Aging and death signalling in mature red cells: from basic science to transfusion practice

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Introduction

The red blood cells (RBCs) aging process is considered as an issue of special scientific and clinical interest. It represents a total of unidirectional, time-dependent but not-necessarily linear series of molecular events that finally lead to cell clearance[1]. Under normal circumstances, all human RBCs live approximately 120±4 days in blood circulation, implying the existence of tightly regulated molecular mechanism(s), responsible for the programming of the lifespan and the nonrandom removal of senescent RBCs[2,3]. Although the RBCs have already been used as a model for aging study[1], the molecular participants, as well as the signalling pathways involved, are not yet completely clarified.

RBCs storage under blood bank conditions is far from being considered analogous to the physiologic *in vivo* aging process. The putative implicated *in vivo* signalling pathways are expected to be more-or-less preserved under *in vitro* conditions, nevertheless slightly modulated, in response to a totally different environment. A storage period of up to 35-42 days at 4 °C probably is not a "congenial interlude" of the physiological maturity process and definitely does not represent an ignorable time period, compared to the RBCs lifespan. Stored RBCs age without the normal adjacency of other cells or plasma, which continuously provide them with survival factors and signals and, moreover, they are obligated to share their living space with their own and other cells' wastes. Since no clearance mechanisms seem to function, senescent RBCs are probably sentenced to "survive" for a longer period than they were probably programmed for. Although there is evidence suggesting that storage disturbs the physiological RBC aging process[4-7], the mechanistic basis of the aging progress inside the blood unit and the functional reactivity of the modified RBCs *in vivo*, remain still elusive.

Given the fundamental need for safe and efficient transfusions, the clinical impact of stored blood, as a function of the storage parameters, has attracted considerable attention. Clinical trials that focus on the potential adverse clinical consequences of transfusing older storage-age RBCs units vs. younger ones have already been reported[8]. Apart from the skepticism around their design and execution[9], these studies indicate the necessity of thorough examination of the storage effect on packed RBCs, before analyzing their potential impact on the clinical outcome.

This review focuses on the current knowledge on aging and death signalling pathways operating in both *in vivo* systems and stored RBCs and suggests future directions in the preservation science, helpful for addressing what seem to be the current critical questions in transfusion medicine.

RBC aging, senescence and *in vivo* death signalling pathways

RBCs experience a range of continuous metabolic and physical damages as they age, such as membrane vesiculation[10], haemoglobin (Hb) modifications and progressive failure of both, cellular homeostasis and antioxidant defenses. The increase in RBCs density[11], the nonenzymatic glycation of Hb[12] and the deamidation of protein 4.1*b* to 4.1a[13,14] have been widely used as sensitive RBC age markers. In fact, numerous post-translational protein modifications, including phosphorylation, oxidation and aggregation are functionally involved in the regulation of RBCs homeostasis and lifespan.

Despite these cumulative events, the senescent signals, namely the molecular measure of RBCs age, do not seem to gradually express in cells. On the opposite, they appear as a snap, rapid and non-linear

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cascade of events at the terminal stage of the aging process, probably shortly before RBCs removal by the reticuloendothelial system[3]. Taken into consideration the inability of mature RBCs to synthesize new proteins, the recognizable markers must derive from modifications in pre-existing molecules. Their generation is most probably the ultimate step of more than one signalling pathways, working in a sophisticated context of molecular interplays. To the current knowledge, the "RBC aging phenotype", namely the repertoire of age-dependent alterations, can be safely associated with a reported decline in metabolic activity, a progressive cell shape transformation, a membrane remodelling, as well as with oxidative injury, microvesiculation and exposure of surface removal markers. The only common feature of these modifications is that they all, directly or indirectly, trigger erythrophagocytosis[15].

Microvesiculation

Membrane microvesiculation is part of the RBCs maturation. It represents a well regulated process that is accelerated in older cells[10]. Depending on the circumstances, it may function against (by contributing to irreversible membrane/Hb loss) or in favour (by carrying away damaged and signalling effective cell components) of the growing RBCs[16]. The exocytosis of non-functional proteins and senescence marks through vesiculation not only protects the RBCs from premature death but also indicates that the same recognition signals mediate the rapid removal of old RBCs and vesicles from the circulation[16]. Through the continuous release of vesicles, RBCs indices change as they age[12] and cell density progressively increases concomitantly to a decrease in cellular deformability and membrane flexibility. These modifications have been appreciated as major determinants of RBCs premature *in vivo* removal.

