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An update on red blood cell storage lesions, as gleaned through biochemistry and omics technologies

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Abstract

Recent mass spectrometry and electron microscopy studies have provided novel insights into the molecular changes underpinning the accumulation of storage lesions to packed erythrocytes in the blood bank.

Red blood cell aging in the blood bank is characterized by the accumulation of a significant number of biochemical and morphological alterations. Biochemical lesions include altered cation homeostasis, reprogrammed energy and redox metabolism, which result in the impairment of enzymatic activity and progressive depletion of high energy phosphate compounds. These factors contribute to the progressive accumulation of oxidative stress, which in turn promotes oxidative-lesions to proteins (carbonylation, fragmentation, hemoglobin glycation) and lipids (peroxidation). Biochemical lesions negatively affect red blood cell morphology, which is marked by progressive membrane blebbing and vesiculation.

These storage lesions contribute to the altered physiology of long stored red blood cells, and promote the rapid clearance of up to one-fourth of long stored erythrocytes from the recipient's bloodstream after 24 hours from administration.

While prospective clinical evidence is accumulating, hints from retrospective clinical trials and omics/morphological approaches both suggest that RBCs stored longer than 14days might not be as safe and effective as fresh ones.

Introduction

In most countries, the shelf-life of packed RBCs is limited to 42days. However, results from retrospective clinical trials have hinted at a correlation between untoward consequences in certain categories of recipients (e.g.traumatized, critically ill or peri-operative patients) and transfusion of packed erythrocytes stored longer than 14days.[1,2]While clinical prospective evidence is still missing or inconclusive,[3] an accumulating body of evidence indicates that biochemical and morphological lesions to stored RBCs tend to accumulate soon after the second week of storage (**Figure 1**).[4-9]The bulk of this evidence comes from the application of mass spectrometry (MS)-based metabolomics, proteomics, and lipidomics to the field of transfusion medicine, as we will discuss in this review.

Omics disciplines are characterized by the systematic determination and quantification of broad classes of molecules, such as metabolites (low MW compounds below 1.5kDa), proteins[8]and lipids.[10]Data from multiple omics platforms can be then integrated through bioinformatic approaches and mathematical modeling to obtain a systems biology level of understanding.[11]

Aging *in vivo* and *in vitro*

A deeper understanding of the molecular mechanisms driving RBC aging in the blood bank should aid in the design of new storage strategies to extend the shelf-life of packed RBCs. However, these mechanisms have been only partially disclosed and are incompletely understood.[4-7]

RBC aging *in vivo* and *in vitro* are characterized by distinct mechanisms.[7,12,13]*In vivo* circulating RBCs have an approximate lifespan of 120days.[13]Approximately 10^{11} erythrocytes are generated every day and cleared from the bloodstream by residential macrophages in the reticuloendothelial system, through a synchronized mechanism underpinning their generation and senescence in peripheral blood.[7,13,14]

Circulating RBCs are characterized by heterogeneous erythrocyte populations,[15,16]and RBC populations are differentially affected by injuries *in vivo*[13,16]or storage in the blood bank.[17]Although up to 25% of the transfused RBCs is rapidly removed from the bloodstream of the recipient, RBCs that survive the first 24 hours in circulation have a normal or near normal survival.[18]This evidence strengthens the case for an increased susceptibility of certain RBC populations to the so-called "storage lesions".[17,18]

While *in vivo* aging of RBCs culminates with senescence, aging *in vitro* has been also associated to eryptosis, a controversial process that closely mimics the programmed cell death of nucleated cells (apoptosis).[14]Eryptotic phenomena *in vivo* result from injury or (oxidative) stress to RBCs.[16]Under blood bank conditions, ensuing of eryptosis is tied to storage lesions(**Figure 2**).[13]

In the following paragraphs we will relate the accumulation of biochemical and morphological storage lesions to the impaired physiology and functionality of RBCs. Focus will be on biochemical and omics studies on oxygen transport, cation homeostasis, energy and redox metabolism. The association of these storage-influenced biochemical parameters to the compromised protein and structural integrity of long-stored RBCs will be explored. Finally, we will discuss the donor variability issues, as they influence transfusion outcomes and hitherto hampered omics investigators from drawing universally valid conclusions.

Oxygen transport

Administration of transfusion therapies in the intensive care setting is associated with the need to restore tissue oxygenation, volemia and blood viscosity in response to hemorrhagic shock. Long-stored human erythrocytes are characterized by a higher oxygen affinity, since pO_2 is essentially unchanged between 3h and 14days, whereas hemoglobin O_2 saturation increases steadily throughout storage duration up to 99% by storage day 42.[19]Consistently, a storage-dependent increase in O_2 affinity was recently confirmed,[20]although *in vitro* interactions with oxygen were largely preserved through 42days of storage.[20]Such effects are promoted by the storage-dependent consumption of high-energy phosphate compounds (adenosine triphosphate-ATP and 2,3-diphosphoglycerate–2,3-DPG).[21]These compounds are known to act as allosteric effectors as they stabilize the "T"(deoxygenated) state of hemoglobin and thus their decrease positively affects haemoglobin-oxygen affinity.[20]However, it is worth noting that oxygen off-loading from hemoglobin is promoted by intracellular acidification (Bohr effect), a condition that is observed during storage in the blood bank,[22]as a result of ongoing glycolysis (Embden-Meyerhoff energy metabolism pathway).[21]Conversely, the decrease in pH has a negative feedback on glycolysis.[23] Storage is also accompanied by deregulated S-nitrosylation of hemoglobin at β93cys, suggesting a likely-compromised ''hypoxic vasodilation'' capacity of longer stored RBCs.[19]

