Red blood cell microparticles and blood group antigens: an analysis by flow cytometry

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Background. The storage of blood induces the formation of erythrocytes-derived microparticles. Their pathogenic role in blood transfusion is not known so far, especially the risk to trigger alloantibody production in the recipient. This work aims to study the expression of clinically significant blood group antigens on the surface of red blood cells microparticles.

Material and methods. Red blood cells contained in erythrocyte concentrates were stained with specific antibodies directed against blood group antigens and routinely used in immunohematology practice. After inducing erythrocytes vesiculation with calcium ionophore, the presence of blood group antigens was analysed by flow cytometry.

Results. The expression of several blood group antigens from the RH, KEL, JK, FY, MNS, LE and LU systems was detected on erythrocyte microparticles. The presence of M (MNS1), N (MNS2) and s (MNS4) antigens could not be demonstrated by flow cytometry, despite that glycophorin A and B were identified on microparticles using anti-CD235a and anti-MNS3.

Discussion. We conclude that blood group antigens are localized on erythrocytes-derived microparticles and probably keep their immunogenicity because of their capacity to bind specific antibody. Selective segregation process during vesiculation or their ability to elicit an immune response in vivo has to be tested by further studies.

Keywords: microparticles, erythrocytes, blood group antigens.

Introduction

Microparticles (MPs) are membrane vesicles released by various cells, including red blood cells (RBCs). They are defined by a size of less than 1 μm[1] and contain proteins derived from their parent cell. Microvesiculation represents a controlled process[2] which is triggered by various stimuli and initiates with externalization of negatively charged phospholipids. Erythrocyte-derived MPs (EMPs) represent the most abundant source of MPs in certain pathological states, such as sickle cell disease[3].

Cell activation or apoptosis promote calcium influx and lead to a cascade of events which breaks the links between the membrane and the cytoskeleton proteins, specifically spectrin and protein 4.1R. Therefore, the membrane becomes unstable, allowing the release of MPs[4,5]. During their lifespan, RBCs lose a certain amount of their haemoglobin content and surface area through this mechanism[6]. As RBCs are exposed to a constant oxidative stress and have a limited capacity for self-repair, vesicle formation certainly represents one of the solution to clear senescent antigens and prevent the exposure of dangerous molecules on their surface[7]. MPs formation has been considered as an integral step of RBC aging, as outlined by two recent models of erythrocyte senescence and is strongly correlated with spectrin oxidation[6,8-16]. Once vesiculation capacity is exceeded, old erythrocytes are removed by the reticuloendothelial system[17,18].

The composition of EMPs has been established through proteomic analysis. EMPs proteome differs from its parental cell by the decrease of cytoskeletal-linked molecules, the enrichment of several components (especially hemoglobin, band 3, glycophorins, complement receptors, glycosyl-phosphatidylinositol-anchored proteins and lipid-raft markers) and the exposure of removal signal molecules such as phosphatidylserin and autologous IgG[6,7,17,19,20]. These differences are explained by a process of selective sorting during microvesiculation process[7].

Under blood bank conditions, RBCs undergo structural and biochemical changes[21,22], described as the "storage lesion"[7,23-26]. This lesion shares similar features with RBC aging, with the peculiarity that oxidation of cytoskeleton proteins plays a central role[7,27-31]. It has been shown that the number of glycophorin A (GPA) positive EMPs increases in blood units over time and that their structure gradually change with accumulation of stomatin, compared to EMPs generated *in vivo*[6,18,20,32]. These observations make likely a raft-based process responsible for vesiculation under these conditions[7,17,22,33]. EMPs structure becomes also gradually more heterogeneous, according to the absence of their immunologic removal in the bag[18,28].