The Band 3-based aging pathway

So far, numerous RBCs aging pathways have been proposed based on cellular changes identified in older RBCs. Among them, the clustering[17] and/or the breakdown of Band 3[18,19] is probably the central step in the major immunologically mediated pathway, leading to the generation of a powerful senescent signal, a senescent-specific neo-antigen, *in vivo*. Similar processes seem to be responsible for premature RBCs clearance in haemoglobinopathies, membrane protein deficiencies, Down's syndrome, Alzheimer's disease etc[17,19]. Upwards, free radical oxidation may be an important factor underlying the formation of the senescent signal. There is evidence connecting the RBC oxidation levels *in vivo* to the breakdown of Band 3 and to the autologous IgG binding[20], as well as the elevated membrane-bound modified Hb to the high incidence of autologous IgG binding[21]. Furthermore, formation of advanced glycation end products[22], binding of oxidative denatured Hb to Band 3[23] and the following modifications in tyrosine phosphorylation[17], may induce the observed topographic redistribution of Band 3. Downwards, the senescence neoantigen appearance induces the binding of both autologous IgGs and probably C3 fraction of the complement to the membrane, triggering erythrophagocytosis[18,24,25].

Calcium homeostasis

Distorted calcium (Ca^{2+}) homeostasis is probably part of another aging-related pathway either as a triggering factor for aging or as its consequence[26]. Although the path's regulation under physiological conditions is obscured, membrane-associated Bcl-X_L and Bak, which form functional interactions with survival factors of the plasma, might mediate it[2]. Calcium influx is clearly correlated to oxidative damage, vesiculation, dehydration and deformability defects suffered by the senescent RBCs[27]. Furthermore, there is an established functional connection between calcium influx and apoptosis-like events in mature erythrocytes[27-30]. That calcium-involved set of pathways working in RBCs in response to stress (oxidative, osmotic etc) has been called eryptosis. Two underlying signalling pathways have been reported: (i) formation of prostaglandin E2 that leads to the activation of Ca^{2+}-permeable cation channels and (ii) phospholipase A2–mediated release of platelet-activating factor that activates a sphingomyelinase, leading to formation of ceramide. Increased intracellular Ca^{2+} and ceramide levels lead to PS exposure. Moreover, calcium activates Ca^{2+}-sensitive K^+ channels, leading to cellular KCl loss and cell shrinkage. In addition, Ca^{2+} stimulates μ-calpain transglutaminase-2 and, occasionally, caspases that degrade/crosslink the cytoskeleton proteins, resulting

in loss of membrane integrity, deformability and blebbing. Finally, Ca^{2+} disrupts the critical interaction between phosphotyrosine phosphatase and Band 3[31]. Eryptosis may be a haemolysis escape mechanism of defective erythrocytes[29,30] but its relevance - if any- to RBC aging process still has to be firmly established by future studies.

Caspase signalling and PS exposure

Erythrocytic procaspase 3 is *in vitro* activated under oxidative stress, leading to Band 3 modifications, PS exposure and erythrophagocytosis[32,33]. Such a mechanism is very likely to have an *in vivo* physiological role in RBCs aging/clearance, as indicated by the active caspase -3 and -8 detection, as well as by the formation of the Fas-signalling death complex in the lipid raft membrane microdomains of aged RBCs[34,35]. Aged RBCs present lower aminophospholipid translocase activity and higher levels of externalized PS, in comparison with younger ones[35]. Activation of caspase 3 during RBCs senescence, under the stimulus of increased oxidative stress, could cleave[34] or modulate[36] Band 3, triggering a cascade which leads to RBC removal. Thus, stimulation of caspases in aged or damaged RBCs could induce their phagocytosis in order to prevent haemolysis.

Most RBC-derived vesicles expose PS[16] and a number of haematologic diseases[29,37] or *in vitro* stressful treatments of human RBCs result in PS exposure[29]. Interestingly, PS externalization reflects the rate at which biotinylated RBCs remove from circulation *in vivo*[37]. However, *in vivo* PS exposure in healthy individuals senescent RBCs is still a matter of debate, mainly because of the scepticism against the techniques used to isolate old RBCs[16,38]. Notably, Bratosin *et al.* have recently detected active caspases in a fraction of PS-exposing senescent RBCs, isolated from blood circulation[39]. Considering the powerful thrombogenic effect of externalized PS, it is more likely that it plays a role in the senescence and removal of normal or stressed RBCs, under certain circumstances, through the activation of different signalling pathways. Nevertheless, this postulation should be revaluated by more sophisticated technical skills.

Other mechanisms of RBC *in vivo* aging

Other mechanisms that have been proposed in order to explain the selective recognition of old RBCs by macrophages, include the time-dependent desialylation of membrane proteins, which leads to the exposure of "senescence factor glycopeptides" on senescent cells[1]. Apart from the mechanism of RBC-bound opsonins, sialic acids and membrane constituents such as CD47, at least in animal models, are suggested to play a further regulative role in the elimination of senescent RBCs, by inhibiting erythrophagocytosis[40,41]. Their normal RBC membrane topology/stoichiometry functions as effective "non-eat-me" signals for macrophages bearing their respective receptors. However, such a role for CD47 is probably species-specific[28], since there is so far no evidence that Rhesus-null human RBCs with reduced CD47 exhibit increased rate of phagocytosis[42].