However, it has recently been concluded that, although fresh RBCs might be superior to long-stored RBCs, increased oxygen affinity of "older" packed erythrocytes may provide a benefit in hemorrhagic shock resuscitation.[24]

Cation transport

Hypothermia during storage in the blood bank is known to negatively influence the activity of cation transporters.[25]Older RBCs display altered Na^+/K^+ fluxes,[9,19]resulting in the supernatant accumulation of potassium, a pitfall compromising transfusions to certain recipients, such as pediatric patients. Impaired potassium homeostasis is also linked to the progressive increase of

intracellular ionic calcium.[21,26,27]Depletion of ATP promotes calcium build up in the cytosol,[21]since internal Ca^{2+} is subjected to metabolic control via an ATP-dependent extrusion mechanism(Ca^{2+} pump). As a consequence, intracellular calcium accumulation triggers the opening of the Ca^{2+}-dependent K^{+} channel, other than the activation of calcium-dependent proteases(such as μ-calpain) while promoting the onset of apoptosis-like phenomena.[3]However, eryptosis in long-stored RBCs is mainly tied to starvation(depletion of high energy phosphate compounds) rather than to calcium alone.[28]Calcium loading also results in dose-dependent decreases in reduced glutathione(GSH) levels in rabbit erythrocytes,[29]and promotes glutathione S-transferase migration to the cell membrane in human RBCs.[30]

Energy and Redox Metabolism

Efficiency of energy metabolism is measured by the rate of high energy phosphate compound generation. These metabolites serve as energy tokens to be spent on the preservation of cellular homeostasis. For example, ATP levels influence membrane stability and thus RBC survival.[31]However, alterations to DPG, ATP and cation imbalances are rapidly reversible upon transfusion of RBCs in the bloodstream of the recipients.[32]

Energy and redox metabolism are intimately connected in RBCs, which can rely on glycolysis to generate approximately 90% of cell energy through anaerobic oxidation of glucose.[7]In response to high oxygen saturation and oxidative stress, glucose catabolites are channeled through the pentose phosphate pathway (PPP) to fuel the generation of NADPH and maintain GSH redox poise. Branching from glycolysis, the Rapoport-Luebering shunt interconverts the 1,3- and 2,3- isoforms of DPG, thereby connecting energy metabolism to hemoglobin-oxygen affinity.[7]Glycolysis also influences the $NADH/NAD^{+}$ ratio. NADH contributes to redox homeostasis by promoting ferric heme iron reduction back to the ferrous state, a reaction catalyzed by the NADH-dependent enzyme cytochrome b5 reductase in the methemoglobin reduction pathway.[7]As the list of proteins in the RBC proteome rapidly expands through modern proteomics approaches(recently extended to 2289 entries and counting),[33,34]novel or hitherto underinvestigated RBC metabolic pathways might emerge in the future as key players in the accumulation of storage-triggered metabolic lesions. In analogy to cancer induced alterations to cell metabolism, examples might be represented by serine and glutamine metabolism, and their indirect role in glutathione homeostasis.[35]

Energy metabolism

RBC storage is characterized by the progressive depletion of ATP and DPG reservoirs, a phenomenon facilitated by the negative influence of hypothermia on enzyme activities. Nevertheless, storage is accompanied by the constant accumulation of lactate in the supernatants.[21]

Storage of packed erythrocytes in CPD-SAGM,[21,36,37]MAP,[38]AS1[39]or PAGGGM[40,41]results in the early accumulation of glycolytic intermediates during the first two weeks of storage and rapid decrease soon afterwards. These observations likely arise from a metabolic modulation that promotes a shift towards the PPP via partial glycolytic blockade. However, the ratio of metabolic intermediates of the PPP and glycolysis[21,36]indicates that such a compensatory mechanism, whether confirmed, might only be transient and progressively impaired[35]from the second storage week onwards.[21]Alkaline additives or rejuvenation solutions are currently under evaluation as they have been reported to better preserve, or to replenish, ATP and DPG reservoirs even upon extended storage.[42]

Redox metabolism

Storage of RBCs results in the progressive deregulation of the redox poise, as it is accompanied by decreased GSH and increased GSSG levels.[21]GSH homeostasis is negatively affected by a decline in GSH anabolism, resulting from a reduced uptake[43]and increased efflux[44]of aminoacid precursors (glutamate, glutamate-precursor glutamine, glycine and cysteine), secondary to a storage-dependent decrease in ATP concentrations.[43]

Reactive oxygen species (ROS) in the form of hydroxyl radicals and superoxide are generated through Haber Weiss and Fenton reactions from heme iron.[45]ROS tend to reach a maximum within the first two weeks of storage, both in leukofiltered and non-leukofiltered units (though to a lesser extent in the former).[8,26,46,47]

