95.
6) Koch CG, Li L, Sessler DI, et al. Duration of red-cell storage and complications after cardiac surgery. N Engl J Med 2008; **358**: 1229-39.
7) Hess JR. Red cell changes during storage. Transfus Apher Sci 2010; **43**: 51-9.
8) Bennett-Guerrero E, Veldman TH, Doctor A, et al. Evolution of adverse changes in stored RBCs. Proc Natl Acad Sci USA 2007; **104**: 17063-8.
9) Greenwalt TJ. The how and why of exocytic vesicles. Transfusion 2006; **46**: 143-52.
10) Bosman GJ, Lasonder E, Luten M, et al. The proteome of red cell membranes and vesicles during storage in blood bank conditions. Transfusion 2008; **48**: 827-35.
11) Mause SF, Weber C. Microparticles: protagonists of a novel communication network for intercellular information exchange. Circulation Res 2010; **107**: 1047-57.
12) Hargett LA, Bauer NN. On the origin of microparticles: from "platelet dust" to mediators of intercellular communication. Pulm Circ 2013; **3**: 329-40.
13) Wolf P. The nature and significance of platelet products in human plasma. Br J Haematol 1967; **13**: 269-88.
14) Ogata N, Nomura S, Shouzu A, et al. Elevation of monocyte-derived microparticles in patients with diabetic retinopathy. Diabetes Res Clin Pract 2006; **73**: 241-8.
15) Piccin A, Murphy WG, Smith OP. Circulating microparticles: pathophysiology and clinical implications. Blood Rev 2007; **21**: 157-71.
16) Soriano AO, Jy W, Chirinos JA, et al. Levels of endothelial and platelet microparticles and their interactions with leukocytes negatively correlate with organ dysfunction and predict mortality in severe sepsis. Crit Care Med 2005; **33**: 2540-6.
17) VanWijk MJ, VanBavel E, Sturk A, Nieuwland R. Microparticles in cardiovascular diseases. Cardiovasc Res 2003; **59**: 277-87.
18) Mantel PY, Hoang AN, Goldowitz I, et al. Malaria-infected erythrocyte-derived microvesicles mediate cellular communication within the parasite population and with the host immune system. Cell Host Microbe 2013; **13**: 521-34.
19) Ramakrishnaiah V, Thumann C, Fofana I, et al. Exosome-mediated transmission of hepatitis C virus between human hepatoma Huh7.5 cells. Proc Natl Acad Sci USA 2013; **110**: 13109-13.
20) Regev-Rudzki N, Wilson DW, Carvalho TG, et al. Cell-cell communication between malaria-infected red blood cells via exosome-like vesicles. Cell 2013; **153**: 1120-33.
21) Rubin O, Canellini G, Delobel J, et al. Red blood cell microparticles: clinical relevance. Transfus Med Hemother 2012; **39**: 342-7.
22) Tissot JD, Rubin O, Canellini G. Analysis and clinical relevance of microparticles from red blood cells. Curr Opin Hematol 2010; **17**: 571-7.
23) Simak J, Gelderman MP. Cell membrane microparticles in blood and blood products: potentially pathogenic agents and diagnostic markers. Transfus Med Rev 2006; **20**: 1-26.
24) Ayers L, Kohler M, Harrison P, et al. Measurement of circulating cell-derived microparticles by flow cytometry: sources of variability within the assay. Thromb Res 2011; **127**: 370-7.
25) Van der Vlist EJ, Nolte-'t Hoen EN, Stoorvogel W, et al. Fluorescent labeling of nano-sized vesicles released by cells and subsequent quantitative and qualitative analysis by high-resolution flow cytometry. Nature Protocols 2012; **7**: 1311-26.
26) Rubin O, Crettaz D, Canellini G, et al. Microparticles in stored red blood cells: an approach using flow cytometry and proteomic tools. Vox Sang 2008; **95**: 288-97.
27) Xiong Z, Cavaretta J, Qu L, et al. Red blood cell microparticles show altered inflammatory chemokine binding and release ligand upon interaction with platelets. Transfusion 2011; **51**: 610-21.
28) Xiong Z, Oriss TB, Cavaretta JP, et al. Red cell microparticle enumeration: validation of a flow cytometric approach. Vox Sang 2012; **103**: 42-8.
29) Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger CJ. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. A novel assay for apoptosis. Immunol Methods 1995; **184**: 39-51.
30) Gkoumassi E, Dijkstra-Tiekstra MJ, Hoentjen D, de Wildt-Eggen J. Hemolysis of red blood cells during processing and storage. Transfusion 2012; **52**: 489-92.
31) Parish CR, Warren HS. Use of the intracellular fluorescent dye CFSE to monitor lymphocyte migration and proliferation. Curr Protoc Immunol 2002; Chapter 4:Unit 4.9.
32) Quah BJ, Warren HS, Parish CR. Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. Nat Protoc 2007; **2**: 2049-56.
33) Giarratana MC, Kobari L, Lapillonne H, et al. Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. Nat Biotechnol 2005; **23**: 69-74.
34) Parish CR. Fluorescent dyes for lymphocyte migration and proliferation studies. Immunol Cell Biol 1999; **77**: 449-508.
35) Lee JS, Gladwin MT. Bad blood: the risks of red cell storage. Nat Med 2010; **16**: 381-2.
36) Sowemimo-Coker SO. Red blood cell hemolysis during processing. Transfusion Med Rev 2002; **16**: 46-60.
37) Nantakomol D, Imwong M, Soontarawirat I, et al. The absolute counting of red cell derived microparticles with red cell bead by flow rate based assay. Cytometry B Clin Cytom 2009; **76**: 191-8.
38) Aras O, Shet A, Bach RR, et al. Induction of microparticle and cell-associated intravascular tissue factor in human endotoxemia. Blood 2004; **103**: 4545-53.
39) Connor DE, Exner T, Ma DD, Joseph JE. The majority of circulating platelet-derived microparticles fail to bind annexin V, lack phospholipid-dependent procoagulant activity and demonstrate greater expression of glycoprotein Ib. Thromb Haemostasis 2010; **103**: 1044-52.
40) Shet AS, Aras O, Gupta K, et al. Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes. Blood 2003; **102**: 2678-83.
41) Rubin O, Crettaz D, Tissot JD, Lion N. Microparticles in stored red blood cells: submicron clotting bombs? Blood Transfus 2010; **8**: 31-8.
42) Last'ovicka J, Budinský V, Spísek R, Bartůnková J. Assessment of lymphocyte proliferation: CFSE kills dividing cells and modulates expression of activation markers. Cell Immunol 2009; **256**: 79-85.
43) Jordan MB, van Rooijen N, Izui S, et al. Liposomal clodronate as a novel agent for treating autoimmune hemolytic anemia in a mouse model. Blood 2003; **101**: 594-601.
44) Donadee C, Raat NJ, Kanias T, et al. Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion. Circulation 2011; **124**: 465-76.

Arrived: 02 June 2014 - Revision accepted: 26 August 2014
Correspondence: Massimo Dominici
Department of Medical and Surgical Sciences of Children & Adults
University Hospital of Modena and Reggio Emilia
Via del Pozzo, 71
41100, Modena, Italy
e-mail: massimo.dominici@unimore.it