TRANSFUSION MEDICINE AND TRANSFUSION COMPLICATIONS

Original article

Red cell proteasome modulation by storage, redox metabolism and transfusion

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Background - Proteasomes are proteolytic complexes with prominent roles in the control of protein homeostasis and cellular viability. However, little is known about the effects of storage and glucose-6-phosphate dehydrogenase deficiency (G6PD$^-$) on the activity and topology of red blood cell (RBC) proteasomes.

Materials and methods - We investigated the concentration (by GeLC-MS proteomics analysis and immunoblotting), activity (by using peptide substrates and proteasome inhibitors), and subcellular/extracellular distribution (following cell fractionation and isolation of extracellular vesicles, respectively) of RBC proteasomes in fresh blood and RBCs from control and G6PD$^-$ donors following storage in leukoreduced units. RBC proteasome activity was also tested in transfusion-mimicking conditions *in vitro*.

Results - Stored RBCs were characterised by decreased cytosolic proteasome activity compared to fresh RBCs but increased membrane activity and protein concentration levels. Active proteasomes along with other "repair or destroy" proteins are recruited to the membrane during storage. A proportion of them is released in the supernatant in soluble form or inside extracellular vesicles. Significantly increased enzymatic activity and release of proteasomes were observed in G6PD$^-$ *vs* control RBCs. Similar variations were observed in stress protein biomarkers at the G6PD$^-$ membrane. The proteasome profile (mainly the caspase-like activity) had significant correlations with the G6PD$^-$ metabolome and quality markers of the RBC units. The storage-induced modifications in the proteasome activities were only partly restored in transfusion-mimicking conditions.

Discussion - Storage conditions and G6PD deficiency affect (individually and in synergy) the abundance, distribution, activity, and release of RBC proteasomes. The partial irreversibility of these effects in transfusion-mimicking conditions demands further investigation of their clinical impact on transfusion outcomes.

Keywords: *red cell storage lesion, G6PD deficiency, proteasome, oxidative stress, proteomics and network analyses.*

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INTRODUCTION

Proteasome is a proteolytic supramolecular complex that selectively degrades damaged, misfolded or aberrant proteins[1]. The 19S regulatory complex of the 26S holoenzyme recognises ubiquitinated substrates and transfers them for proteolysis into the catalytic

20S core particle. The proteolytic chamber of the 20S subcomplex contains active sites with different proteolytic specificities, the caspase-like (CASP-like), trypsin-like (TR-like), and chymotrypsin-like (CH-like), located in the β1 (PSMB6), β2 (PSMB7), and β5 (PSMB5) subunits, respectively. In contrast to the 26S proteasome, the 20S core can degrade substrates in an ubiquitin and ATP-independent manner[2], and is involved in multiple cellular pathways[3]. Proteasomes can change in response to different physiological or pathophysiological conditions, including increased oxidative stress[4]. By preventing cytotoxicity, they are key regulators of processes including stress response and signal transduction. Consequently, inhibition or overactivation of proteasomes have dramatic effects on cellular functionality, viability, ageing and cancer progression.

Red blood cells (RBCs) have been used as a source for proteasomes, in spite of containing substantially fewer particles compared to the nucleated cells[5]. Proteasomes are retained, however, within RBCs while most other organelles are lost, suggesting a functional role at the later stage of developmental maturation of the erythroid lineage. Previous studies involving high resolution and confocal microscopies[6], proteomics[7], and functional assays provided evidence that *in vivo*[6] and under storage at blood banks[5] RBCs contain functional proteasomes in the cytosol[8,9] and the membrane[10-14], with a >10 fold excess of 20S core particles compared to the 26S holoenzymes[5]. This is probably related to the fact that the 20S proteasomes are more resistant to oxidative stress[15] and more effective against it[16]. Variations in the proteasome activity and concentration have been detected in RBCs of patients with Alzheimer's disease[17], congenital RBC aplasia[18], unexplained haemolytic anaemia[19], multiple myeloma, during organismal ageing[20] and in human obesity[21], among others.

Here, we define variations in proteasomal topology and activity in RBCs and extracellular vehicles (EVs) released by them in the context of blood storage, by analysing units donated by healthy volunteers, with no underlying conditions or with asymptomatic deficiency of glucose 6-phosphate dehydrogenase (G6PD) activity. G6PD deficiency is the most common enzymopathy in humans[22]. It constrains the capacity to generate nicotinamide adenine dinucleotide phosphate (NADPH), a reducing equivalent involved in redox homeostasis and recycling of oxidised antioxidant small molecules (e.g., reduced glutathione) and enzymes[23]. Stored RBCs from G6PD-deficient donors exhibit increased oxidative stress[24] and decreased recovery in children with sickle cell disease[25] as well as in healthy G6PD-deficient recipients (often <75% of threshold) following autologous transfusion of end-of-storage units[26]. However, G6PD-deficient subjects (who, in some areas, may account for up to 12.3% of the total donor population[27]) are routinely accepted as blood donors. As such, we hypothesised that increased proteasomal activity could represent a compensatory mechanism in the RBCs of G6PD-deficient subjects, and that such activity would manifest itself in the context of storage-induced oxidative stress.