The progressive loss of metabolic modulation is attributable to lesions targeting the band 3

The anion exchanger 1-band 3(AE1) is the most abundant RBC membrane protein (≈1x10^6 copies/cell). AE1 lies at the crossroads between anion homeostasis, gas transport and metabolic modulation.[7]The main role of this protein is to promote the so-called "chloride shift", a process resulting in the exchange of cellular HCO_3^- with plasma Cl^-. In so doing, AE1 participates with carbonic anhydrase (CA) to modulate gas transport (O_2 release and CO_2 uptake). By favoring the conversion of the weak acid H_2CO_3 to the strong acid HCl, AE1 contributes to the acidification of the intracellular pH. The transient acidification triggered by AE1 activity boosts O_2 release from hemoglobin (Bohr effect), and oxygen supply to those tissues producing more CO_2 (lactate rich acidic districts). However, AE1 is not only tied to gas transport homeostasis, since its N-terminal cytosolic domain provides a docking site for a series of structural proteins and glycolytic enzymes (e.g.phosphofructokinase, aldolase, glyceraldehyde-3-phosphate and lactate dehydrogenases-**Figure 3A**).[48,49]These interactions result in the assembly of a multi-protein complex often referred to as the "respiratory metabolon". Biochemical studies have highlighted a role for the negatively charged

residues at the N-terminal cytosolic domain in mediating enzyme-AE1 interactions. These interactions are further promoted by phosphorylation of tyrosine 8 and 21.[50]Negative charges in this region also serve to stabilize deoxy-hemoglobin,[49]whose binding to AE1 triggers the release and thus reactivation of otherwise bound-inhibited glycolytic enzymes (**Figure 4**), providing an oxygen-dependent metabolic modulation.[49]

AE1 is also involved in redox homeostasis, as it also interacts with peroxiredoxin2,[51]a scavenger of low-level hydrogen peroxide.[52]Progressive translocation of peroxiredoxin2 to the RBC membrane during storage in the blood bank has been documented,[52]both in leuko- or non-leukoreduced units,[8,26,53]and proposed to be a biomarker of autologous blood transfusions as illicit doping practices for endurance sport athletes.[17]

Clustering of the extracellular regions of AE1 might contribute to the removal of transfused erythrocytes from the bloodstream of the recipient, by stimulating binding of anti-band 3 antibodies and removal by the spleen and liver macrophages.[54]Additionally, alterations to the oligomeric state of AE1 have been reported to precede membrane phospholipid loss during storage of RBCs in the blood bank.[55]Oligomerization of AE1 might be promoted by oxidative stress, since oxidized and poorly-glycosylated AE1 is selectively phosphorylated by Syk kinase, which in turn promotes the formation of large membrane clusters in normal and glucose 6-phosphate dehydrogenase-deficient RBCs.[50]

Finally, AE1 is targeted by intracellular proteases(such as caspases) and ROS, which results in the generation of distinct fragments of AE1(24 and 35kDa, respectively – **Figure 3B**).[56]Of note, caspase-3 activation is consistent with the storage-dependent triggering of a Fas/caspase-driven death program.[57]

Oxidative damage to proteins: fragmentations, carbonylations and non-enzymatic glycation

Storage-dependent oxidation of proteins results in at least three main documented events: (i)increased protein fragmentation, membrane migration or externalization;[8,46,47,58-62](ii)increased protein carbonylation;[8,61-64](iii)increased (non-)enzymatic glycosylation of cytosolic[65]and membrane proteins.[66]

Alterations to RBC cytosolic and membrane proteins during storage have been extensively documented through proteomics technologies,[8,46,58-62]and include: (i) the fragmentation of structural proteins (spectrin, ankyrin, AE1, band 4.1 and 4.2-either triggered by proteases or ROS); (ii) membrane accumulation of hemoglobin, antioxidant enzymes(peroxiredoxin2) and chaperones; (iii) cytosolic decrease of transglutaminase-2, beta actin, and copper chaperone for superoxide dismutase.[8,46,57-62]Remodeling of the cytoskeleton has been appreciated through the observed

relocation of vesicle-associated membrane fusion proteins (SNAPs)[8]and the decrease in RBC membrane content of lipid raft-associated proteins flotillins and stomatin.[60]Stored RBCs also tend to exocytose the otherwise functional proteasome,[67]which is likely indicative of an impaired capacity of the ubiquitination system in older RBCs,[68]thus limiting the removal of irreversibly damaged proteins.

Aging of RBCs, both *in vivo* and *in vitro,* also promotes a conformational change to CD47, a"do not eat me"signal for erythrocyte phagocytosis as it interacts with the inhibitory immunoreceptor SIRPα expressed by macrophages.[69]Senescent and long-stored erythrocytes display CD47 that has undergone a conformational change which triggers its binding to thrombospondin-1(TSP-1). This promotes RBC phagocytosis by human red pulp macrophages and is thus associated with a shortened survival of transfused older RBCs.[69]

Carbonylation of RBC proteins, a hallmark of oxidative lesions, increases until the fourth week of storage[8,61,64](especially in non-leukodepleted units),[26]and then decreases by the end of the storage period. The decrease is either due to proteasome activity or vesiculation,[63,70]two likely self-protective/age-dependent mechanisms that will be further discussed below.

