Time-course investigation of SAGM-stored leukocyte-filtered red bood cell concentrates: from metabolism to proteomics

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background
Results from recent, highly debated, retrospective studies raised concerns and prompted considerations about further testing the quality of long stored red blood cells from a biochemical standpoint.

Design and Methods
We performed an integrated mass spectrometry-based metabolomics and proteomics time-course investigation on SAGM-stored red blood cells. In parallel, structural changes during storage were monitored through scanning electron microscopy.

Results
We detected increased levels of glycolytic metabolites over the first 2 weeks of storage. From day 14 onwards, we observed a significant consumption of all metabolic species, and diversion towards the oxidative phase of the pentose phosphate pathway. These phenomena coincided with the accumulation of reactive oxygen species and markers of oxidation (protein carbonylation and malondialdehyde accumulation) up to day 28. Proteomics evidenced changes at the membrane protein level from day 14 onwards. Changes included fragmentation of membrane structural proteins (spectrin, band 3, band 4.1), membrane accumulation of hemoglobin, antioxidant enzymes (peroxiredoxin-2) and chaperones. While the integrity of red blood cells did not show major deviations at day 14, at day 21 scanning electron microscope images revealed that 50% of the erythrocytes had severely altered shape. We could correlate the scanning electron microscopy observations with the onset of vesiculation, through a proteomics snapshot of the difference in the membrane proteome at day 0 and day 35. We detected proteins involved in vesicle formation and docking to the membrane, such as SNAP alpha.

Conclusions
Biochemical and structural parameters did not show significant alterations in the first 2 weeks of storage, but then declined constantly from day 14 onwards. We highlighted several parallelisms between red blood cells stored for a long time and the red blood cells of patients with hereditary spherocytosis.

Key words: red blood cell, storage, mass spectrometry, proteomics, metabolomics.

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Introduction

Red blood cell (RBC) concentrates may be stored for up to 42 days under controlled conditions before transfusion. However, there are still concerns about the suitability of older blood units for transfusion purposes. This is especially true for certain categories of recipients, such as traumatized, post-operative and critically ill patients.[1] Despite accumulating retrospective evidence of reduced blood viability after the first 2 weeks of storage,[2] definitive results from prospective, randomized, double-blind clinical trials are still lacking[3,4] or inconclusive. These problems are mainly due to the intrinsic statistical limitations of the experimental models[4] or the lack of common methods and shared standards between laboratories.[5] A frequent conclusion to the debate has underscored radical questions about the balance of risks and benefits of RBC transfusion.[6] Nevertheless, attempts to produce prospective clinical evidence have led to the quasi-philosophical statement that "available data do not support an adequate suspicion that long-stored RBCs may be associated with common adverse morbidity and/or mortality outcomes, so as to justify exposing experimental subjects to the other known or probable, albeit rare, risks of old RBCs".[7]

It is beyond the scope of this article to provide a proper clinical answer to the critical question on the quality of long-stored RBC. Here, however, we have performed a multi-faceted investigation of RBC storage trying to support and expand existing knowledge from a mere biochemical perspective. Biochemical approaches have already provided convincing evidence that refrigerated storage causes alterations to RBC, which are only reversible to some extent; these alterations are an array of phenomena which are collectively called the "storage lesion".[8-10]

Numerous changes occur in RBC during storage which may irreversibly alter their biological functions, including delivery of oxygen to cells.[11] Increases in O_2 affinity in stored RBC have been well documented;[11,12] these reflect progressive decreases in 2,3-diphosphoglycerate (2,3-DPG) levels over the weeks of storage.[12] However, the O_2 delivery capacity of transfused RBC that have been stored is deficient even early after processing and before a significant decline in 2,3-DPG.[14] Further lesions occur in stored RBC, which have been shown to lose potassium, have depleted ATP stores, and altered lipids and membranes. These lesions result in more rigid cells and reduced oxygen off-loading.[8-10] The suspending fluid becomes enriched with free hemoglobin and biologically active lipids, along with large quantities of negatively charged microvesicles.[14,15] Membrane protein fragmentation[16] and accumulation of membrane biomarkers[17,18] have also been reported to correlate with storage duration.

In the present study we report an integrated overview of the biochemical processes taking place in RBC during storage. New tests such as metabolomics[19] have been performed for the first time on RBC stored in a solution of saline, adenine, glucose and mannitol (SAGM). Recent literature only covers RBC stored in mannitol-adenine-phosphate.[20]

Design and Methods

This study was approved by the Italian National Blood Centre. The design and methods of the study are outlined below, but further details are provided in the *Online Supplementary Design and Methods*.

Sample collection

Whole blood (450 mL ± 10%) was collected from healthy volunteer donors into citrate-phosphate-dextrose anticoagulant (63 mL) and leukodepleted. After separation of plasma by centrifugation, RBC were suspended in 100 mL of SAGM additive solution. We studied RBC units collected from eight donors [male = 4, female = 4, age 45±11.5 (mean ± SD)]. RBC units were stored under standard blood bank conditions (1-6 °C) and samples were removed aseptically for the analysis every week from day 0 until day 42 of storage.