Role of the oxidative stress

The RBCs lifespan dependence on an adequate oxidative stress response, imposed by human diseases has been previously established[43]. The construction of protein-protein interaction networks in the RBC interactome confirmed that RBCs likely suffer of exacerbated oxidative stress and continuously strive against protein and cytoskeletal damage, recruiting a number of alternative pathways related to protein repair, vesiculation or apoptosis[44,45]. Although it currently constitutes an active topic of research, accumulative data suggest a central position for oxidative stress in the RBCs aging signalling. Apart from its impact on Band 3-derived neo-antigen formation and the activation of pro-apoptotic components, oxidative stress also affects Hb and its interactions with membrane components[21,27] and caspase-3[46]. Although the major feature of the oxidatively distorted RBC is the binding of oxidized Hb to high affinity sites on Band 3, the irreducible complexation of Hb with spectrin is also a prominent and probably prior marker of *in vivo* RBCs aging process, tightly correlated with increased RBC rigidity, decreased deformability, echinocytosis and erythrophagocytosis[47-50]. This complex might well promote structural modifications in Band 3 by disturbing the cohesion of the cytoskeleton to the bilayer. Moreover, its formation may threaten the normal assembly of the spectrin tetramer and the phospholipid oxidation via a Ca^{2+}-promoted

quasi-lipoxigenase activity of the oxidized Hb, leading to PS exposure and signalling recognition by the CD36 macrophage receptor[27].

In vivo aging remarks

At the present, it is difficult to assign the relative contribution of each of the various speculative mechanisms to aging and removal of senescent RBCs, but it is likely that all of them play a role in this, apparently complex and tightly regulated, process[1]. The new era of RBC proteomics revealed an incredible array of RBC proteins involved in intracellular signalling cascades[51,52], while the available refined models of RBC membrane organization suggest the involvement of other molecules or mechanisms in the aging process. The newly discovered metabolon of Band 3 and glycolytic enzymes complex[53,54], the macrocomplex of 4.1R, which may contribute to the remodelling of RBC surface[55], the Band 3-to-skeleton bridge of adducin with its possible role in the membrane mechanics and vesiculation[56] and the proposed structural role of glucose transporter-1 in RBCs[57] (the membrane expression of which is augmented under RBCs storage in blood banks[4]), are some examples of well organized -by multiple signalling pathways- elements. Once again, the "whether, how and when" of these factors into the aging process deserve to be the object of future endeavours.

Aging, senescence and death signalling pathways in stored RBCs

RBC storage lesion

Biochemical and biomechanical changes in RBCs function and integrity during storage, which affect *in vivo* survival and function, can be summarized by the term "RBC storage lesion". Easily recognizable biochemical storage effects are the reduction of adenosine triphosphate (ATP), 2,3-diphosphoglycerate (2,3-DPG), pH and glycolysis rate, the accumulation of lactic acid and the increase in Ca^{2+} intracellularly. 2,3-DPG depletion leads to increased oxygen affinity but after transfusion the 2,3-DPG levels are restored in RBCs. ATP depletion follows the reduction in glycolysis rate, leads to further energetic compromise and is associated -directly or secondary- with a series of biophysical alterations, including cellular shape, membrane stability/deformability and vesiculation[7]. Although membrane deformability has been correlated with RBCs viability after transfusion[58], there are no direct mechanical fragility measurements as a storage period function. Moreover, the contribution of ATP depletion into the storage lesion and the post-transfusion survival of RBCs have been challenged. The main biophysical effect of storage is probably the loss of membrane and Hb through the progressively increased vesiculation (Figure 1) and the subsequent changes in RBCs mechanical and rheological properties[59]. On the basis