ROS-mediated non-enzymatic glycosylation of proteins (i.e.glycation) has been reported to target the most abundant cytosolic (i.e. hemoglobin alpha and beta chains)[65]and membrane proteins.[66]Such phenomena might be exacerbated by the excessive glucose found in anti-coagulation and additive solutions. Indeed, by the end of the storage period, glucose levels in the supernatants of CPD-SAGM-stored packed erythrocytes are approximately of 12±1mM,[41]which is still higher than circulating glucose in diabetic patients (subjects with a consistent glycemia above 7mM are generally deemed to be diabetic).[71]

Increased rates of enzymatic glycosylation of RBC membrane proteins is a potentially adverse event,[66]as membrane-associated carbohydrate structures contribute to alter rheological properties and the pro-immunogenic potential of transfused erythrocytes.

Oxidative stress to lipids

Aging of RBCs results in the progressive accumulation of oxidative stress markers in the lipid fraction (in the form of malondialdehyde[8,72]or prostaglandins, such as 8-isoprostane).[21]Again, a factor contributing to lipid peroxidation might be represented by the elevated glucose loading in collection and storage solutions, which fuels glucose autoxidation.[73]Accumulation of high levels of prostaglandins or oxidized lipids in the supernatants of long-stored erythrocyte concentrates are likely to promote adverse events (e.g. TRALI) or inflammatory responses in the recipients.[74,75]

Like the proteome, the RBC lipidome is diet (and thus donor)-dependent and subject to stability, in that mature erythrocytes are devoid of *de novo* long-chain fatty acid synthesis enzymes.[76]In this view, alterations to the lipidome are regarded to be irreversible. One of the earliest observed alterations to the RBC lipidome during storage in the blood bank is the progressive increase in membrane phospholipid asymmetry, owing to consumption of ATP reservoirs, which results in the apoptosis-like[14]externalization of phosphatidylserine(PS) to the outer leaflet of the plasma membrane.[77]

Recent lipidomics studies[78,79]indicated that long-stored RBCs display higher levels of ceramides, that are released from cell membrane sphingomyelins by an acid sphingomyelinase.[80]The stimulation of sphingomyelinase is dependent upon the platelet-activating factor (PAF), which is in turn generated from cell membrane lipids by an osmotic shrinkage-dependent phospholipase.[14]

Morphological changes and membrane exovesiculation

Physiological shape, protein interactions and surface area-to-volume ratio jointly determine the biomechanical properties of circulating RBCs that are critical for their survival.[81]Unlike the *in vivo* aged RBCs, that progressively become smaller and more dense,[13]storage in the blood bank is associated with an early and probably reversible increase in erythrocyte volume.[26,82]This phenomenon theoretically affects cell deformability.[82]Visible shape abnormalities appear soon after the first week of storage and occur in about one third of the original RBC population by the end of the storage period.[9,26,83]These changes result in loss of the smooth biconcave disc morphology and acquisition of altered morphologies, either reversible or irreversible (echinocytes and stomatocytes, or spherocytes).[9,83]

Reversibility of morphological alterations is inversely proportional to storage duration, and is dependent upon membrane-cytoskeletal interactions.[84]These in turn are modulated by cellular metabolism, ATP levels, and cation homeostasis. Morphological variation seems to be closely associated with storage-related disturbances in cellular deformability,[85]osmotic fragility,[9]mechanical fragility[86]and rheological properties.[83,87]To some extent, morphological lesions can be prevented by leukoreduction[88]or reversed by rejuvenation.[86]

Irreversible morphological alterations are those involving loss of significant portions of the RBC membrane through exovesiculation. Microvesicles are released from the tips of the echinocytic spines of RBCs transformed beyond the early spheroechinocyte stage.[89]Microvesicles are a measure of RBC damage during storage as well as a potential source of mediators that lead to adverse post-transfusion effects. In extreme spherocytosis, the loss of all extra surface implies critically

compromised cell surface-to-volume ratio and deformability, which aggravate both in-bag hemolysis and post-transfusion recovery.[90]

RBC-shed microparticles have also been considered as biomarkers of storage quality. Indeed, they are important carriers of extracellular Hb[60,62,91,92]and can contribute to immunogenic, proinflammatory, procoagulant, thrombogenic and NO scavenging activities.[93-98]Vesicles shed from apoptotic RBCs are characterized by phosphatidylserine externalization.[99]

The rate of RBC vesiculation increases after the second week of storage.[62]Interestingly, not only the extent but also the nature of RBC vesiculation mechanisms may vary with storage time,[60]either in terms of size, structure,[62]protein composition[60,62,85,99-103]or PS exposure.[85]In addition, microvesiculation exhibits dependence on RBC age,[66]as well as on manufacturing method and storage settings, including the presence of additive solutions, leukoreduction[26,104]and the plasticizer material used in blood bags.[105]In particular, pre-storage leukoreduction reduces the total levels of both RBC-derived macroparticles and microparticles-mediated procoagulant and inflammatory markers.[96,98,101]

Mass spectrometry-based proteomics studies of RBC membrane and vesicles suggested that vesiculation *in vitro* is a different process than vesiculation *in vivo*.[60,102]Indeed, *in vivo* vesiculation is suggested to be an integral part of the cellular homeostasis and physiologic aging process[106]for the efficient disposal of damaged or dangerous RBC components.[92]However, storage-related disturbances in cellular metabolism (energy depletion), biomechanical properties, calcium, ceramide and PS-exposure levels further exacerbate the formation of microparticles.[95,107,108] Immunoblotting,[62]flow cytometric[103]and proteomic analysis[60,99-102]suggested that several factors may influence the vesicles release profile during storage, including the storage-dependent acceleration of RBC protein breakdown and oxidation, as described above.