Metabolomics

Samples from the eight units were extracted and treated as extensively reported in the *Online Supplementary Design and Methods*[19] which also includes details of the high performance liquid chromatography (HPLC) settings, mass spectrometry settings and statistical analysis of the metabolomics results. Results were plotted as fold-change variations upon normalization to day 0 control values, as described by Nishino *et al.*[20]

Oxidative stress

Reactive oxygen species

Reactive oxygen species (ROS), protein carbonylation and accumulated malondialdehyde levels were calculated spectrophotometrically, as extensively described in the *Online Supplementary Design and Methods*.

Proteomics

Red blood cell protein extraction

Human erythrocyte membrane and cytosol proteins were extracted on day 0, day 14 and day 35 as previously described,[16] with minor modifications, including either the presence or absence of N-ethylmaleimide (NEM) in the extraction protocol to prevent artifactual oxidation of thiol groups[21] (further details are provided in the *Online Supplementary Design and Methods*).

Two-dimensional electrophoresis

Protein precipitates were prepared as previously reported,[16] in the presence or absence of NEM. A total of 250 μL of the resulting protein solution was then used to perform two-dimensional electrophoresis [13 cm long IPG 3-10 NL (Amersham Biosciences) for the first dimension and a 5-16% T gradient sodium dodecylsulfate polyacrylamide gel]. Further experimental details are provided in the *Online Supplementary Design and Methods*. Proteins were visualized by staining with Coomassie Brilliant Blue G-250 stain.

Image statistical analysis

Ninety-six stained gels (3 technical replicates × 8 biological replicates × 2 groups x 2 periods – day 0 and 35 – with or without NEM) were digitalized and elaborated for among-group comparisons (ANOVA) as previously reported[16] and extensively described in the *Online Supplementary Design and Methods*. Differential protein expression was considered statistically significant for P values less than 0.05 and the change in the photodensity of protein spots between day 0, day 14 and day 35 samples (with or without NEM) had to be more than 2-fold. Moreover, as protein fragments were the main changes to be expected,[16] we took into account only protein spots with an apparent molecular weight of below approximately 60 kDa.

In-gel digestion and protein identification by mass spectrometry

Protein spots were carefully excised from stained gels and sub-

jected to in-gel trypsin digestion, as previously reported.[16] Peptide mixtures were separated using a nanoflow-HPLC system (Ultimate; Switchos; Famos; LC Packings, Amsterdam, The Netherlands). Mass spectrometry settings and bioinformatic identification details are provided in the *Online Supplementary Design and Methods*.

Structural analyses

Scanning electron microscopic studies of RBC were performed by means of a JEOL JSM 5200 electron microscope. Samples were prepared as described in full in the *Online Supplementary Design and Methods*. The different cell shapes were identified using Bessis' classification.[22] The percentages of discocytes, echinocytes, sphero-echinocytes, stomatocytes, sphero-stomatocytes, and spherocytes were evaluated by counting 1000 to 1500 cells in randomly chosen fields. Although there is still an open debate about reversible and irreversible shape classification, shape changes were classified as according to Berezina *et al.*[23] According to this classification, RBC manifesting echinocyte and stomatocyte shapes are capable of returning to the discocyte shape under certain conditions. Thus, these RBC shape changes are considered potentially reversible transformations. In contrast, RBC assuming sphero-echinocyte, sphero-stomatocyte, spherocyte, ovalocyte or degenerated shapes are irreversibly changed cells.

Results

Metabolomics, proteomics and scanning electron microscopy (SEM) analyses were performed simultaneously on eight leukodepleted RBC units (residual white blood cell count < 1×10^6/unit).[24] At the end of the storage (42 days), hemolysis was less than 0.8%[24] for all the tested products.

It is worth mentioning that the possibility of performing multi-faceted investigations covering both proteomic and metabolomic aspects stems from the sensitivity (down to the fmol level) and specificity of the HPLC and mass spectrometry techniques. Although these approaches are not used routinely in the clinical setting, they are becoming widespread in research laboratories given their versatility and robustness. For example, the integrated metabolomic analysis here was used to perform relative quantification analyses of 12 different metabolites through HPLC-mediated elution of 20 μL of the original sample into the mass spectrometer.

Time-course metabolomics

Fold-change variation values upon normalization to day 0 controls are presented in *Online Supplementary Figure S1*.

Glycolytic phosphate precursors apparently accumulated slightly over the first 2 weeks of storage, as observed for glucose-6-phosphate (G6P)/fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (G3P) (*Online Supplementary Figure S1*). On the other hand, fructose-1,6-diphosphate (FDP) accumulated significantly at day 7. From day 14 onwards the trend was inverted and metabolites were rapidly consumed so that by the end of the storage period they were below (75.6±3.4% for G6P/F6P) or far below (51.4±2.1% for FDP and 29.2±1.4 % for G3P) the initial concentration.