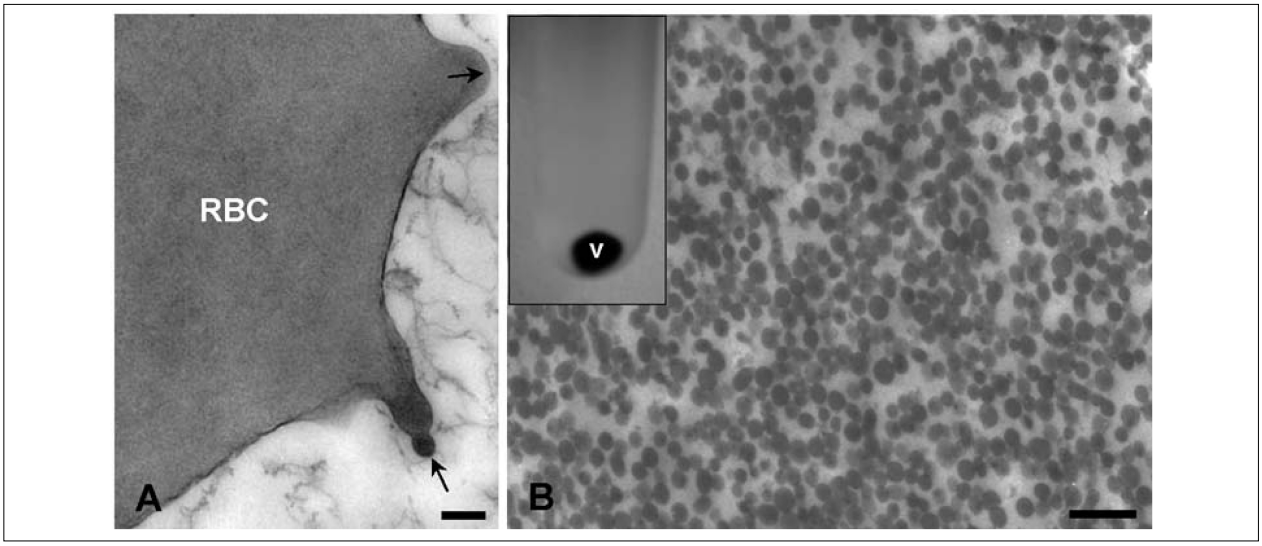

Figure 1 - Conventional electron microscopy of RBCs and vesicles released after prolonged storage (35 days) in CPD-A. (A) Membrane blebbing in a RBC that has undergone echinocytic (arrows) transformation. (B) Ultrastructural appearance of a vesicle preparation. Insert: the sediment of the vesicles (v) collected at the final step of the isolation protocol (bars, 0.5 μm).

of thrombogenetic[7] and nitric oxide scavenging[60] potential of vesicles, their transfusion is thought to be connected with adverse clinical outcomes.

The release of leukocyte-associated enzymes, cytokines and oxygen radicals have been associated with the storage lesion. Comparative proteomic analysis in stored samples showed predominant accumulation of several bioactive proteins in the supernatant of non-leukofiltered units[61]. Furthermore, the oxygen-dependent metabolic modulation is progressively altered during storage and is strongly associated with modifications in Band 3 protein[62]. In fact, the storage results in remarkable RBC membrane remodelling and vesicular protein variability[4,63,64]. Comparative analysis of RBC membranes during the progress of storage revealed changes in the presence/amount of proteasomes, chaperones, proteases, kinases, and phosphatases at the membrane[4,63]. Growing evidence portrays a time-dependent oxidative assault to Hb, membrane and cytoskeleton components, indicating that oxidative injury is a key part of the physiology of stored RBCs[5,64-67]. Interestingly, the "onset" of the storage impact in the membrane-cytoskeleton network is detected in an earlier time-point than previously appreciated[4,5,63,66,68], pointing out that any optimization attempt should be applied in the first weeks of the storage period. Moreover, recent studies showed that aged, stored RBCs have reduced ability to produce nitric oxide, while they suggested that reduction in nitric oxide bioavailability at the endothelium -via the reactivity of the cell-free Hb in stored blood- may underlie the storage lesion. The later may be connected to post-transfusion pathological outcomes, such as microvascular vasoconstriction, platelet activation and pro-oxidant and pro-inflammatory effects[60].

Band 3-related aging machinery and membrane vesiculation during storage

As expected, some profile differences between *in vivo* and *ex vivo* aging have been noticed, including the reverse change in Mean Corpuscular Volume (MCV) index[7] and the variation in size and shape of the released vesicles. Nevertheless, stored RBCs progressively express some of the typical marks of senescence and erythrophagocytosis[6,15]. More importantly, all the hallmarks of the Band 3-related aging machinery have been documented in stored RBCs: early and progressively increased accumulation of oxidized/denatured Hb to the membrane and the cytoskeleton, early complication of spectrin with Hb, aggregation of Band 3 at the membrane level and IgG deposition[4,63,66], while removal of transfused RBCs was remarkably delayed after complement aphaeresis during storage[69]. The storage-related vesiculation is a raft-based process that is exacerbated over time in the cold[7,59]. Analysis of the vesicles documented the presence of processed/aggregated Band 3 and denatured/oxidized Hb, IgGs and complement components[4,5,64], verifying the beneficial role of vesiculation in the survival of stored RBCs. It should be noted that the lasting coexistence of vesicles with their cells of origin represents another "novelty" of the *ex vivo* storage system, the consequences of which to the RBCs are still obscure.

Eryptosis/apoptosis signalling in stored RBCs

The vesicles shed by the stored RBCs expose PS[6,7] but there is still no clear consensus whether this also applies for the RBCs *per se*[28,70-72]. This matter is probably analogous to the previously stated argumentation of *in vivo* aging, further augmented in *ex vivo* conditions, because of the limited storage duration and the smaller RBC population that contains proportionally less prone to clearance cells. Notably, the storage-dependent remodelling of the RBC membrane includes the Ca^{2+}-promoted binding of sorcin and synexin[63]. Moreover, prolonged storage has been associated with modifications in Fas-associated proteins and caspase activation in both, RBCs[5,63] and vesicles[5]. This element is significant, considering that WBC-derived functional soluble Fas ligand has been detected in the RBC supernatants after prolonged storage[73]. Furthermore, semi-quantitative proteomic analysis verified the differences in the expression of lipid rafts-associated proteins between RBC membrane and vesicles[4,7], suggesting that alterations in membrane lipid organization are involved in vesicle formation and in storage-associated increase in PS exposure[6].