Clarifying the root causes of RBCs vesiculation *in vitro* is critical for improvement of current blood component processing and storage strategies. For example, it has recently been reported that, in a murine model of transfusion, addition of antioxidants to stored RBCs units results in a significant decrease in microparticle formation as well as improved RBC 24-hour post-transfusion recovery and recipient alloimmunization.[109]Additional beneficial effects might derive from the introduction of filters designed to remove immunoglobulins, cytokines, and other bioactive proteins from aged RBC supernatants.[110]

miRNAs

Micro RNAs (miRNAs) are known to be involved in post-transcriptional/translational control, which should be absent in anucleated ribosome-free RBCs. However, when assaying 52miRNAs in

stored packed RBCs, Kannan and Atreya detected a significant alteration in the levels of miR-96, miR-150, miR-196a, and miR-197, which increased during the first 20days of storage and decreased thereafter.[111]These miRNAs might derive from residual leukocytes and platelets in the unit. Indeed, modern pre-storage leuko- and platelet-reduction filters only remove 3-3.5logs and ~2logs white blood cells and platelets, respectively.[112]Nucleated white blood cells and platelets contain machinery to process pre-miRNAs into mature miRNAs, and specific platelet miRNA levels have been found to correlate with platelet reactivity.[113]Functional studies in the future will determine whether and to what extent miRNAs accumulating over storage might affect transfusion recipients.

Post-translational modifications

Although hitherto underinvestigated, protein post-translational modifications might represent a key biological mediator of molecular signaling events triggered by storage-dependent variables. For example, kinases such as AMPK and PKC might be activated by ATP consumption and intracellular calcium accumulation, and thus mediate downstream signaling. Phosphorylation of downstream targets might thus affect enzyme activities or the stability of structural proteins. Of significance, control of the RBC shape and membrane dynamics are both consequences of dynamic cytoskeleton alterations at spectrin junctions, a process requiring ATP hydrolysis.[114]This local remodeling of the membrane is likely related to ATP-driven phosphorylation of specific structural proteins, a reoccurring theme in the regulation of membrane stability. Indeed, coupling between the phospholipid bilayer and the spectrin/actin network governs the deformability of RBCs through complex protein-protein interactions that are modulated by phosphorylation.[115,116]Interestingly, recent studies have documented changes in the phosphorylation status of membrane proteins in sickle red cells[117-119]and associated these events to morphology-related abnormalities typical of diseased erythrocytes. Similar studies on stored RBCs are still missing, although one of the hallmarks of storage lesions is the irreversible alteration of the shape phenotype, which is mediated by membrane perturbations and cytoskeleton dysfunctions, as discussed above. By means of phosphoproteomics technologies, we recently observed a storage duration-dependent increase in the Ser/Thr phosphorylation status of some crucial RBC membrane proteins(*e.g.* AE1, spectrin, ankyrin, band 4.1 and adducin).[120]In line with available models of the RBC membrane organization, these preliminary data confirm a pivotal role in the regulation of membrane mechanics and erythrocyte surface remodeling of the 4.1R macrocomplex and the adducin-to-cytoskeleton bridged complex, which contains the membrane-spanning proteins AE1 and glycophorin C. The reduced

survival of transfused RBCs might thus be attributable, in part, to deformability-linked phosphorylation-events.[120]

Other post-translational modifications, such as the above-discussed (non)-enzymatic glycosylation might also be deleterious to RBC function. For example, advanced glycation end products of proteins from stored RBCs increase endothelial ROS generation through the interaction with the receptor for advanced glycation end products in the recipient.[121]

Donor variability and pre-analytical issues

Sample heterogeneity is a critical issue in omics studies.[42]Variability in donated units arises from biological (donor) and technical factors, also referred to as pre-analytical issues.[122]These variables complicate *in vitro* studies of RBCs storage, limit RBC storage system development and confound studies aimed at determining the impact of storage lesions on transfusion outcomes.[123]