There are 31 RBC group systems identified so far[34], five of which hold antigens with carbohydrate structures (ABO, H, GLOB, P, LE) while the others consist of proteins[35]. Up to half of the RBC transmembrane proteins carry blood group antigens, including proteins that are major constituents of the RBC membrane, such as band 3 (ABO and DI), GPA and glycophorin B (MNS), glycophorins C and D (GE), Rhesus polypeptides (RH) and Aquaporin 1 (CO)[36,37]. These highly-expressed molecules are of two types: either transporters proteins with multiple membrane spanning domains (band 3, RH, CO) or sialylated glycoproteins with a single transmembrane domain (GPA, GPB). Due to their density, it would be expected that they play important structural or functional roles[37]. RBC cytoskeleton network is essential for membrane stability and consists of spectrin backbone anchored to the lipid membrane through interaction with actin, protein 4.1R and ankyrin[38]. Some of these erythrocyte proteins, such as the antigens of the RH and FY systems, belong to macromolecular complexes and contribute to membrane stabilization, through their link with protein 4.1R[39].

During storage, RBCs show a decrease of blood group antigens expression, concomitant to the progressive increase of soluble blood group concentration in the plasma[40,41]. Evidence of blood group antigens activity on MPs originated from erythrocyte concentrates (ECs) has been reported by some authors, using agglutination/inhibition tests, Western blotting, immunoelectron microscopy or radio-labeled anti-D antibodies[22,41-44]. In a previous study on EMPs, we identified RH peptides by mass spectrometry from SDS-PAGE of proteins isolated from EMPs[20]. Thus, our work aims to study the presence of clinically significant blood group antigens on MPs. We induced microvesiculation of RBCs stored in ECs after activation with calcium and performed the flow cytometry analysis with antibodies routinely used in immunohematology laboratories for agglutination tests.

Materials and methods

Whole blood collection, processing and storage

Whole blood was collected from normal volunteer donors, attending the Service Régional Vaudois de Transfusion Sanguine and held in bags containing an anticoagulant solution (citrate-phosphate-dextrose) for a maximum of 24 hours at room temperature, prior to processing. The bags were centrifuged, plasma was removed and the packed RBCs were suspended in 100 mL of SAG-M (sodium-adenine-glucose-mannitol) additive solution, afterwards leukocytes were removed by filtration. The ECs were finally stored at 4 °C.

Donor selection was based on homozygous expression of RH, JK, FY or MNS antigens except for KEL3 (Kp^a) and LU1 (Lu^a). Only ECs that did not satisfy quality criteria for transfusion were dedicated to this study (particularly those from donors with elevated liver enzymes).

Labeling

The experiment consists in an indirect staining of RBCs with specific blood group antibodies, followed by the generation of RBC-derived MPs via calcium ionophore activation and their analysis by flow cytometry. Samples with anti-RH antibodies undergo a double labelling with anti-CD235a (anti-GPA).

Samples of stored ECs were washed in phosphate-buffered saline solution (PBS) and centrifuged at 1,000 × *g* for 30 seconds at 4 °C. The residual RBC pellets were incubated with antibodies against blood group antigens according to their phenotype (Human IgM Monoclonal anti-RH2 [anti-C], anti-RH4 [anti-c], anti-RH3 [anti-E], anti-RH5 [anti-e], anti-MNS3 [anti-S]; Human IgM Polyclonal anti-JK1 [anti-Jk^a], anti-JK2 [anti-Jk^b]; Human IgG Polyclonal anti-KEL3 [anti-Kp^a] anti-KEL4 [anti-Kp^b], anti-FY1 [anti-Fy^a], anti-FY2 [anti-Fy^b], anti-MNS4 [anti-s], anti-LU1 [anti-Lu^a], anti-LU2 [anti-Lu^b];

Mouse IgM Monoclonal anti-MNS1 [anti-M], anti-MNS2 [anti-N], anti-LE1 [anti-Le^a], anti-LE2 [anti-Le^b]; all from Biotest, Dreieich Germany), adjusted to 100 µL of PBS and mixed constantly for 90 minutes. Excess of antibody was removed by two washing steps in PBS.