The effect of RBC membrane remodelling[39] and Hb oxidation[46] on caspase activation has already been suggested. Until today, it is not clear whether caspase activation is related or not to apoptosis/eryptosis in stored RBCs. Interestingly, their stimulation coincides with the detection of membrane/cytoskeleton

modifications in stored RBCs[5]. Those presumptive caspase-induced membrane modifications might interfere with the mechanical properties of the membrane[74], the extent of vesiculation[7] and the signalling of the aging *per se*[75]. The apoptotic death of RBCs during storage might work as a beneficial alternative to haemolysis, provided that apoptosis is not exacerbated by the storage. Otherwise, apoptosis of RBCs inside the storage bag will be a direct measure for *in vitro* age/stress-related RBCs changes, with a clear consequence on RBCs recovery and survival, following RBCs transfusion.

CD47-related phagocytosis of stored RBCs

Regarding other signalling pathways, loss of CD47 markers has been documented in RBCs, especially in the older ones[71], during the storage period[4,64,72,76]. Apparently, this modification may render RBCs more susceptible to clearance when transfused, but the above association has to be firmly established. CD47 has also been detected in the released vesicles[4,64], showing an ongoing interplay among their various phagocytosis-related signals.

Oxidative stress relevance to the RBC ex vivo aging

As storage progresses, antioxidant defence and oxidative injures on RBC membrane, cytoskeleton and cytoplasm components are getting worse[5,65,66,77]. Oxidative mechanisms that lead to normal *in vivo* aging (see above) are currently considered as the underlying contributor to storage lesions[67] and are probably related to accelerated and/or aberrant aging of stored RBCs. There is evidence that oxidative stress plays significant role in storage-related vesiculation[78] and in protein degradation, especially of cytoskeleton proteins[68]. The oxidative state of Hb and the incidence of Hb-induced membrane damage are modulated as a function of the storage period, signifying the key role of Hb oxidation not only in the physiology of stored RBCs but also in the progression of irreversible signalling mechanisms[5,64,66,67]. All these factors might contribute to trigger the formation of neoantigens in blood units. Although the direct link between the oxidative stress response and the RBC senescence/removal in stored RBCs remains to be firmly established, there is evidence that RBCs stored under the antioxidant and membrane-stabilizing effect of mannitol exhibit a different expression pattern of senescence marks[5]. In the same context, the storage-related variation in proteasome or protein repair molecules[4,5], likely represents responsive mechanisms against the aging-related assault in major RBC membrane proteins. In the absence of clearance mechanisms inside the blood bag, the final effect of the above functional mechanisms might be multiplied in the recipients, especially in massively transfused ones.

Concluding remarks

Under the continuous pressure of raised clinical questions concerning the safety and efficacy of stored RBCs, there is a strong need for a more thorough and updated investigation of the RBCs storage issue. Clinical practice demands for recruitment of novel biomarkers, as accurate *in vitro* predictors, not only for RBC *in vivo* survival but also for functional capability and effects. The proteomic and *in silico* approaches are valuable for performing a global screening of the storage- and aging- related RBC modifications, their course during the storage period and the impact of storage variations on it. The currently provided data on RBCs aging indicate that RBCs are highly dynamic blood components and provide the basis for further proteomic experiments with a direct impact on transfusion medicine. The future research efforts in RBCs preservation science should be enriched with emerging principles, techniques and knowledge regarding the RBCs interactome, the formation of lipid rafts and other functionally important multiprotein complexes, the repair/destroy mechanisms, the response to oxidative stress, the post-translational processing, the protein sorting into the vesicles and the membrane as a place of execution of the senescence-related apoptosis-like events. This advanced, sophisticated approach would benefit the understanding of the mechanisms that define life and death of RBCs *in vivo*, in the plastic bags and in the circulation after transfusion. It also represents the safer way to the successful optimization of RBCs preservation protocols and transfused RBCs.

We very much regret that many important studies which made significant contributions to red cell research could not be cited or discussed in this review article due to strict space limitations.

Keywords: aging, senescence, death, red blood cell signalling, proteome, apoptosis.