Diphosphoglycerate (DPG) levels followed trends already reported in literature,[11,20] with early moderate accumulation up to day 7 and then a constant decrease until day 21 and onwards, at which point the concentrations were as low as 0.05±0.01% of the original day 0 values.

On the other hand, phosphoenolpyruvate (PEP) followed an anomalous trend, with a constant decrease until day 28, at which point we detected a 1.6 ± 0.1 fold increase in almost all the tested units, followed by a reversion so that the levels were lower than day 0 control values by day 42 (0.65 ± 0.04 %).

Lactate, which is a frequently measured parameter in metabolic analyses of RBC,[11,20], constantly accumulated as storage progressed, reaching a final 20.39±0.9 fold increase in comparison to day 0 control levels.

In parallel, we observed a moderate, albeit constant accumulation of pentose phosphate pathway intermediates: 6-phosphogluconic acid reached a plateau at day 28, while NADPH levels continued to rise. At day 42 the levels of both metabolites had doubled in comparison to day 0 control levels.

ATP appeared to increase up to day 7 and was then rapidly consumed soon after the first week, so as to reach initial values and below. However, the ATP consumption rate decreased from day 14 to day 21, and from day 21 onwards.

NAD^+ accumulated until day 7, then it decreased constantly until the end of the storage, when we recorded levels 59.99±0.2% lower than the day 0 control level.

Finally, the levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) followed opposing trends. The former decreased constantly over storage so that its levels had halved by day 42, while the latter increased constantly from day 14 onwards (1.56±0.02 fold increase at the end of the storage).

Oxidative stress

Reactive oxygen species

ROS accumulated over the first 3 weeks of storage, to reach a plateau at 21 days (252.4±12.5 units) (*Online Supplementary Figure S2A*).

Protein carbonylation

Protein carbonylation increased constantly and uniformly in all the tested samples from day 0 (41.3±2.4 mmol/mL) until the fourth week of storage (62.1±2.5 mmol/mL). From the 28th day onwards carbonylation trends seemed to be reversed. Detected protein carbonyls were reduced in almost all of the tested subjects at the end of the storage (48.2 mmol/mL), while in others it reached a plateau with only slight decreases (59 mmol/mL), in agreement with previous observations by Papassideri's group[18] (*Online Supplementary Figure S2B*).

Malondialdehyde

The malondialdehyde assay demonstrated a progressive and constant accumulation of oxidized lipid markers from 3.4±0.3 mmol/mL at day 0 up to 7.1±0.6 mmol/mL at day 42 (*Online Supplementary Figure S2C*).

Proteomics

In our previous study on RBC fragmentation during storage we observed an increase of fragments between the second and the fifth weeks. However, that study had some major limitations since the analyses were performed on RBC units that were neither buffy coat-depleted nor leukofiltered.[16] Early fragments might, therefore, have been provoked by white blood cells in the unit. On

the other hand, when testing leukocyte-filtered RBC concentrates, protein fragments accumulated in the period between the third and fifth weeks, as we could eventually assess. Indeed, in the present study, 2-DE differential analyses between day 0 and day 14 samples highlighted the presence of a limited number of statistically significant differential spots (spot numbers 320, 327 and 342, all identified as protein band 4.1; and spots 389, 402 and 417, identified as peroxiredoxin 2 (Figure 1A2 and *Online Supplementary Table 1A*). Conversely, significant levels of fragments were observed at day 35 in all the tested units. We, therefore, performed a targeted 2-DE analysis of samples from the eight leukocyte-filtered RBC units at day 0 and day 35 mainly aimed at determining significant variations [$P<0.05$ ANOVA, $|\text{fold-change}|> 2.0$] in the low molecular weight region, below 60 kDa. Since protein fragments have been supposed to be masked or triggered by artifactual oxidation during sample preparation,[21] we decided to perform the 2-DE analysis both in the presence and absence of the alkylating agent NEM. Results for the two arms of the study are reported in *Online Supplementary Figure S1A and S1B*, respectively.

Results obtained without NEM included the observation of 15 different highly statistically significant protein spots, which were further identified as eight distinct proteins (*Online Supplementary Table 1B*).

In the presence of NEM, the overall number of spots (at day 0 in comparison to samples without NEM) visibly increased. Although it is beyond the scope of this article to understand the reasons underlying this biochemical phenomenon, it cannot be excluded that NEM-modified proteins could have been hydrolysed to the corresponding N-ethylsuccinimide cysteine residue. This may provide additional protease cleavage sites and thus promote enzyme-mediated protein fragmentation.[25] However, the effect was the same in day 0, day 14 and day 35 samples and thus it did not compromise differential analyses. Besides, fragments evidenced at day 35 were the same both in the presence and absence of NEM, while NEM addition only enabled resolution of further non-fragmented proteins, as could be deduced from the relation between the theoretical and the observed molecular weights (Figure 1, and *Online Supplementary Table S1*). On the other hand, no significant fragmentation was observed in day 14 samples with NEM in comparison to day 0 controls treated with NEM (*data not shown*).