As the primary antibodies are not fluorochrome-conjugated, the remaining RBC pellets were incubated with a secondary antibody (100 µL of diluted fluorescein isothiocyanate (FITC)-conjugated antibody), namely FITC-conjugated anti-human IgM (Caltag Laboratories, Burlingame, CA, USA), FITC-conjugated anti-human IgG (Chemicon, Temecula, CA, USA) or FITC-conjugated anti-mouse IgM (BD Biosciences, Franklin Lakes, NJ, USA). The samples were agitated continuously for 60 minutes in the dark. The excess of antibody was removed as described above with centrifugation and washing steps.

Generation of microparticles

Calcium inophore solution (2.6 µg of Ca ionophore A23187; Sigma-Aldrich, St-Louis, MO, USA) and 5 µL of $CaCl_2$ (Fluka, Buch, Switzerland) in 1 mL of PBS were added to the remaining pellet and agitated in the dark for 60 minutes, at 37 °C. Five µL of phycoerythrin (PE)-conjugated anti-human CD235a, diluted 1/10 (BD Pharmingen, San Diego, CA, USA) were added only to the samples labelled with anti-RH antibodies and these were agitated 20 minutes in the dark at room temperature. All the samples were then diluted with 50 µL of PBS and spun down at 1,000 × *g* for 30 seconds to remove RBCs and keep the supernatant containing EMPs. The supernatants were transferred to 5 mL plastic tubes (Falcon, BD Biosciences, Franklin Lakes, NJ, USA).

Flow cytometry

Supernatants were diluted in PBS. MPs size was determined using flow cytometric light scatter (FACScalibur, BD Biosciences, Franklin Lakes, NJ, USA). The flow cytometer was calibrated using beads (CaliBRIT™ 3 kit, BD Biosciences, Franklin Lakes, NJ, USA) to ensure standard instrument settings for each analysis. MPs were defined as particles less than 1 µm. Sizing beads of 1-1.4 µm (Spherotech, Lake Forest, IL, USA) were used to verify the appropriateness of the gate. EMPs were gated on the basis of their forward scatter and side scatter signals with logarithmic fluorescence scales (Figure 1). Results of blood group antigens expression were analysed on dot plots and histograms (Figure 2) with negative threshold defined by EMPs obtained from RBCs lacking the corresponding antithetic antigen.