MATERIALS AND METHODS

Donor recruitment and blood processing

We studied twenty male regular blood donors (Caucasian race, 25-30 years old). Ten of them had G6PD deficiency (G6PD$^-$) of class II Mediterranean variant. RBC storage analysis was performed in pre-storage log4 leukoreduced units (Haemonetics, Braintree, MA, USA) containing citrate-phosphate-dextrose (citrate-phosphate-dextrose[CPD]/saline-adenine-glucose-mannitol [SAGM]). RBC concentrates were prepared by centrifugation in an automated blood processing device (Compomat G4, Fresenius HemoCare, Bad Homburg, Germany) before adding 100 mL SAGM solution (0.9 g dextrose monohydrate, 0.877 g sodium chloride, 0.0169 g adenine, 0.525 g D-mannitol). Fresh blood samples from each individual donor and stored samples, collected aseptically after 7 days of storage and weekly thereafter, were used for the experiments. The study was submitted to and approved by the Scientific Ethics Board of the General Hospital of Nikea-Piraeus "Agios Panteleimon" (n. 13702, 03/20/2014). Investigations were carried out in accordance with the principles of the Declaration of Helsinki.

Red blood cell fractionation and extracellular vehicles isolation

Red blood cell membranes and cytosols were isolated by hypotonic lysis in phosphate buffer (pH 8.0) supplemented by protease inhibitors. EVs were obtained from day 42 supernatants through a 3-step ultra-centrifugation (37,000 *g*) protocol for 1 hour (h) each at 4°C[28].

Proteomics and immunoblotting analyses

Proteomics analyses were performed in samples of RBC units on storage days 2, 21 and 42 (s2, s21 and s42) through a GeLC-MS approach[29], as extensively described in prior technical notes[30]. Isolated membrane proteins (30 μg) were further immune-probed for a variety of proteasomal and other proteins by using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence development (GE Healthcare, Chicago, IL, USA and Millipore, Burlington, MA, USA). Monoclonal antibodies against proteasome 20S core subunits (α7, β1, β2, β5), 19S Rpt1/S7 and Rpn10 (s5a) subunits, as well as mono- and polyubiquitinylated conjugates and DJ-1 (D29E5) were obtained by Enzo Life Sciences (Osaka, Japan), Cell Signaling Technology (Danvers, MA, USA) and Novus Biologicals (Centennial, CO, USA). Antibodies against peroxiredoxin-2 (Prdx2, SP5464, Acris, Luzern, Switzerland), human Hb (CR8000GAP, Europa Bioproducts, Wicken, UK), phosphatase PTP1b (clone FG6, Millipore), human IgGs, band 3 (Sigma Aldrich, St. Louis, MO, USA), and caspase-3 (Cell Signaling Technology) were also used. Monoclonal antibody (mAb) against stomatin was kindly provided by Prof. R. Prohaska (Department of Medical Biochemistry, Max F. Perutz Laboratories, Medical University of Vienna, Vienna, Austria). Protein bands were identified and quantified by scanning densitometry using the Gel Analyzer v.1.0 image-processing program.

Proteasome activity and reactive oxygen species assays

Proteasomal activities in plasma, supernatant, RBC membrane and cytosol fractions and EVs were determined by fluorescence assays as described previously[31]. Briefly, 100-200 μg of protein samples in 20 mmol/L Tris-HCl (pH 7.5 or 8.0) were incubated with the fluorogenic peptide substrates Suc-Leu-Leu-Val-Tyr-aminomethylcoumarin (AMC) (for CH-like activity), z-Leu-Leu-Glu-AMC (for CASP-like activity) and Boc-Leu-Arg-Arg-AMC (for TR-like activity) for 1-3 h at 37°C in the dark, in the presence and absence of inhibitors (10 μmol/L bortezomib, 400 μmol/L MG-132, 100 mmol/L lactacystin and 500 μmol/L N-Acetyl-Leu-Leu-Methional). All substrates and inhibitors were procured from Enzo Life Sciences. Fluorescence intensity was measured using a fluorescence reader (VersaFluor, BIO-RAD Hercules, CA, USA) with an excitation filter of 365 nm and an emission filter of 460 nm. Reactive oxygen species (ROS) were detected by using the redox-sensitive and membrane-permeable probe 5-(and-6)-chloromethyl-2',7'-dichloro-dihydro-fluorescein diacetate, acetyl ester (CM-H2DCFDA, Invitrogen, Molecular Probes, Eugene, OR, USA)[32].