References

1) Aminoff D, Rolfes-Curl A, Supina E. Molecular biomarkers of aging: the red cell as a model. Arch Gerontol Geriatr 1992; **15** Suppl 1: 7-15.
2) Walsh M, Lutz RJ, Cotter TG, O'Connor R. Erythrocyte survival is promoted by plasma and suppressed by a Bak-derived BH3 peptide that interacts with membrane-associated Bcl-X L. Blood 2002; **99**: 3439-48.
3) Franco RS. The measurement and importance of red cell survival. Am J Hematol 2009; **84**: 109-14.
4) 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.
5) 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.
6) Bosman GJ, Lasonder E, Groenen-Döpp YA, et al. Comparative proteomics of erythrocyte aging in vivo and in vitro. J Proteomics 2010; **73**(3): 396-402.
7) 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.
8) Koch CG, Li L, Sessler DI. Duration of red-cell storage and complications after cardiac surgery. N Engl J Med 2008; **358**: 1229-39.
9) Dzik W. Fresh blood for everyone? Balancing availability and quality of stored RBCs. Transfus Med 2008; **18**: 260-5.
10) Willekens FLA, Roerdinkholder-Stoelwinder B, Groenen-Döpp YAM, et al. Hemoglobin loss from erythrocytes in vivo results from spleen-facilitated vesiculation. Blood 2003; **101**: 747-51.
11) Piomelli S, Seaman C. Mechanism of red blood cell aging: relationship of cell density and cell age. Am J Hematol 1993; **42:** 46-52.
12) Bosch FH, Werre JM, Roerdinkholder-Stoelwinder B, et al. Characteristics of red blood cell populations fractionated with a combination of counterflow centrifugation and Percoll separation. Blood 1992; **79**: 254-60.
13) Mueller TJ, Jackson CW, Dockter ME, Morrison M. Membrane skeletal alterations during in vivo mouse red cell aging. J Clin Invest 1987; **79**: 492-9.
14) Lutz HU, Stammler P, Fasler S, et al. Density separation of human red blood cells on self forming Percoll gradients: correlation with cell age. Biochim Biophys Acta 1992; **1116**: 1-10.
15) Bosman GJCGM, Werre JM, Willekens FLA, Novotný VMJ. Erythrocyte ageing in vivo and in vitro: structural aspects and implications for transfusion. Transfus Med 2008; **18**: 335-47.
16) Willekens FLA, Werre JM, Groenen-Dopp YAM, et al. Erythrocyte vesiculation: a self-protective mechanism? Br J Haematol 2008; **141**: 549-56.
17) Pantaleo A, Giribaldi G, Mannu F, et al. Naturally occurring anti-band 3 antibodies and red blood cell removal under physiological and pathological conditions. Autoimmun Rev 2008; **7:** 457-62.
18) Kay MM, Bosman GJ, Johnson GJ, Beth AH. Band-3 polymers and aggregates, and hemoglobin precipitates in red cell aging. Blood Cells 1988; **14**: 275-95.
19) Kay MMB. Immunoregulation of cellular life span. Ann N Y Acad Sci 2005; **1057**: 85-111.
20) Kay MM, Bosman GJ, Shapiro SS, et al. Oxidation as a possible mechanism of cellular aging: Vitamin E deficiency causes premature aging and IgG binding to RBCs. Proc Natl Acad Sci U S A 1986; **83**: 2463-7.
21) Rettig MP, Low PS, Gimm JA, et al. Evaluation of biochemical changes during in vivo erythrocyte senescence in the dog. Blood 1999; **93**: 376-84.
22) Ando K, Beppu M, Kikugawa K, et al. Membrane proteins of human erythrocytes are modified by advanced glycation end products during aging in the circulation. Biochem Biophys Res Commun 1999; **258**: 123-7.
23) Low PS, Waugh SM, Zinke K, Drenckhahn D. The role of haemoglobin denaturation and band 3 clustering in red blood cell aging. Science 1985; **227**: 531-3.
24) Kay MM, Goodman SR, Sorensen K, et al. Senescent cell antigen is immunologically related to band 3. Proc Natl Acad Sci U S A 1983; **80**: 1631-5.
25) Lutz HU, Stammler P, Fasler S. Preferential formation of C3b-IgG complexes in vitro and in vivo from nascent C3b and naturally occurring anti-band 3 antibodies. J Biol Chem 1993; **268**: 17418-26.
26) Samaja M, Rubinacci A, Motterlini R, et al. Red cell aging and active calcium transport. Exp Gerontol 1990; **25**: 279-86.
27) Kiefer CR, Snyder LM. Oxidation and erythrocyte senescence. Curr Opin Hematol 2000; **7**: 113-6.
28) Bosman GJCGM, Willekens FLA, Were JM. Erythrocyte aging: a more than superficial resemblance to apoptosis? Cell Physiol Biochem 2005; **16**: 1-8.
29) Lang F, Lang KS, Lang PA, et al. Mechanisms and significance of eryptosis. Antioxid Redox Signal 2006; **8**: 1183-92.
30) Lang KS, Lang PA, Bauer C, Duranton C, Wieder T, Huber SM, and Lang F. Mechanisms of suicidal erythrocyte death. Cell Physiol Biochem 2005; **15**: 195-202.
31) Zipser Y, Piade A, Barbul A, et al. Ca2+ promotes erythrocyte band 3 tyrosine phosphorylation via dissociation of phosphotyrosine phosphatase from band 3. Biochem J 2002; **368**(Pt 1): 137-44.
32) Mandal D, Moitra PK, Saha S, Basu J. Caspase 3 regulates phosphatidylserine externalization and phagocytosis of oxidatively stressed erythrocytes. FEBS Lett 2002; **513**: 184-8.
33) Miki Y, Tazawa T, Hirano K, et al. Clearance of oxidized erythrocytes by macrophages: Involvement of caspases in the generation of clearance signal at band 3 glycoprotein. Biochem Biophys Res Commun 2007; **363**: 57-62.