As for the present study, we determined a significant increase at day 35 against day 0 control gels of 27 differential spots which enabled identification of 33 distinct protein entries (*Online Supplementary Table S1B*). Notably, all of the protein entries identified without NEM could still be identified upon addition of the alkylating agent, allowing us to confirm that observations made so far on protein fragments[16] were not artifactual results from oxidation of thiol groups during preparation steps.

The addition of NEM favored the separation and identification of an increased number of protein spots, which accumulated in the membrane fraction at day 35 in comparison to day 0 controls. Indeed, a comparison of proteins found with NEM and those found without NEM revealed a series of new protein entries. These mainly included proteins which were characterized by thiol groups in their functional/catalytic domain [for example, thioredoxin-like fold (IPR012336) in TXNL1, CLIC1, GSTO1, and GPX1].

These proteins were destined to artifactual over-oxidation during the extraction and their fluctuations ended up being under-estimated in the absence of the alkylating agent NEM, in agreement with previous observations.[21,26]

Since RBC lack the capacity to synthesize new proteins, the increased number (and increased photodensity) of protein spots in the low molecular weight range of the membrane fraction could be due to either increase in fragmentation events involving higher molecular weight proteins or the migration to the membrane fraction of cytosolic proteins [soluble fraction (GO:0005625)].

Fragmentation of high molecular weight proteins implies the experimental individuation through 2-DE of protein spots, the molecular weight of which might not be lower than expected theoretically from online available databases. A series of proteins could be included in this group (*Online Supplementary Table S1*): (i) oxidative stress-related enzymes with oxidoreductase activity (GO:0016491) (glutamate—cysteine ligase catalytic subunit isoform a, stress-induced-phosphoprotein 1); (ii) structural proteins [spectrin alpha and beta chains, band 3 and protein 4.1 isoform 6 - structural constituent of cytoskeleton (GO:0005200)]; and (iii) apoptosis-related proteins (ALG-2 interacting protein 1).

Another group of proteins of cytosolic origin became increasingly represented at the membrane fraction of day 35 samples. This group included: (i) structural proteins (stomatin and flotilin-2); (ii) vesicle-related proteins [alpha-soluble NSF attachment protein, alpha SNAP, 55 KDa erythrocyte membrane protein isoform 1 – belonging to the BioCarta pathway "Synaptic Proteins at the Synaptic Junction (h_PDZsPathway)"]; (iii) oxidative stress-related enzymes [glutathione S-transferase omega-1 isoform 1, glutathione peroxidase, thioredoxin-like protein 1 - glutathione metabolism (hsa00480); 6-phosphogluconolactonase, nicotinate phosphoribosyltransferase-like protein, biliverdin-IX beta reductase isozyme I – KEGG pathway: pentose phosphate pathway (hsa00030) and gene ontology: cofactor metabolic process (GO:0051186)]; (iv) chaperones (stress-induced-phosphoprotein 1, DNA-damage inducible protein 2, KIAA0002, T-complex protein 1 subunit beta isoform 1, peptidyl-prolyl cis-trans isomerase FKBP4, HSPC263, Hsc70-interacting protein); and (v) ion channels (nuclear chloride cannel).

Structural analyses: scanning electron microscopy

SEM images were collected and analyzed following the Bessis classification[22] and reversible and irreversible membrane shape alterations during storage (from day 7 to day 42) were differentiated as described by Berezina *et al.*[23] The results are reported in Table 1, as mean ± SD of discocytes, cells with reversibly and irreversibly-altered membrane, as percentages of the overall number of cells taken into account in randomly chosen fields (ranging from 1000 to 1500 per subject). While at day 7 the percentage of discocyte RBC was high (75.3±4.1) and the fraction of RBC displaying irreversible modification of the membrane averaged below 10% (9.2±3.5), SEM images on day 42 showed a substantial percentage of RBC with reversible (45.3±3.8) and irreversible (31.0±2.9) membrane alterations (Table 1). Figure 2 shows a detail and an overview of a RBC sample stored for 42 days (Figure 2A and 2C) in comparison with one stored for 28 days (Figure 2B).

Discussion

Metabolic parameters change rapidly over the first 2 weeks of storage

In this study we simultaneously tested eight units of RBC stored in SAGM under refrigeration for metabolism, oxidative stress and protein parameters and monitored them throughout their shelf-life (42 days).

Sugar-phosphates and their glycolytic metabolites seemed to accumulate during the first week and then began to decrease as storage progressed, so as to reach low or extremely low levels at the end of the third week (*Online Supplementary Figure S1*).