Variability in erythrocyte storage characteristics among healthy donors is a long-recognized phenomenon that goes by the name of "storability". Storability has been a major unsolved problem throughout the history of blood banking. The variability issue was firstly recognized in the '60s by Dern et al.[124]and remains a concern in modern storage strategies. Indeed, under the same conditions, different blood donors have markedly different RBC pre- and post-transfusion capacities. Unknown donor-related factors not only affect a range of physiological properties of stored cells, but they have also been shown to represent the most significant contributing factors influencing in-bag hemolysis and RBC recovery.[91,124-126]A major factor affecting these parameters is the donor-dependent RBC capacity to cope with oxidative injury, as gleaned through recent investigations on post-storage viability either in inbred mouse strains or small groups of humans.[125]Several RBC storage-dependent physiological parameters have been found to display donor-dependence as well, including leukoreduction-associated hemolysis,[26]RBCs age upon donation,[17,127]metabolic rate and metabolite concentrations (e.g.ATP),[128]fragility profiles,[129]membrane vesiculation degree,[26]susceptibility to oxidative stress[53,130]and many more clinically significant properties of stored RBCs (such as vascular effects observed in the recipients).[131]Moreover, storage lesions appear to be dependent upon donor gender, age and smoking habit,[132-134]and can be influenced by the genetic background of the donor. The genetic background either implies beta thalassemia traits, glucose-6-phosphate dehydrogenase deficiency,[135]intrinsic variation in erythrocyte HbA1c[136]or PS-exposure levels.[137]

Within this framework, the currently ongoing Recipient Epidemiology and Donor Evaluation Study (REDS)-III has been designed to test whether genetic characteristics of the donors underlie the inter-donor variability observed in storage-related hemolysis.[138]Identifying the critical factors

influencing "storability" may ease the assessment of donated blood quality, and help tailor manufacturing strategies that could cope with the variability issue.

Other than donor variability, pre-analytical issues[122]and processing strategies (leukofiltration, pathogen inactivation, additive solutions, rejuvenation, etc.)[139]influence the phenotype of packed RBCs. Examples of variability include supernatant K^+ levels and hemolysis,[140]inflammatory response mediators,[141]oxygen transport,[142]phosphatidylserine exposure,[137]eicosanoid mediators,[125]as well as the amount and composition of microparticles in the supernatant.[143]These parameters are often a function of specific storage settings and manufacturing strategies.[139]Leukofiltration and additive solutions significantly alter biochemical profiles of RBC units, as gleaned through omics investigations.[8,26,101]Affected parameters include the rate and extent of hemolysis, erythrophagocytosis, vesiculation and oxidative stress management of the cells.[123,132,138,140]Finally, overnight hold of whole blood at room temperature before component processing affects several *in vitro* measures (ATP, 2,3-DPG, hemolysis)[144]and membrane properties(osmotic resilience, vesiculation).[145]

Conclusion

Transfusion of packed RBCs still represents one of the most valuable life-saving treatments in many areas of modern medicine. Despite controversial retrospective clinical studies, prospective evidence recommending against the use of packed RBC units stored longer than two weeks as an issuable blood-derived therapeutics is still missing or inconclusive. On the other hand, an accumulating body of evidence from biochemical, morphological and omics investigations suggests that RBCs stored longer than 14days are characterized by the accumulation of a series of lesions that make them qualitatively different from fresh RBCs. As of now, it is unclear whether and to what extent these lesions might end up compromising the safety and effectiveness of the transfusion therapy.

However, the hereby reviewed literature can help pave the way for the development of alternative storage strategies aimed at abrogating the potential risk factors associated with the transfusion of older units. In this view, omics technologies can guide the development and testing of currently available or future alternatives to routine storage. Examples include, but are not limited to, the introduction and optimization of alternative storage strategies (cryostorage[146,147]or deoxygenation[148,149]) or (additive/rejuvenation[150]) solutions, such as those envisaging the implementation of alkaline pH[151]or anti-oxidants[109,152]in the storage unit. Additionally, future biochemical and omics studies should be applied to emerging technologies in the field of transfusion medicine, such as stem cell-derived *ex vivo*-generated RBCs.[153]

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Figure legends

Figure 1 - Dynamics of the main biochemical lesions during RBC storage.

Time course Z-score normalized quantitative changes of biochemical and morphological parameters during storage durations. Heat maps (left) and sparklines (right) have been graphed based upon re-elaboration of original results from references no. 8, 9, 19, 21, 26, 59, 64, 65. Quantitative changes are graphed either in blue (decrease) or red (increase) against normalized values.

Figure 2 – An overview of the main biochemical changes of in vitro aging red blood cells (RBCs) under blood bank conditions.

In clockwise order:

(1) Cation homeostasis dysregulation (K^+, Ca^{2+}) is influenced by low temperatures and progressive depletion of high energy phosphate reservoirs (adenosine triphosphate – ATP and 2,3-diphosphoglycerate - DPG).

(2) Glucose (additive solution) is internalized through GLUT transporters and consumed through the Emden-Meyerhof glycolytic pathway, as to produce ATP, lactate (LAC) and promote pH lowering.

(3) However, low temperatures and the progressive accumulation of oxidative stress (likely triggered by Hb–mediated Fenton reactions) promote a metabolic divertion towards the pentose phosphate pathway, as to produce the oxidized glutathione (GSSG)-reducing cofactor NADPH.

(4) Alterations to calcium (Ca^{2+}) homeostasis (and of other second messenger signaling molecules, such as cAMP and AMP) promote the activation of specific kinases (e.g. PKC, PKA, AMPK) or rather activate proteolytic enzymes (such as calpains) that start digesting structural and functional proteins at the cytosol and membrane level, above all band 3 (AE1).