In vitro model of transfusion

To evaluate the proteasome activity of stored RBCs under simulated *in vivo* conditions, day-35 stored RBCs were incubated at 35-40% haematocrit for increasing time periods with fresh plasma (2,500 g for 15 min at 4°C) from healthy (control or G6PD$^-$) recipients of the same ABO blood group (n=4), pre-diluted with supernatant of the same RBC unit, in a ratio corresponding to transfusion of two RBC units per recipient. Shortly (30-40 min) before reconstitution, the RBC unit was transferred at room temperature to better simulate common transfusion practice. Post mixing, the samples were incubated for 3, 6, 12 and 24 h at 37°C in 5% CO_2-air along with aliquots of non-reconstituted packed RBCs. To avoid settling, the reconstituted RBCs had been under constant gentle agitation throughout the incubation period. The subsequent assays were performed in paired, mixed and unmixed samples, treated under the same conditions[24].

Statistical analysis

All experiments were performed in triplicate, unless otherwise stated. For statistical analysis, computer software (Statistical Package for Social Sciences, IBM SPSS [Armonk, NY, USA] version 22.0 for Windows, administered by NKUA) was used. All variables were analysed for normal distribution profile and the presence of outliers (Shapiro-Wilk test and detrended normal Q-Q Plots) in order to minimise the false discovery rate that is associated with the small size of our cohort. Between groups analysis was performed by independent t-test or Mann-Whitney test as appropriate. Time course analysis was performed by repeated measures ANOVA, corrected by a Bonferroni-like adjustment for multiple comparisons. Pearson's and Spearman's tests were performed to assess correlations between variables. Outputs of these analyses were used for the construction of biological networks connecting variables of fresh donor's blood (*in vivo* state) with those of stored RBCs (*ex vivo* state) by significant and repeated correlations present at every time point of the storage

period, as previously described[33]. Those correlations were topologically represented in undirected biological networks by using the Cytoscape version 3.7.1 application. The length of each edge was inversely proportional to the r value (the shortest the edge, the higher the r); p<0.050 was considered statistically significant.

RESULTS

Binding of proteasome subunits to the membrane is storage time- and donor-dependent

We first asked whether the composition of RBC membrane in proteasome components varies as a function of storage age and G6PD deficiency. To answer this, we performed proteomics analysis of pooled membrane samples from control or G6PD$^-$ donors at early, middle and late storage. We identified 42 proteasome or proteasome-associated components, including the alpha (α) and beta (β) subunits of the 20S core proteasome particle (PSMs) and the regulatory subunits (ATPases/PSMCs and non-ATPases/PSMDs) of the 19S proteasome (*Online Supplementary* **Table SI**). Semi-quantitative analysis revealed increased membrane presentation of proteasome subunits at mid-storage compared to early or late storage, in both control and G6PD$^-$ RBCs, with several subunits being more abundant in the G6PD$^-$ membrane (**Figure 1A**).

Peroxiredoxins and other proteins (including the valocin-containing protein-VCP ATPase, catalase and E3 ubiquitin-protein ligase-UBR4) also exhibited maximum levels at mid-storage (*Online Supplementary* **Figure S1**).

To explore inter-donor variability in the membranous proteasomes within each donor group we performed immunoblotting analysis of individual RBC membrane samples (**Figure 1B** and **C**). That analysis revealed: (i) substantial differences among donors in the levels of proteasome components before and throughout storage, (ii) increased membrane binding of proteasome subunits in stored *vs* fresh RBCs around middle storage (verifying the proteomics data); and (iii) a trend for higher concentration of individual proteasome subunits (e.g., subunit α1 on s28; p=0.058) in the G6PD$^-$ compared to the control samples. By further comparing the two groups of donors, we found statistically significant increases in Prdx2, IgG species, PTP1b (**Figure 1C**) and Band 3 fragmentation (on day 42; p=0.023, *data not shown*), but lower levels of ubiquitinated components (day 42) in the G6PD$^-$ membranes (**Figure 1C**). These results show that there was increased membrane binding of proteasome and other cytosolic components around middle storage compared to fresh blood, and occasionally in the G6PD$^-$ over control RBCs. Following treatment of RBCs with the thiol reagent N-ethylmaleimide (NEM) to block sulfhydryl groups, bands of Prdx2-oligomers were mostly detected in the membrane of old stored G6PD$^-$ *vs* control RBCs (*Online Supplementary* **Figure S2**). Proteasomes were not a major component of the EVs compared to the RBC membrane (*Online Supplementary* **Table SI**), as opposed to Prdx2, caspase-3 and, interestingly, DJ-1 (PARK7) proteins, in both donor groups (*Online Supplementary* **Figure S3**).

Storage and G6PD deficiency further affect proteasome activity in RBC compartments

We next asked whether the above-mentioned membrane variations were associated with differences in the proteasome activity levels. By using functional assays, we measured greater proteasomal activity in the membrane of stored *vs* fresh RBCs (with the exception of chymotrypsin [CH]-like activity in controls) at various storage periods (**Figure 2A**).