Consumption of both 1,3 and 2,3 DPG (we could not discriminate between the isomers through mass spectrometry) had already been reported and had been related to the impaired oxygen delivery capacity of RBC upon transfusion.[26] Indeed DPG, in association with pH and HCO_3^-/CO_2 modulates the position and shape of the oxygen dissociation curve.[27]

The rate of glycolysis decreases as pH falls, which is known to occur over storage.[14] Another limiting factor should be complete reduction of NAD^+ to NADH. This was confirmed to some extent by our observations, which included both a constant decrease of pH (*data not shown*) and a decrease of NAD^+ as storage progressed (although we also noted a significant increase of NAD^+ levels in the first week) (*Online Supplementary Figure S1*). Lactate accumulated over the whole period of storage, suggesting that glycolysis did not stop, or at least was diverted towards the pentose phosphate pathway, since NADPH and 6PG accumulated.

On the other hand, ATP depletion was constant over the whole 42-day period, testifying to an inefficient rate of ATP production. This is relevant in the light of the role of ATP in the maintenance of electrolyte balance by powering sodium-potassium cation pumps. However, Na^+/K^+ pumps are known to be turned off at 4°C.[28] In the present study ATP depletion was associated with impairment of parameters of cell shape, in agreement with other published data.[29]

Overall, we could conclude that, if metabolism plays a role in RBC storage, it is to sustain RBC energy production during the first 2 weeks and then it is switched to producing metabolites involved in anti-oxidant responses from day 14 onwards.

Oxidative stress: from metabolism to accumulation of reactive oxygen species

Several authors have proposed that oxidative stress might underpin *ex vivo* aging of RBC.[8,9,16] In this study, we found that ROS accumulated, reaching a plateau at day 21 (*Online Supplementary Figure S2A*). Protein carbonyls and MDA (*Online Supplementary Figure S2B,C*) also accumulated, in parallel to a decrease in GSH and increase in GSSG levels (*Online Supplementary Figure S1*).

Conversely, we observed an increase of pentose phosphate pathway oxidative phase metabolic intermediates NADPH and 6PG as storage progressed. 2-DE analyses of NEM-extracted samples indicated relocation to the membrane of the 6PG-limiting enzyme (6-phosphogluconolactonase – *Online Supplementary Table S1*) and nicotinate phosphoribosyltransferase-like protein, which catalyzes the conversion of nicotinic acid to nicotinic acid mononucleotide and is essential for nicotinic acid to be able to increase cellular NAD^+ levels and prevent oxidative stress of the cells.[30] As we detected higher levels of the product metabolites of these enzymes, it is likely that they relocate to the membrane, where they are needed the most.

Other proteins relocated to the membrane were GSH-homeostasis-related enzymes, such as glutathione S-transferase, glutathione peroxidase and thioredoxin-like protein 1 (hsa00480 – KEGG pathway annotation).

Since G3P levels did not increase again with 6PG and NADPH accumulation, we could assume that the non-oxidative phase of the pentose phosphate pathway is somehow inhibited, while the oxidative phase alone is over-activated. This assumption is coherent with the observation by Giardina's group[31] that the ratio of G3P which is produced through glycolysis and the pentose phosphate pathway does not show major fluctuations over storage, though their interesting conclusions were drawn from nuclear magnetic resonance-based data about (^{13}C-2 and ^{13}C-3 derived from ^{13}C-glucose) G3P levels alone instead of a whole subset of metabolites.

In conclusion, oxidative stress appeared to increase markedly from day 14 to day 21, when it reached maximum levels.

Alterations of red blood cell membrane shape and vesiculation: a timely snapshot through proteomics

Through proteomics we were able to record an increase of oxidative stress-related enzymes in RBC membranes. We detected proteins involved in GSH-homeostasis, which relocated to the membrane already at day 14 (peroxiredoxin 2 – Figure 1A2 and *Online Supplementary Table S1*) or later on, at 35 days (Figure 1A,B; *Online Supplementary Table S1*). Most of these oxidative stress-related proteins, as well as most of the chaperones individuated have been indicated as key regulatory nodes in the protein-protein interaction analysis of the RBC proteome and interactome in the 2010 study by our group,[32] as they mapped to the very heart of the "Save or Sacrifice - SOS" sub-network. The "SOS" sub-network represented the top-score result of a bioinformatic processing cataloguing all the RBC proteins discovered so far based on the relevance of their role in sustaining RBC survival and functionality.