34) Mandal D, Baudin-Creuza V, Bhattacharyya A, et al. Caspase 3-mediated proteolysis of the N-terminal cytoplasmic domain of the human erythroid anion exchanger 1 (Band 3). J Biol Chem 2003; **278**: 52551-8.
35) Mandal D, Mazumder A, Das P, et al. Fas-, caspase 8- and caspase 3-dependent signaling regulate the activity of the aminophospholipid translocase and phosphatidylserine externalization in human erythrocytes. J Biol Chem 2005; **280**: 39460-7.
36) Ficarra S, Tellone E, Giardina B, et al. Derangement of erythrocytic AE1 in beta-thalassemia by caspase 3: pathogenic mechanisms and implications in red blood cell senescence. J Membr Biol 2009; **228**: 43-9.
37) Boas FE, Forman L, Beutler E. Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. Proc Natl Acad Sci USA 1998; **95**: 3077-81.
38) Connor J, Pak CC, Schroit AJ. Exposure of phosphatidylserine in the outer leaflet of human red blood cells. J Biol Chem 1994; **269**: 2399-404.
39) Bratosin D, Tcacenco L, Sidoroff M, et al. Active Caspases-8 and -3 in Circulating Human Erythrocytes Purified on Immobilized Annexin-V: A Cytometric Demonstration. Cytometry A 2009; **75A**: 236-44.
40) Ensinck A, Biondi CS, Marini A, et al. Effect of membrane-bound IgG and desialysation in the interaction of monocytes with senescent erythrocytes. Clin Exp Med 2006; **6**: 138-42.
41) Oldenborg PA, Zheleznyak A, Fang YF, et al. Role of CD47 as a marker of self on red blood cells. Science 2000; **288**: 2051-4.
42) Arndt PA, Garratty G. Rh null red blood cells with reduced CD47 do not show increased interactions with peripheral blood monocytes. Brit J Haematol 2004; **125**: 412-4.
43) Tsantes AE, Bonovas S, Travlou A, Sitaras NM. Redox imbalance, macrocytosis and RBC homeostasis. Antioxid Redox Signal 2006; **8**: 1205-16.
44) Goodman SR, Kurdia A, Ammann L, et al. The human red blood cell proteome and interactome. Exp Biol Med 2007; **232**: 1391-408.
45) D'Alessandro A, Righetti PG, Zolla L. The red blood cell proteome and interactome: an update. J Proteome Res. 2010; **9**: 144-63.
46) Tellone E, Ficarra S, Giardina B, et al. Oxidative effects of gemfibrozil on anion influx and metabolism in normal and beta-thalassemic erythrocytes: physiological implications. J Membr Biol 2008; **224**: 1-8.
47) Fortier N, Snyder LM, Garver F, et al. The relationship between in vivo generated haemoglobin skeletal protein complex and increased red cell membrane rigidity. Blood 1988; **71**: 1427-31.
48) Snyder LM, Leb L, Piotrowski J, et al. Irreversible spectrin-haemoglobin crosslinking in vivo: A marker for red cell senescence. Br J Haematol 1983; **53**: 379-84.
49) Snyder LM, Garver F, Liu SC, et al. Demonstration of haemoglobin associated with isolated, purified spectrin from senescent human red cells. Br J Haematol 1985; **61**: 415-9.
50) Snyder LM, Fortier NL, Trainor J. Effect of hydrogen peroxide exposure on normal human erythrocyte deformability, morphology, surface characteristics, and spectrin-hemoglobin cross-linking. J Clin Invest 1985; **76**: 1971-7.
51) Kakhniashvili DG, Bulla LA Jr, Goodman SR. The human erythrocyte proteome: analysis by ion trap mass spectrometry. Mol Cell Proteomics 2004; **3**: 501-9.
52) Pasini EM, Kirkegaard M, Mortensen P, et al. In-depth analysis of the membrane and cytosolic proteome of red blood cells. Blood 2006; **108**: 791-801.
53) Bruce LJ, Beckmann R, Ribeiro ML, et al. A band 3-based macrocomplex of integral and peripheral proteins in the RBC membrane. Blood 2003; **101**: 4180-8.
54) Campanella ME, Chu H, Low PS. Assembly and regulation of a glycolytic enzyme complex on the human erythrocyte membrane. Proc Natl Acad Sci USA 2005; **102**: 2402-7.
55) Salomao M, Zhang X, Yang Y, et al. Protein 4.1 R-dependent multiprotein complex: New insights into the structural organization of the red blood cell membrane. Proc Natl Acad Sci U S A 2008; **105**: 8026-31.
56) Anong WA, Franco T, Chu H, et al. Adducin forms a bridge between the erythrocyte membrane and its cytoskeleton and regulates membrane cohesion. Blood 2009; **114**: 1904-12.
57) Khan AA, Hanada T, Mohseni M, et al. Dematin and adducin provide a novel link between the spectrin cytoskeleton and human erythrocyte membrane by directly interacting with glucose transporter-1. J Biol Chem 2008; **283**: 14600-9.
58) Card RT, Mohandas N, Mollison PL. Relationship of post-transfusion viability to deformability of stored red cells. Br J Haematol 1983; **53**: 237-40.
59) Greenwalt TJ. The how and why of exocytic vesicles. Transfusion 2006; **46**: 143-52.
60) Gladwin MT, Kim-Shapiro D.B. Storage lesion in banked blood due to hemolysis-dependent disruption of nitric oxide homeostasis. Curr Opin Hematol 2009; **16**: 515-23.
61) Annis AM, Glenister KM, Killian JJ, Sparrow RL. Proteomic analysis of supernatants of stored red blood cell products. Transfusion 2005; **45**: 1426-33.
62) Messana I, Ferroni L, Misiti F, et al. Blood bank conditions and RBCs: the progressive loss of metabolic modulation. Transfusion 2000; **40**: 353-60.
63) Kriebardis AG, Antonelou MH, Stamoulis KE, et al. Storage-dependent remodeling of the red blood cell membrane is associated with increased immunoglobulin G binding, lipid raft rearrangement, and caspase activation. Transfusion 2007; **47**: 1212-20.
64) 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.