Though the G6PD$^-$ membrane exhibited high average values (compared to CTRL) mainly at mid-storage, the large inter-donor variation rendered these differences not statistically significant (multiple comparison analysis).

In striking contrast to the membrane, proteasome activities decreased in the cytosol of stored RBCs at mid-storage (control RBCs) or early storage (CASP- and TR-like activities in the G6PD$^-$ RBCs) compared to the *in vivo* state (**Figure 2B**), with higher levels measured occasionally in the G6PD$^-$ *vs* control RBCs (**Figure 2B**). Comparative analysis of the activity levels in RBC membrane *vs* cytosol, revealed a shift towards the membrane by storage. While in circulating RBCs proteasome activities were mostly cytosolic or equally distributed in cytosol and membrane, substantially higher (p<0.050) proteasome activities were detected in the membrane *vs* cytosol of stored control (all activities) and G6PD$^-$ (CASP-like activity) RBCs at mid- and/or late storage (**Figure 2C**).

Release of active proteasomes by red blood cells during storage

To find out whether variation in the proteasome activities of stored RBCs was further due to release of active proteasome components through vesiculation,

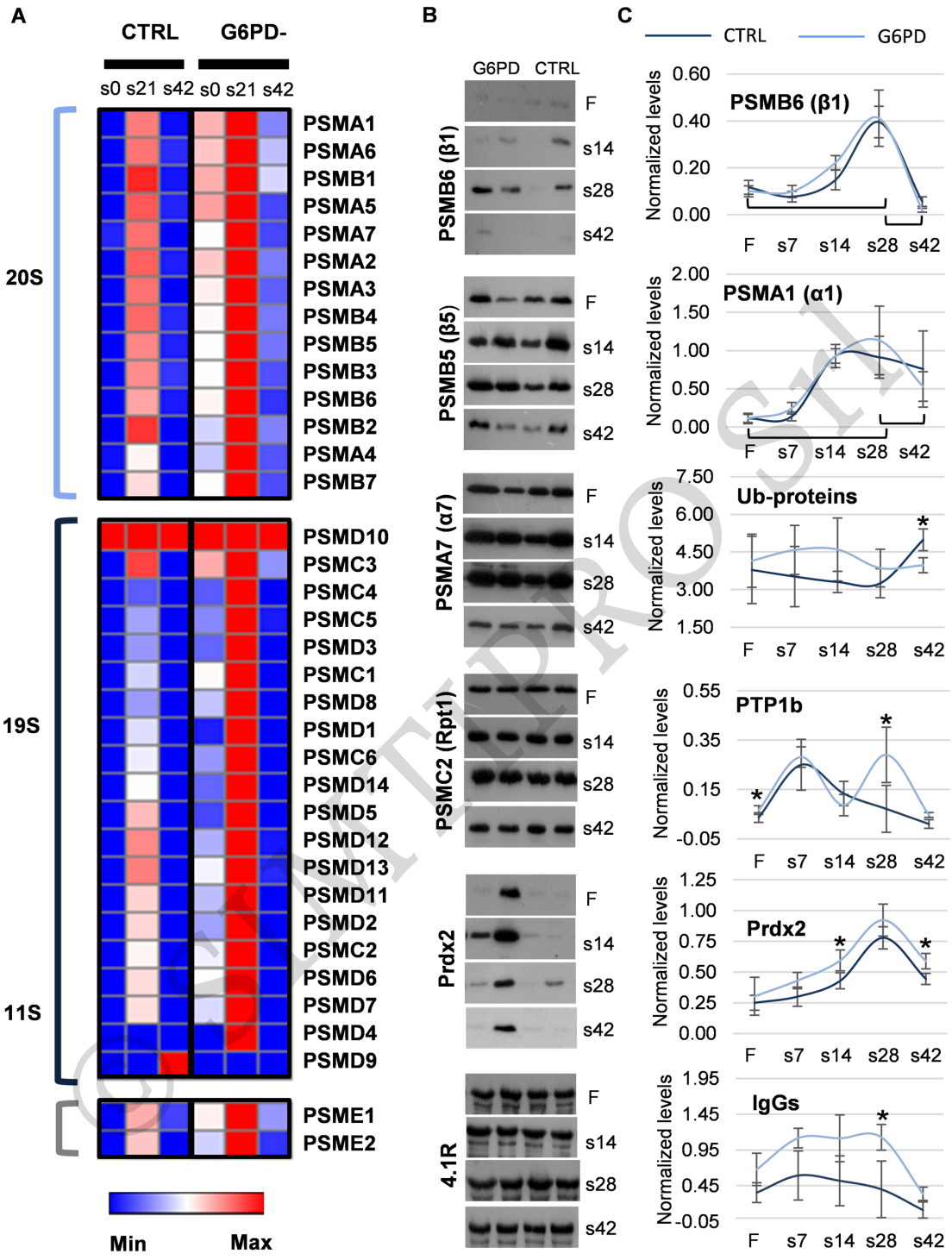

Figure 1 - Protein expression patterns through proteomics and immunoblotting analysis
(A) Heat map (GeLC-MS proteomics) analysis of proteasome subunits in pooled red blood cell (RBC) membrane samples of control (CTRL) and glucose 6-phosphate dehydrogenase negative (G6PD$^-$) donors (n=6), showing variations as a function of storage age and G6PD deficiency. s0-s42: storage day 0-42. (B) Representative immunoblots of Prdx2 and four proteasome subunits in the membrane of two G6PD$^-$ and two control donors. 4.1R was used as protein loading control. (C) Time-course analysis of variation in membrane protein levels of stored RBCs. Data are presented as mean±standard deviation. F: fresh blood; s7-s42: storage day 7-42; *CTRL *vs* G6PD$^-$ p<0.050; horizontal lines: p<0.050.