We could not conclude whether this enrichment of oxidative stress-related proteins might be due to an increased necessity to face oxidative stress in the membrane region or rather these no-longer functional proteins are relocated to the membrane through the docking of vesicles prior to their extrusion from the cell. However, the former hypothesis seems reasonable in the light of recent observations by our group which clearly indicate that membrane peroxiredoxin-2 in RBC that have been stored for a long time is still functional.[17]

The likely effect of oxidative stress at the membrane protein level appeared not only to be limited to an increase in carbonyl levels (*Online Supplementary Figure S2B*), but also to an increase in fragmentation of structural proteins [spectrin alpha and beta chains, band 3 and protein 4.1 isoform 6 - structural constituent of cytoskeleton (GO:0005200)]. Fragments of band 4.1 were found at the membrane already at day 14 (Figure 1A2). This is in agreement with SEM analyses, which highlighted that significant membrane alterations had accumulated already by day 14 (approximately 45% of RBC showed membrane

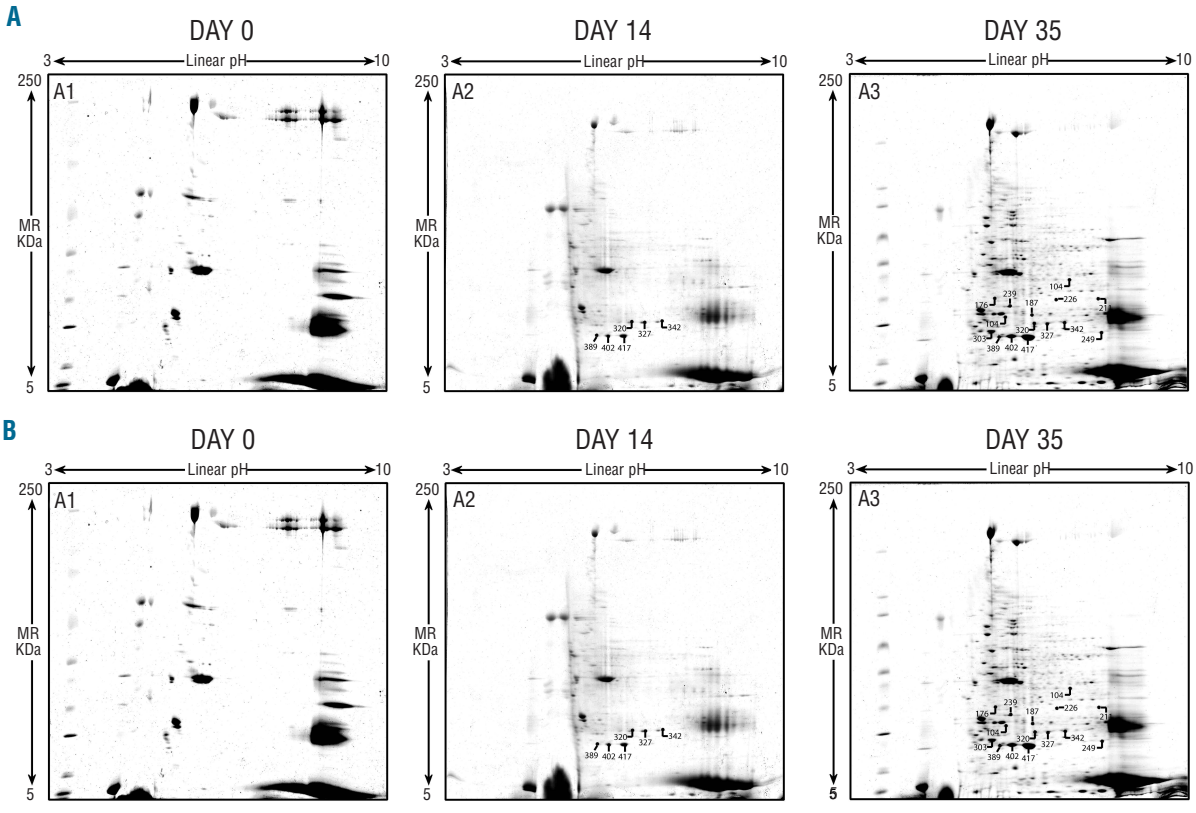

Figure 1. In (A) Two-dimensional gel electrophoresis (extraction protocol without incubation with the alkylating agent N-ethyl maleimide - NEM) of day 0 (A1) *versus* day 14 (A2) and 35 (A3) RBC. First dimension IEF pI values linearly span between 3 and 10, while molecular weights are indicated on the left. Spots characterized by different photodensities in the low molecular weights region were individuated and further identified through mass spectrometry (Table 1A). (B) Two-dimensional gel electrophoresis (extraction protocol including incubation with NEM of day 0 (B1) *versus* day 35 (B2) RBC. First dimension IEF p*I* values linearly span between 3 and 10, while molecular weights are indicated on the left. Spots displaying different photodensities in the low molecular weight region were individuated and further identified through mass spectrometry (Table 1B).

alterations) and continued to accumulate as storage progressed (by day 35 over 65% of the examined RBC displayed relevant alterations of membrane shape).