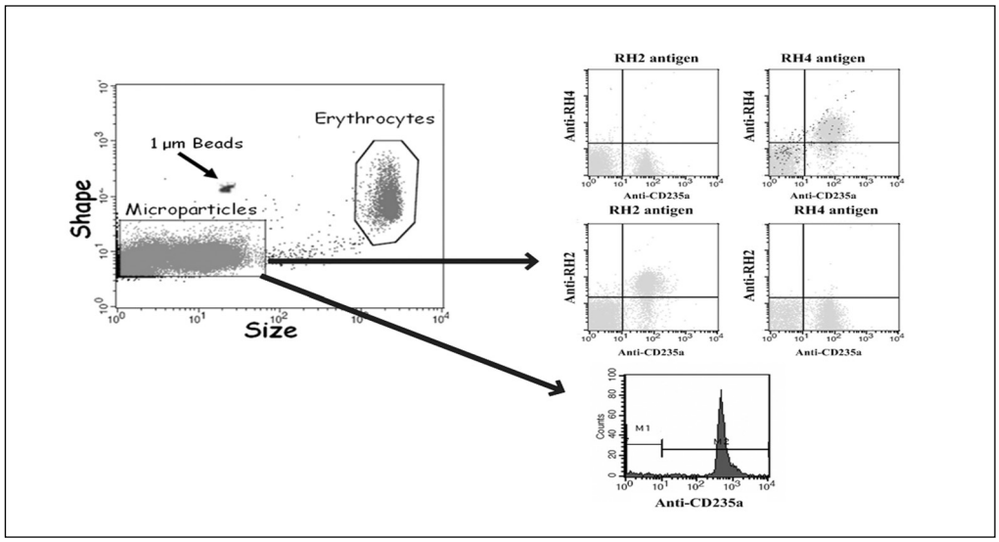

Figure 1 - Flow cytometry analysis of microparticles in erythrocyte concentrates.
Size of events is defined by calibration beads of 1 µm. Two gating regions are represented: on the left, the events smaller than 1 µm which contains the microparticles and on the right, the erythrocytes. The expression of Rhesus antigens RH2 (C) and RH4 (c) (depending on the Rhesus phenotype of the donors) and glycophorin A (anti-CD235a) is illustrated.

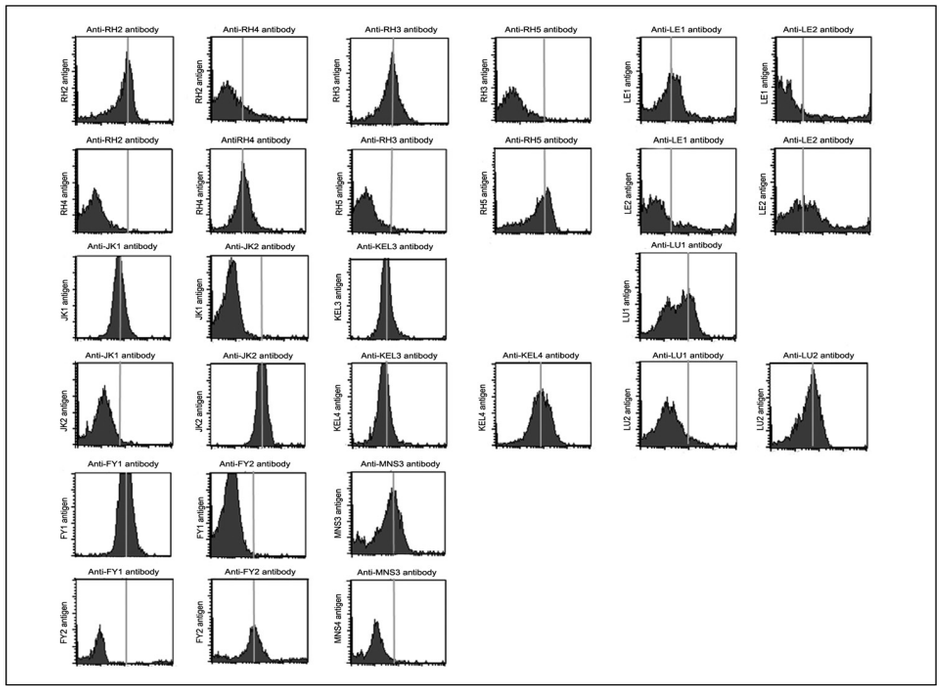

Figure 2 - Detection of clinically significant blood group antigens on microparticles derived from erythrocyte concentrates.
The red blood cells were selected according to their homozygous expression of blood group antigens (vertical axis), except for KEL3 (Kp^a) and LU1 (Lu^a). Flow cytometric histograms show the expression of RH2 (C), RH3 (E), RH4 (c), RH5 (e), KEL3 (Kp^a), KEL4 (Kp^b), JK1 (Jk^a), JK2 (Jk^b), FY1 (Fy^a), FY2 (Fy^b), MNS3 (S), LU1 (Lu^a) and LU2 (Lu^b) antigens on the erythrocyte-derived microparticles. The absence of the antithetic antigen on microparticles originated from homozygous erythrocytes served as negative control.