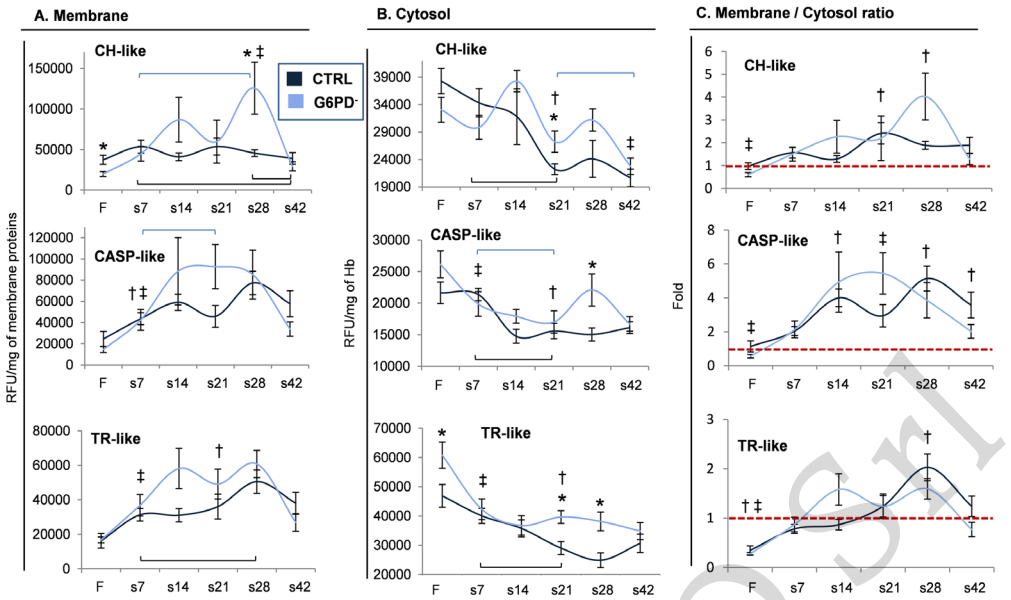

Figure 2 - Proteasome activity levels (relative fluorescence units [RFU]) in the membrane (A) and the cytosol (B) of fresh (F) and stored red blood cells (RBCs) from control (CTRL) and glucose 6-phosphate dehydrogenase negative (G6PD$^-$) donors (n=10 per group) following incubation of RBC fractions with fluorogenic peptide substrates and proteasome inhibitors
The horizontal bars connect statistically significant differences (p<0.050) inside storage. *p<0.050 CTRL *vs* G6PD$^-$. †p<0.050 F *vs* storage in the CTRL samples. ‡p<0.050 F *vs* storage in the G6PD$^-$ samples. (C) Graphical presentation of membrane-to-cytosol ratios of proteasome activities per protein mass unit. Dotted line: activity levels in the cytosol. †p<0.050 membrane *vs* cytosol in the CTRL samples. ‡p<0.050 membrane *vs* cytosol in the G6PD$^-$ samples. Error bars: ±standard error of the mean. s7-s42: storage days 7-42.

we measured CH-like levels in the RBC supernatant and EVs. Extremely low activity (approx. 40-140-fold less than cytosol) per extracellular protein mass unit was detected in fresh plasma and supernatant (n=6 per group) (**Figure 3A**). Despite this, the extracellular levels increased as a function of storage age in both donor groups (p=0.043 and 0.026 s2 *vs* s28, for CTRL and G6PD$^-$ samples, respectively), with higher values (p<0.050) in the G6PD$^-$ compared to the control units. The s42 EVs had greater activity per protein mass unit compared to the s42 supernatant in both donor groups (n=3 each) (**Figure 3B**), while the G6PD$^-$ EVs had significantly greater activity vs control EVs, in spite of similar s42 intracellular levels (**Figure 3B**, right panel). Those results suggested that both storage and G6PD deficiency promote release of active proteasomes by RBCs mainly through EVs.