In the presence of the alkylating agent NEM during the extraction we detected, through 2-DE, differential photodensities for those protein spots containing oxidation-sensitive thiol groups, which are otherwise artifactually oxidized during the extraction protocol.[21,25,26] Notably enough, a series of proteins involved in vesiculation were detected, such as alpha SNAP and 55 kDa erythrocyte membrane protein isoform 1 (h_PDZsPathway - Biocarta annotation). The former, in particular, is known to play a pivotal role in the process of exocytosis, as it mediates vesicle docking to the membrane also, but not uniquely, in a calcium-dependent manner.[33] Since internal Ca^{2+} is subjected to metabolic control via an ATP-dependent extrusion mechanism (Ca^{2+} pump),[33,34] the decreased ATP content occurring during red cell aging should lead to a raised cellular Ca^{2+}. This has been reported in the literature from studies that used the fluorescent probe Fura-2 in cells separated on Percoll density gradients, which revealed that *in vivo* aged RBC (senescent) contained a higher content of free Ca^{2+} (almost four times higher) than the younger cells.[35] The consistency of the role of Ca^{2+} in the frame of RBC storage is strengthened by the consideration about the role of this ion in modulating the Ca-dependent K channel[36] and the influence on RBC membrane shape.[37,38]

Most of the proteins detected through our 2-DE approach, both in the presence and absence of NEM, had already been reported to accumulate in RBC-leaked micro- and nano-vesicles, for example stomatin, ankyrin, biliverdin reductase, and 14-3-3 zeta/delta (*Online Supplementary Table S1* in comparison to the findings of Bosman *et al.*[15]), just to mention few.

The list included fragments of structural proteins, among which the cytosolic domain of band 3 deserves a special mention since it is known to represent a "respiratory metabolon" docking site at the RBC membrane,[39] and is also found in RBC vesicles.[15]

Red blood cell storage: parallelism with hereditary spherocytosis

Among the observed membrane anomalies that we could relate to the progression of storage, we evidenced an increased osmotic fragility of long-stored RBC (*data not shown*). It has long been known that hereditary spherocytosis is correlated with elevated osmotic fragility.[40] Hereditary spherocytosis is a hemolytic anemia characterized by the production of sphere-shaped rather than biconcave disk-shaped RBC; these sphere-shaped RBC have

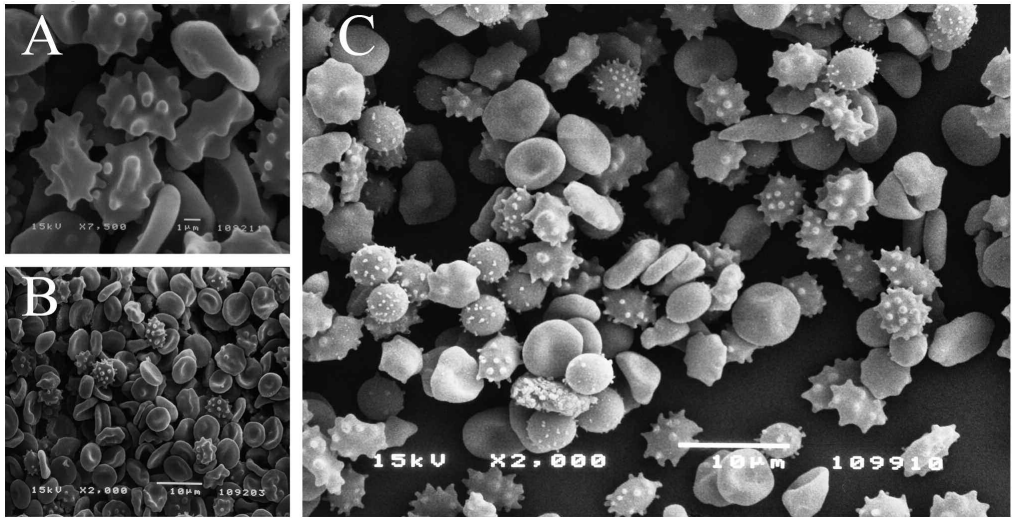

Figure 2. Scanning electron images (JEOL JSM 5200 scanning electron microscope) of long-stored RBC. A detail of a 42-day echinocyte (7,500x; scale bar = 1 µm) (A). A panoramic view of a 28-day RBC sample (2,000x; scale bar = 10 µm) (B). A 2,000x field of 42-day RBC (scale bar = 10 µm) (C).

Table 1. SEM classification of RBC shape.

Day	Discocyte (%)	Reversibly* changed RBC (%) (echinocyte and stomatocyte shape)	Irreversibly* changed RBC (%) (spheroechinocyte, spherostomatocyte, spherocyte, ovalocyte, and degenerated shapes)
7	75.3±4.1	15.5±1.9	9.2±3.5
14	55.8±2.7	29.1±2.4	15.1±0.9
21	51.0±4.0	32.6±2.6	16.4±1.4
28	45.6±3.3	35.6±1.7	18.8±1.6
35	35.2±1.9	42.3±2.2	22.5±3.1
42	23.7±2.5	45.3±3.8	31.0 2.9

**Reversible and irreversible changes were classified based on the classification by Berezina* et al.[23] *However, details of the Bessis'[22] shape classification are provided as well.*

an increased tendency to hemolysis because of a decreased surface/volume ratio, which results in a reduced capacity to face increased osmotic stresses.