65) Kriebardis AG, Antonelou MH, Stamoulis KE, et al. Membrane protein carbonylation in nonleukodepleted CPDA-preserved red blood cells. Blood Cells Mol Dis 2006; **36**: 279-82.
66) Kriebardis AG, Antonelou MH, Stamoulis KE, et al. Progressive oxidation of cytoskeletal proteins and accumulation of denatured hemoglobin in stored red cells. J Cell Mol Med 2007; **11**: 148-55.
67) Kanias T, Acker JP. Biopreservation of red blood cells-the struggle with haemoglobin oxidation. FEBS J 2010; **277**: 343-56.
68) D'Amici, Rinalducci S, Zolla L. Proteomic analysis of RBC membrane protein degradation during blood storage. J Proteome Res 2007; **6**: 3242-55.
69) Szymanski IO, Odgren PR, Valeri CR. Relationship between the third component of human complement (C3) bound to stored preserved erythrocytes and their viability in vivo. Vox Sang 1985; **49**: 34-41.
70) Geldwerth D, Kuypers FA, Bütikofer P, et al. Transbilayer mobility and distribution of red cell phospholipids during storage. J Clin Invest 1993; **92**: 308-14.
71) Sparrow RL, Healey G, Patton KA, Veale MF. Red blood cell age determines the impact of storage and leukocyte burden on cell adhesion molecules, glycophorin A and the release of annexin V. Transfus and Apher Sci 2006; **34**: 15-23.
72) Stewart A, Urbaniak S, Turner M, Bessos H. The application of a new quantitative assay for the monitoring of integrin associated protein CD47 on red blood cells during storage and comparison with the expression of CD47 and phosphatidylserine with flow cytometry. Transfusion 2005; **45**: 1496-503.
73) Ghio M, Contini P, Mazzei C, et al. Soluble HLA class I, HLA class II and Fas ligand in blood components: a possible key to explain the immunomodulatory effects of allogeneic blood transfusions. Blood 1999; **93**: 1770-7.
74) Suzuki Y, Ohkubo N, Aoto M, et al. Participation of caspase-3-like protease in oxidation-induced impairment of erythrocyte membrane properties. Biorheology 2007; **44**: 179-90.
75) Pietraforte D, Matarrese P, Straface E, et al. Two different pathways are involved in peroxynitrite-induced senescence and apoptosis of human erythrocytes. Free Radic Biol Med 2007; **42**: 202-14.
76) Anniss AM, Sparrow RL. Expression of CD47 (integrin-associated protein) decreases on red blood cells during storage. Transf Apher Sci 2002; **27**: 233-8.
77) Dumaswala UJ, Wilson MJ, Wu YL, et al. Glutathione loading prevents free radical injury in red blood cells after storage. Free Radic Res 2000; **33**: 517-29.
78) Wagner GM, Chiu DT, Qju JH, et al. Spectrin oxidation correlates with membrane vasiculation in stored RBC. Blood 1987; **69**: 1777-81.

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