Proteasome activities of stored red blood cells change in transfusion-mimicking conditions

We then looked for probable modifications in the proteasome activities under transfusion-mimicking conditions. To that purpose we used an *in vitro* protocol (**Figure 4A**) that phenocopies the impact of recipient plasma and body temperature on stored RBCs, as previously described[24]. The physiological temperature promoted the TR- and CASP-like catalytic activities in the cytosol over baseline levels in the cold (grey horizontal bars in **Figure 4B**), though both were partly attenuated by plasma. On the contrary, the CH-like cytosolic activity in the reconstituted RBCs remained lower than storage levels, despite increasing temperature. Reactive oxygen species (ROS) levels in the cytosol were also lower than baseline.

The membrane of reconstituted RBCs (**Figure 4C**) was characterised by substantially lower levels of CASP-like activity, in comparison to both baseline membrane levels of stored RBCs (s35 RBCs at 4°C) and cytosolic levels of reconstituted RBCs (**Figure 4B**) (p<0.003). Membrane binding of proteasomal subunits did not increase over baseline in spite of the triggering effect of temperature. Indeed, significant increases in the levels of ubiquitinated proteins and Prdx2 oligomers were measured over the incubation period (**Figure 4C**). Consequently, the individual proteasome activities of old-stored RBCs were different at transfusion-mimicking conditions compared to those at blood bank conditions.

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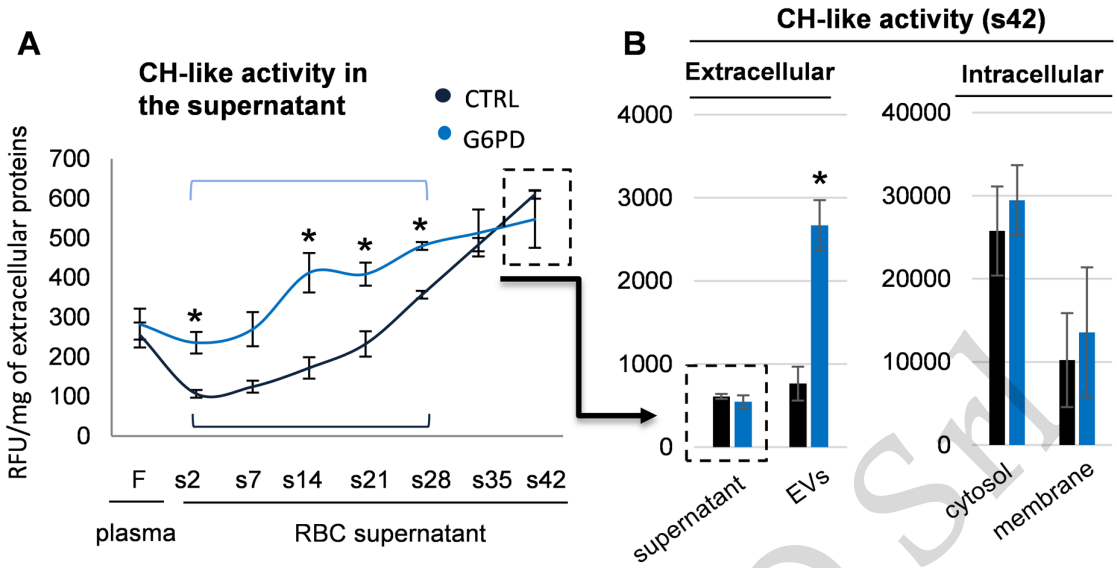

Figure 3 - Chymotrypsin (CH)-like activity levels in fresh plasma (F), supernatant and extracellular vehicles (EVs) isolated from red blood cell (RBC) units throughout storage
(A) The extracellular proteasome activity increased progressively by storage in both donor groups. *p<0.050 control (CTRL) *vs* glucose 6-phosphate dehydrogenase negative (G6PD$^-$) (n=6 per group). Horizontal bars: statistically significant differences within storage; s2-s42: storage days 2-42. (B) Comparative presentation of intracellular and extracellular proteasome activity levels on s42. Higher levels of proteasomal activity per protein mass unit were detected in the EVs (n=3 per group) *vs* s42 supernatant. *p<0.050 CTRL *vs* G6PD$^-$. Error bars: ±standard deviation.

Proteasome networking in G6PD deficiency revealed a cross-talk between protein breakdown and redox/energy metabolism

Though correlation does not imply causation, it can be suggestive of potential mechanistic links between the proteasome profile and the metabolic modulation in G6PD deficiency. As such, consistent with methods and metabolomics data described in our previous studies[24], we performed statistical and network analyses before and following storage of G6PD$^-$ RBCs. Notably, the fresh *vs* stored RBC network shown in **Figure 5** is developed around a core of membrane-specific proteasome activities.