Results

Activation of RBCs with calcium allows the generation of MPs which are less than 1 μm (Figure 1). The erythrocyte origin of the MPs is confirmed by the anti-GPA staining. Intensities of detection of blood group antigens are shown on flow cytometric histograms (Figure 2). Using this method, the following antigens were detected on EMPs: RH2 (C), RH3 (E), RH4 (c) and RH5 (e) antigens, KEL3 (Kp^a) and KEL4 (Kp^b) antigens, JK1 (Jk^a) and JK2 (Jk^b) antigens, FY1 (Fy^a) and FY2 (Fy^b) antigens, MNS3 (S), Le^a (LE1) and Le^b (LE2), and finally LU1 (Lu^a) and LU2 (Lu^b) antigens. The presence of M (MNS1), N (MNS2), and s (MNS4) antigens could not be demonstrated because of agglutination of RBCs after the addition of the primary antibody, despite successive dilutions of the latter. However, glycophorin A was clearly identified on the surface of EMPs, either by flow cytometry or immunoblot analyses[20]. Gylcophorin B was detected with MNS3 antibodies (Figure 2).

Discussion

This study shows that MPs generated from ECs express clinically significant blood group antigens. Detecting their expression by flow cytometry indicates that most of them keep the property to react with the corresponding antibodies despite cytoskeletal dissociation during vesiculation. The activities of M (MNS1), N (MNS2), and s (MNS4) antigens have not been demonstrated owing to technical difficulties. For caution, these findings are based on artificially induced microvesiculation in blood bags and request verification in human models.

Our results did not determine whether the antigenic sites density on MPs corresponds to those of the parent cell. Thus, the question of the selective segregation (or even enrichment) of blood group antigens into MPs cannot be answered. Prior imunoelectron microscopy analysis of EMPs has ruled out the clustering of FY monomeric chains formation during vesiculation[42]. However, antigen

selection could arise in MPs, as suggested by the loss of specific RBC antigens during autoimmune hemolytic anemia or transfusion of incompatible blood[45, 46]. In these examples, antibody binding could induce disruption of membrane organization. Therefore it is reasonable to speculate that selective loss of antigen may occur through RBC vesiculation, although the exact mechanism involved has not been elucidated so far[47].

Duffy protein, also named DARC protein, is a receptor for chemokines. RBCs without DARC protein have lost the Duffy-dependent chemokine binding capacity. This may partly explain the tendency of FY:-1,-2 sickle cell patients to chronic inflammation and alloimmunization[37]. Some authors have shown maintenance of blood group antigens function on EMPs, especially the chemokine binding activity of DARC protein[42]. The hypothetic role of Duffy positive MPs in clearing local inflammation mediators has to be further investigated.

As their antigenic activity is oriented outwards and recognized by specific monoclonal antibodies, blood group antigens located on MPs may elicit an immune response *in vivo*. EMPs are easily phagocytised by antigen-presenting cells and despite their small size and low total volume, may represent a significant immunogenic load during transfusion[17, 18, 48]. Recently, Rhesus immunization originating from EMPs was suspected in an aphaeresis platelet recipient[49]. However, the capacity of EMPs to induce alloimmunization after transfusion needs to be demonstrated, especially whether they are more immunogenic and over which threshold they may trigger antibody formation.

The level of EMPs in blood of sickle cell patients is significantly elevated and strongly correlates with the degree of intravascular hemolysis and premature aging of RBCs[3, 50, 51]. Red cell antibody development is a well recognized complication of chronic transfusion in sickle cell patients, with an incidence of 10-40% in prior published reports[52-54]. Transfusion of RBCs with phenotype matching for C (RH2), c (RH4), E (RH3), e (RH5) and K (KEL1) antigens have reduced to six fold the immunization rate[55,56]. Several factors contribute to RBC alloimmunization in these patients. Therefore it will be important to define if EMPs contained in blood units will participate to the stimulation of the immune system and which strategies need to be developed by blood banks for its prevention.

Conclusion

Our work has brought further insights in the composition of EMPs generated during ECs storage upon calcium stimulation and raised questions about their potential immunogenicity. Nevertheless, the MPs capacity of immunization during transfusion needs animal models to investigate the immune effectors side. As well, determining antigen density on EMPs using antibody calibration kit will provide further information about the hypothesis of selective sorting during erythrocyte microvesiculation[57].