Our SEM results, in agreement with data in the literature,[23] support the characterization of progressive membrane-targeting storage lesions, which end up severely altering RBC shape (*Online Supplementary Table S1*). Moreover, a recent proteomic investigation on RBC from patients suffering from hereditary spherocytosis concluded that these patients' cells were characterized by altered redox-regulation, nucleotide metabolism, protein aggregation and/or degradation, cytoskeletal disorganization and severe oxidative stress.[41] In particular, Rocha *et al.*[42] reported that peroxiredoxin 2 located at the membrane in RBC from patients with hereditary spherocytosis concomitantly with an increase in oxidative stress. As we had previously observed[17] through western blotting studies, and confirmed here by 2-DE (Figure 1A, *Online Supplementary Table S1A*), peroxiredoxin-2 relocated progressively at the membrane level from the third week to the end of the storage.

Hemoglobin chains have been shown to bind to the RBC membrane in hereditary spherocytosis,[43] but also in long-stored RBC in a previously published study[9] and in the present study (*Online Supplementary Table S1A,B*).

Protein degradation at the membrane level was previously reported to begin at day 14 and reach a maximum at day 33.[16] Here we observed a significant accumulation of fragments of a series of structural proteins at day 35 (spectrin alpha and beta chains, band 3 and protein 4.1 isoform 6 - structural constituent of cytoskeleton – *Online Supplementary Table S1A,B*), as a further parallelism between prolonged storage and hereditary spherocytosis in which mutations/fragmentation events targeting these very same proteins have been reported.[44,45]

Finally, oxidative stress seems to represent another pattern in common with hereditary spherocytosis, as we noted increases in both ROS and protein carbonylation (*Online Supplementary Figure S2A,B*), peaking at days 21 and 28, respectively. Lipid oxidation was also observed, through the accumulation of MDA (*Online Supplementary Figure S2C*).

Conclusion

Storage lesions stem from a domino of events leading to the accumulation of irreversible alterations in long-stored RBC. While clinical concerns about the safety and effectiveness of such RBC are still under evaluation, biochemical information is broadly available, although not conclusive. In this context, we performed the current integrated metabolomic and proteomic study to further explore the dynamics of the phenomena underlying RBC storage.

(i) Storage progression corresponded to a phenomenon which could be roughly described as a progressive modulation of RBC metabolism, which was in part coherent with previous *in silico* models and in part expanded nuclear magnetic resonance-based observations about the ratio of G3P generation rate through glycolysis and the pentose phosphate pathway, available in the literature.[31,45] Taken together, our results indicate that the oxidative steps of the pentose phosphate pathway might be over-activated while, if a blockade exists,[31] it is at the non-oxidative steps.

(ii) If on the one hand RBC metabolism was active within the first 14 days of storage at most, the rapid accumula-

tion of ROS resulted in a significant increase in oxidized proteins and lipids.

(iii) Oxidative stress appeared to represent the trigger to changes at the protein level especially in the membrane fraction, to a lesser extent until day 14, while these changes accumulated significantly by day 35. These changes included fragmentation of structural proteins, relocation of anti-oxidant enzymes from the cytosol to the membrane, and promotion of vesiculation through proteins associated with vesicle formation and docking to the membrane.

(iv) Vesiculation results in membrane loss and thus in the final acquisition of the spherocytic phenotype, as confirmed by SEM.

The present study shows that, from biochemical and molecular standpoints, the parameters defining the integrity of SAGM-stored leukodepleted RBC might be still acceptable within the first 14 days of storage but then begin to decline slowly from day 21 and onwards. This information might be relevant with regards to clinical transfusion practices, further fueling the debate about the safety and effectiveness of RBC concentrates stored for longer than 14 days.[2] In other terms, if we evaluated RBC-based transfusion therapies with the same strict rules regulating drug interventions, in the light of present findings we could easily conclude that RBC units as a therapeutic product do not preserve their integrity over the whole duration of the storage period, especially from day 14 onwards, this likely resulting in compromised safety and effectiveness.

All the tested parameters appeared to be modified during storage as they are in RBC from patients with hereditary spherocytosis. Indeed, membrane structural protein fragmentation, accumulation of anti-oxidant enzymes and chaperones in the membrane district, as well as the increase in ROS, protein carbonylation and MDA accumulation are all representative of significant, prolonged exposure to oxidative stress in *ex vivo* RBC. Based on this evidence, it will be interesting to evaluate alternative storage strategies which envisage cold storage of RBC concentrates under anaerobic conditions in order to tackle the accumulation of oxidative stress.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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