Firstly, the proteasome profiles *in vivo* had several significant correlations with the metabolic profile of stored RBCs. For example, the membrane levels of CASP-like activity were found to be directly correlated with central metabolites of glycolysis and pentose phosphate pathways in stored cells, while the cytosolic levels correlated well with the hypoxanthine levels at mid-storage. Moreover, the CASP- and TR-like activities of each donor's RBCs were negatively associated with the levels of plasticizers (phthalate and ethylexyl phthalate) in the RBC units throughout storage. Secondly, several metabolic variables of the donor, especially those of lipid metabolism, correlated with variations in the proteasome activity mainly at the membrane compared to the cytosol of stored RBCs (35 *vs* 6 statistically significant connections, respectively) and with the CASP-like over the other two activities (21, 7, and 7 connections, respectively). Those findings suggested that the G6PD$^-$ specific metabolic defects (in glycolysis, PPP, GSH metabolism, IMP/AMP, hypoxanthine, etc.) have the greatest influence on the CASP-like activity of the stored RBC membrane.

DISCUSSION

The cellular topology and activity of proteasomes may change in response to oxidative stress[31,34] and various diseases[35]. Several proteolytic processes take place at RBC membrane,[36] where proteasomes move during *in vivo* ageing[13]. In the present study, we characterised how proteasomes change during RBC storage. We report that both protein and activity levels vary in the membrane, cytosol and RBC-derived EVs as a function of *ex vivo* ageing, G6PD$^-$ dependent metabolic diversity, and conditions mimicking the bloodstream of the recipient.

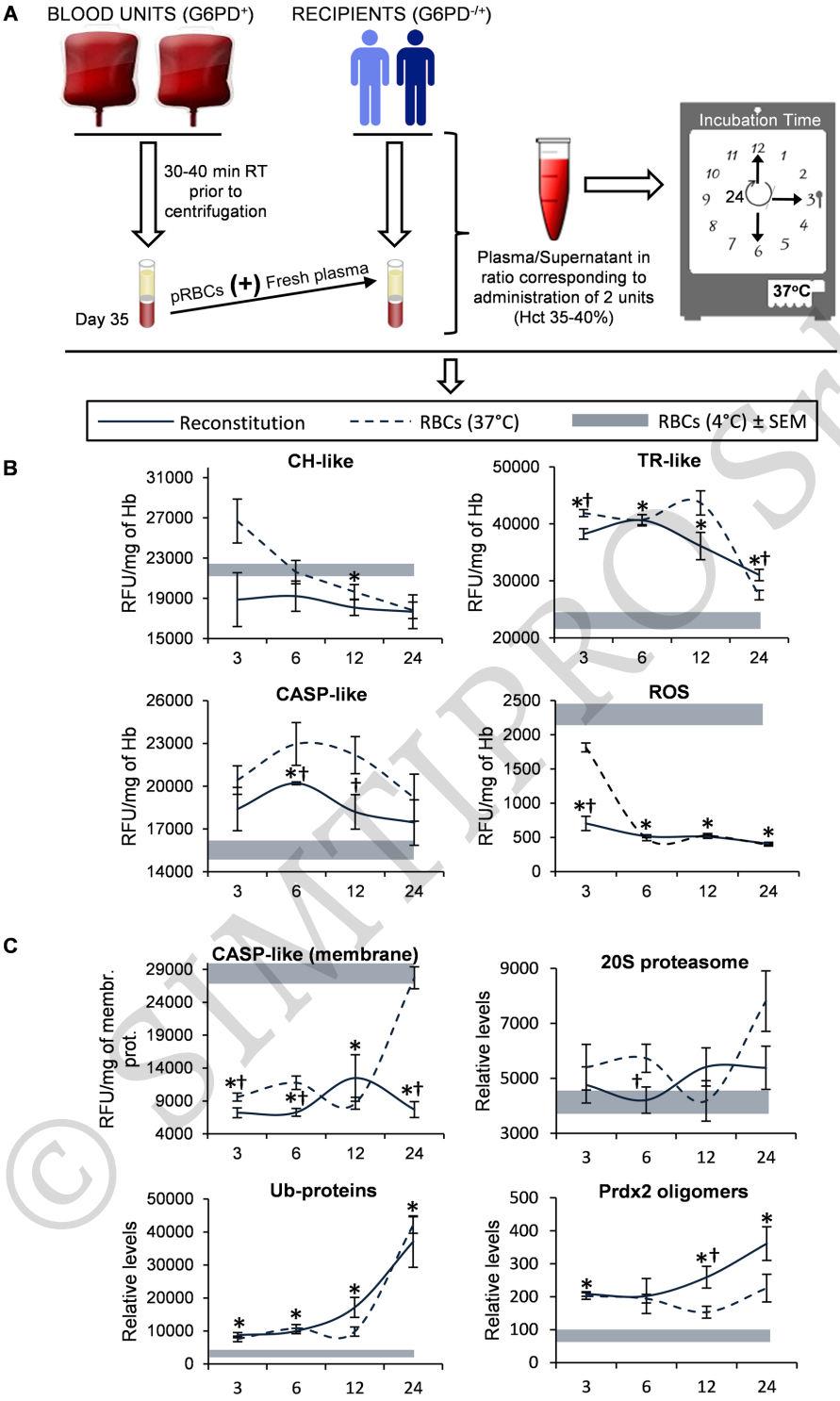

Figure 4 - Time-course variation in the proteasome activities of s35 red blood cells (RBCs) reconstituted with healthy plasma at 35-40% haematocrit at body temperature (n=4)