Although EMPs contributes to erythrocyte homeostasis in eliminating sorted oxidized proteins[18], they represent a "two-edged sword" with the capacity to induce adverse clinical outcomes in transfused recipients through their immune effects (immuno-modulation and alloimmunization), thrombogenic activities and negative impact on the micro-circulation[3,32, 58-60]. The analysis of EMPs generated during blood processing and aphaeresis techniques will permit to improve the quality of blood products[61,62]. Understanding the real impact of EMPs and minimizing their formation during blood processing represent a future challenge in the field of transfusion.

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References

1) Diamant M, Tushuizen ME, Sturk A, Nieuwland R. Cellular microparticles: new players in the field of vascular disease? Eur J Clin Invest 2004; **34**: 392-401.
2) 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.
3) van Beers EJ, Schaap MC, Berckmans RJ, et al. Circulating erythrocyte-derived microparticles are associated with coagulation activation in sickle cell disease. Haematologica 2009; **94**: 1513-9.
4) An X, Guo X, Sum H, et al. Phosphatidylserine binding sites in erythroid spectrin: location and implications for membrane stability. Biochemistry 2004; **43**: 310-5.
5) Manno S, Takakuwa Y, Mohandas N. Identification of a functional role for lipid asymmetry in biological membranes: Phosphatidylserine-skeletal protein interactions modulate membrane stability. Proc Natl Acad Sci USA 2002; **99**: 1943-8.