(5) Anion exchanger 1/band 3 (AE1) is responsible for the chloride shift, whereby bicarbonate (HCO_3^-) is exchanged for chloride (Cl^-), thus modulating anion homeostasis, intracellular pH and, indirectly, Hb-oxygen affinity and thus gas exchanges. Fragmentation of the cytosolic domain of AE1 (mediated by reactive oxygen species – ROS, calpain and caspases) promotes displacement of glycolytic enzymes (thereby bound/inhibited) and structural proteins (ankyrin – ANK, band 4.2 and 4.1).

(6) Enhanced oxidation of cytosolic proteins is partly challenged by antioxidant defenses (SOD1, PRDX2) and chaperone molecules (heat shock proteins – HSPs), while they progressively result in the accumulation of redox modifications to proteins (carbonylations, glycation of hemoglobin – HbA1c, protein fragmentation) and lipids (lipid peroxidation, accumulation of prostaglandins in the supernatant).

(7) A role in the process is also mediated by alternative degradation strategies to proteins (proteasome, eventually extruded in the supernatant) and lipids (sphingomyelinase-dependent accumulation of ceramides).

(8) At the membrane level, storage results in the accumulation of AE1 clusters, exposure of phosphatidylserine (PS) in the outer leaflet, and lipid raft formation that could alter the RBC pro-immunogenic potential.

(9) Taken together, these alterations affect membrane deformability, increase osmotic fragility and promote vesiculation events, a process through which micro- and nanovesicles are shed as to eliminate irreversibly altered proteins (among which traces of glycolytic enzymes.

(10) Exocytic vesicles are indeed enriched with hemoglobin, lipid raft proteins and membrane portions (also exposing common rheological antigens – CD47, Rh, RhAG, glycophorin A-GPA).

Figure 3 – Band 3 and the transport metabolon

In A, an overview of the binding sites for the glycolytic and structural enzymes on the CDB3 and the relative binding sites (red bold font). In B, aminoacid sequence targeted by ROS (red bold font), caspase (blue bold font) and μ-calpain (green bold font) during storage in the blood bank, either from the literature or in silico predicted via GPS-CCD (score > 0.8 – freely available at http://ccd.biocuckoo.org/down.php). ROS, caspase and calpain generate three different fragment of CDB3 of 34, 24 and 43 kDa, respectively.

Figure 4 – Oxygen-dependent metabolic modulation by band 3 and storage lesions

Oxygen-dependent metabolic modulation by band 3 is mediated by the competitive binding of deoxyhemoglobin and glycolytic enzymes phosphofructokinase (PFK), aldolase (ALDOA) and glyceralhdehyde 3-phosphate dehydrogenase (GAPDH) for the cytosolic domain of band 3 (CDB3). In **A**, glycolytic enzymes are bound to the CDB3 and thereby inhibited in response to high oxygen saturation conditions and oxidative stress. This mechanisms promotes a metabolic channeling of glucose catabolites to the pentose phosphate pathway (PPP). In **B**, deoxyhemoglobin binding to the CDB3 dislocates glycolytic enzymes and thus promotes their activation, which in turn results in the promotion of metabolic fluxes through the Embden-Meyerhof glycolytic pathway. In **C**, fragmentation of the CDB3 is triggered by the activation of caspases, calpain and reactive oxygen species (ROS). ROS are produced through Fenton and Haber Weiss reactions from hemoglobin iron. Fragmentation of CDB3 results in the impairment of the oxygen-dependent metabolic modulation of RBCs. Enzymes are either shown in red (**A -** inhibited), green (**B** – active), or orange (**C** – enzyme activity potentially influenced by storage lesions, among which low pH, post-translational modifications or CDB3 fragments generated either by ROS, calpain or caspase).

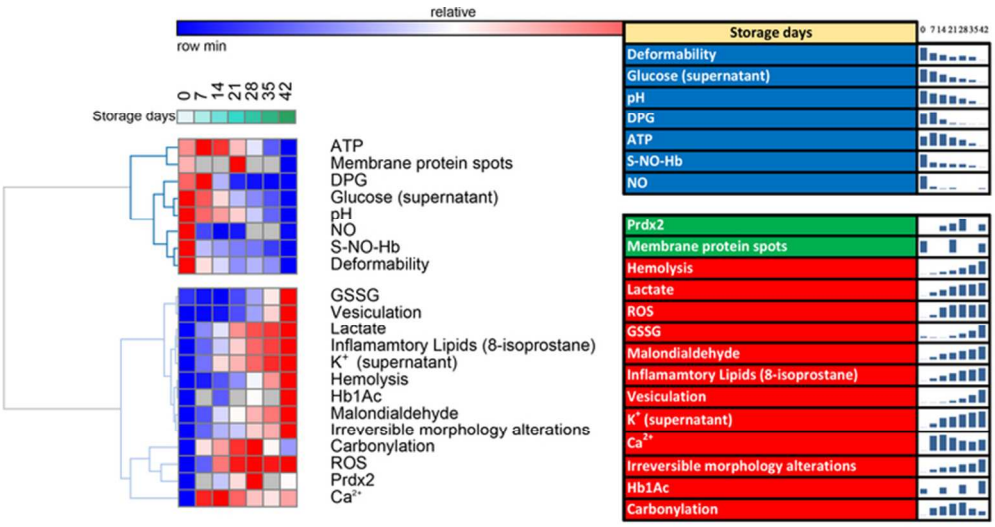

Figure 1 - Dynamics of the main biochemical lesions during RBC storage.
Time course Z-score normalized quantitative changes of biochemical and morphological parameters during storage durations. Heat maps (left) and sparklines (right) have been graphed based upon re-elaboration of original results from references no. 8, 9, 19, 21, 26, 59, 64, 65. Quantitative changes are graphed either in blue (decrease) or red (increase) against normalized values.
66x35mm (300 x 300 DPI)