6) Bosman GJ, Werre JM, Willekens FL, Novotny VM. Erythrocyte ageing in vivo and in vitro: structural aspects and implications for transfusion. Transfus Med 2008; **18**: 335-7.
7) 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.
8) Arese P, Turrini F, Schwarzer E. Band 3/complement-mediated recognition and removal of normally senescent and pathological human erythrocytes. Cell Physiol Biochem 2005; **16**: 133-46.
9) Kay M. Immunoregulation of cellular life span. Ann N Y Acad Sci 2005; **1057**: 85-111.
10) Lang F, Gulbins E, Lerche H, et al. Eryptosis, a window to systemic disease. Cell Physiol Biochem 2008; **22**: 373-80.
11) Lion N, Crettaz D, Rubin O, Tissot JD. Stored red blood cells: a changing universe waiting for its map(s). J Proteomics 2010; **73**: 374-85.
12) Minetti M, Malorni W. Redox control of red blood cell biology: the red blood cell as a target and source of prooxidant species. Antioxid Redox Signal 2006; **8**: 1165-9.
13) Wagner GM, Chiu DT, Qju JH, et al. Spectrin oxidation correlates with membrane vesiculation in stored RBCs. Blood 1987; **69**: 1777-81.
14) Gov NS, Safran SA. Red blood cell membrane fluctuations and shape controlled by ATP-induced cytoskeletal defects. Biophysical journal 2005; **88**: 1859-74.
15) Sens P, Gov N. Force balance and membrane shedding at the red-blood-cell surface. Phys Rev Lett 2007; **98**: 018102.
16) Gov N CJ, Sens P, Bosman GJCGM. Cytoskeletal Control of Red Blood Cell Shape: Theory and Practice of Vesicle Formation. In: Leitmannova Liu A, Iglic A, editors. *Advances in Planar Lipid Bilayers and Liposomes*. Burlington: Academic Press, Elsevier 2009; **10**: 95-119.
17) Willekens FL, Werre JM, Groenen-Dopp YA, et al. Erythrocyte vesiculation: a self-protective mechanism? Br J Haematol 2008; **141**: 549-56.
18) Kriebardis AG, Antonelou MH, Stamoulis KE, et al. RBC-derived vesicles during storage: ultrastructure, protein composition, oxidation, and signaling components. Transfusion 2008; **48**: 1943-53.
19) Rubin O, Crettaz D, Tissot JD, Lion N. Pre-analytical and methodological challenges in red blood cell microparticle proteomics. Talanta 2010; **82**: 1-8.
20) 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.
21) Karon BS, Hoyer JD, Stubbs JR, Thomas DD. Changes in Band 3 oligomeric state precede cell membrane phospholipid loss during blood bank storage of red blood cells. Transfusion 2009; **49**: 1435-42.
22) Salzer U, Zhu R, Luten M, et al. Vesicles generated during storage of red cells are rich in the lipid raft marker stomatin. Transfusion 2008; **48**: 451-62.
23) Bennett-Guerrero E, Veldman TH, Doctor A, et al. Evolution of adverse changes in stored RBCs. Proc Natl Acad Sci U S A 2007; **104**: 17063-8.
24) Gevi F, D'Alessandro A, Rinalducci S, Zolla L. Alterations of red blood cell metabolome during cold liquid storage of erythrocyte concentrates in CPD-SAGM. J Proteomics 2012; DOI 10.1016/j.jprot.2012.03.012
25) Ferru E, Giger K, Pantaleo A, et al. Regulation of membrane-cytoskeletal interactions by tyrosine phosphorylation of erythrocyte band 3. Blood 2011; **117**: 5998-6006.
26) Pantaleo A, Ferru E, Carta F, et al. Irreversible AE1 tyrosine phosphorylation leads to membrane vesiculation in G6PD deficient red cells. PloS one 2011; **6**: e15847.
27) Antonelou MH, Kriebardis AG, Stamoulis KE, et al. Red blood cell aging markers during storage in citrate-phosphate-dextrose-saline-adenine-glucose-mannitol. Transfusion 2010; **50**: 376-89.
28) Bosman GJ, Lasonder E, Groenen-Dopp YA, et al. Comparative proteomics of erythrocyte aging in vivo and in vitro. J Proteomics 2010; **73**: 396-402.
29) Karon BS, Van Buskirk CM, Jaben EA, et al. Temporal sequence of major biochemical events during Blood Bank storage of packed red blood cells. Blood Transfusion 2012; DOI 10.2450/2012.0099-11.
30) D'Alessandro A, D'Amici GM, Vaglio S, Zolla L. Time-course investigation of SAGM-stored leukocyte-filtered red bood cell concentrates: from metabolism to proteomics. Haematologica 2012; **97**: 107-15.
31) Chaudhary R, Katharia R. Oxidative injury as contributory factor for red cells storage lesion during twenty eight days of storage. Blood Transfusion 2012; **10**: 59-62.
32) Sadallah S, Eken C, Schifferli JA. Erythrocyte-derived ectosomes have immunosuppressive properties. J Leukoc Biol 2008; **84**: 1316-25.
33) Greenwalt TJ. The how and why of exocytic vesicles. Transfusion 2006; **46**: 143-52.
34) Helias V, Saison C, Ballif BA, et al. ABCB6 is dispensable for erythropoiesis and specifies the new blood group system Langereis. Nature genetics 2012; **44**: 170-3.
35) Daniels G, Reid ME. Blood groups: the past 50 years. Transfusion 2010; **50**: 281-9.
36) Reid ME, Mohandas N. Red blood cell blood group antigens: structure and function. Semin Hematol 2004; **41**: 93-117.
37) Anstee DJ. The functional importance of blood group-active molecules in human red blood cells. Vox Sang 2011; **100**: 140-9.

38) Mohandas N, Gallagher PG. Red cell membrane: past, present, and future. Blood 2008; **112**: 3939-48.
39) Pantaleo A, De Franceschi L, Ferru E, et al. Current knowledge about the functional roles of phosphorylative changes of membrane proteins in normal and diseased red cells. J Proteomics 2010; **73**: 445-55.
40) Michel FW. The Occurrence of Blood-Group Specific Material in the Plasma and Serum of Stored Blood. Vox Sang 1964; **9**: 471-5.
41) Cole WF, Rumsby MG, Longster GH, Tovey LA. Changes in the inhibition of specific agglutination by plasma due to microvesicles released from human red cells during storage for transfusion. Vox Sang 1979; **37**: 73-7.
42) 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.
43) Oreskovic RT, Dumaswala UJ, Greenwalt TJ. Expression of blood group antigens on red cell microvesicles. Transfusion 1992; **32**: 848-9.
44) Victoria EJ, Branks MJ, Masouredis SP. Immunoreactivity of the Rho(D) antigen in cytoskeleton-free vesicles. Transfusion 1987; **27**: 32-5.
45) Zimring JC, Hair GA, Chadwick TE, et al. Nonhemolytic antibody-induced loss of erythrocyte surface antigen. Blood 2005; **106**: 1105-12.
46) Powers A, Mohammed M, Uhl L, Haspel RL. Apparent nonhemolytic alloantibody-induced red-cell antigen loss from transfused erythrocytes. Blood 2007; **109**: 4590.
47) Head DJ, Lee ZE, Swallah MM, Avent ND. Ligation of CD47 mediates phosphatidylserine expression on erythrocytes and a concomitant loss of viability in vitro. Br J Haematol 2005; **130**: 788-90.
48) Kumpel B. Are weak D RBCs really immunogenic? Transfusion 2006; **46**: 1061-2.
49) Kitazawa J, Nollet K, Morioka H, et al. Non-D Rh antibodies appearing after apheresis platelet transfusion: stimulation by red cells or microparticles? Vox Sang 2011; **100**: 395-400.
50) Bosman GJ. Erythrocyte aging in sickle cell disease. Cell Mol Biol (Noisy-le-grand) 2004; **50**: 81-6.
51) Westerman M, Pizzey A, Hirschman J, et al. Microvesicles in haemoglobinopathies offer insights into mechanisms of hypercoagulability, haemolysis and the effects of therapy. Br J Haematol 2008; **142**: 126-35.
52) Rosse WF, Gallagher D, Kinney TR, et al. Transfusion and alloimmunization in sickle cell disease. The Cooperative Study of Sickle Cell Disease. Blood 1990;**76**: 1431-7.
53) Cox JV, Steane E, Cunningham G, Frenkel EP. Risk of alloimmunization and delayed hemolytic transfusion reactions in patients with sickle cell disease. Arch Intern Med 1988; **148**: 2485-9.
54) Vichinsky EP, Earles A, Johnson RA, et al. Alloimmunization in sickle cell anemia and transfusion of racially unmatched blood. N Engl J Med 1990; **322**: 1617-21.
55) Vichinsky EP, Luban NL, Wright E, et al. Prospective RBC phenotype matching in a stroke-prevention trial in sickle cell anemia: a multicenter transfusion trial. Transfusion 2001; **41**: 1086-92.
56) Godfrey GJ, Lockwood W, Kong M, et al. Antibody development in pediatric sickle cell patients undergoing erythrocytapheresis. Pediatr Blood Cancer 2010; **55**: 1134-7.
57) de Isla NG, Riquelme BD, Rasia RJ, et al. Quantification of glycophorin A and glycophorin B on normal human RBCs by flow cytometry. Transfusion 2003; **43**: 1145-52.
58) Twomley KM, Rao SV, Becker RC. Proinflammatory, immunomodulating, and prothrombotic properties of anemia and red blood cell transfusions. J Thromb Thrombolysis 2006; **21**: 167-74.
59) 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.
60) Lee JS, Gladwin MT. Bad blood: the risks of red cell storage. Nat Med 2010; **16**: 381-2.
61) Veale MF, Healey G, Sparrow RL. Effect of additive solutions on red blood cell (RBC) membrane properties of stored RBCs prepared from whole blood held for 24 hours at room temperature. Transfusion 2011; **51** (Suppl. 1): S25-33.
62) Kim-Shapiro DB, Lee J, Gladwin MT. Storage lesion: role of red blood cell breakdown. Transfusion 2011; **51**: 844-51.

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