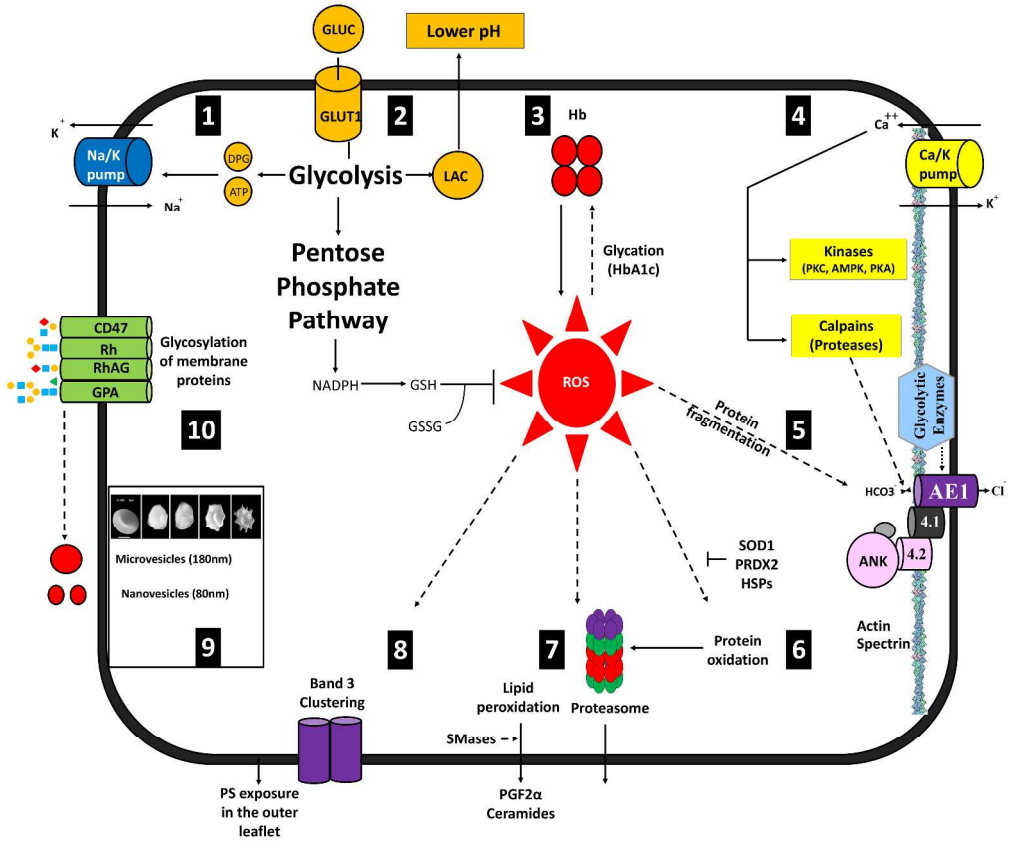

Figure 2 – An overview of the main biochemical changes of in vitro aging red blood cells (RBCs) under blood bank conditions.

In clockwise order:

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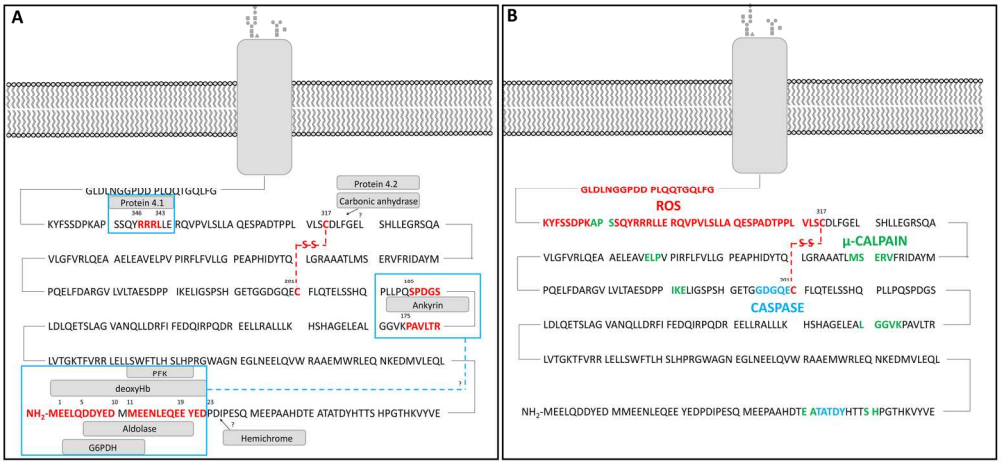
450x377mm (300 x 300 DPI)



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180x83mm (300 x 300 DPI)