Experimental design (A) and measurements in cytosol (B) and membrane (C) samples. Baseline levels of non-reconstituted RBCs in the cold (4°C) and under incubation for 24 hours at 37°C are shown by grey horizontal bands (band thickness is representative of the standard error of mean [SEM]) and dotted lines, respectively. *p<0.050 reconstituted RBCs *vs* baseline RBCs in the cold; †p<0.050 reconstituted RBCs *vs* non-reconstituted RBCs at body temperature. Error bars: ±standard error of mean. min: minutes.

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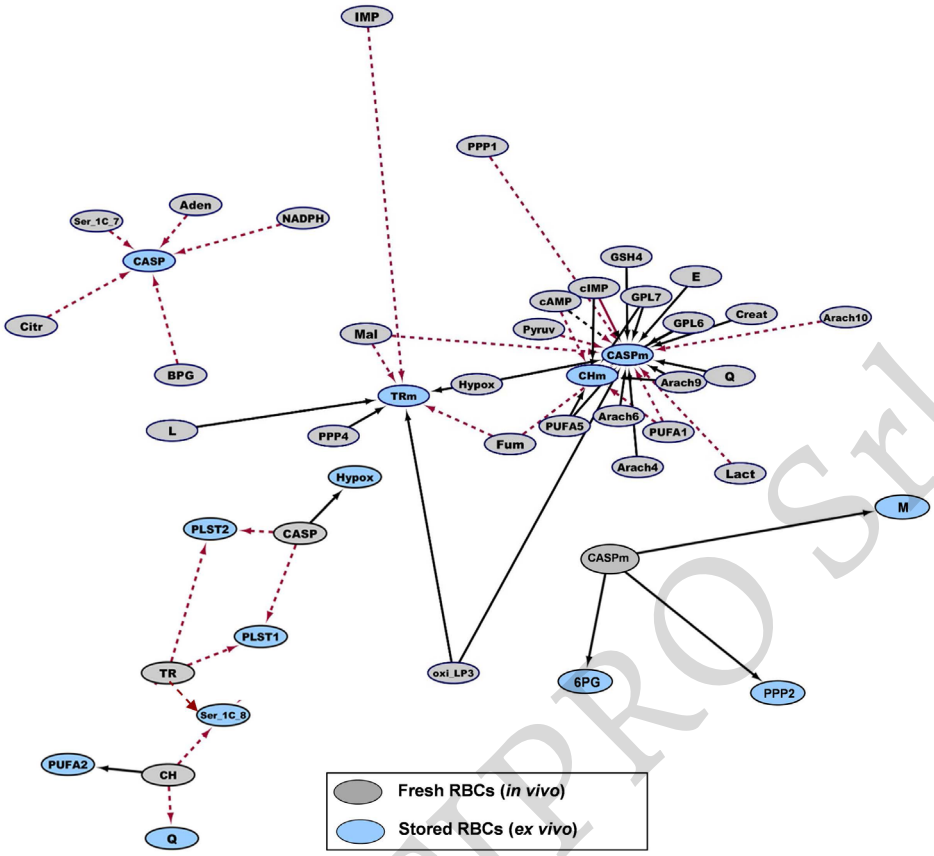

Figure 5 - The metabolome of fresh glucose 6-phosphate dehydrogenase negative (G6PD$^-$) red blood cells (RBCs)[24] exhibits interesting correlations with the proteasome activity levels in stored RBCs throughout storage, (i.e. repeatedly at every time point of storage) and vice versa, as shown in this biological network (n=6)

Most of the pairs include proteasome activities at the membrane *vs* the cytosol of stored RBCs and mainly the CASP-like activity over the other two. Continuous black lines: positive correlations; dashed red lines: negative correlations. The list of abbreviations used for the node names of the variables is presented in the *Online Supplementary* **Table SII**.

Proteasome variations in stored red blood cells

Previous studies in whole RBC lysates showed a progressive decrease in the CH-like activity by storage[5]. Our analysis revealed that not only the CH-like, but all proteasomal activities substantially decreased in the cytosol of control RBCs mainly after the middle of the storage period, which may well explain the previously reported low carbonylation of soluble RBC proteins at early storage[37]. Loss of cytosolic activity might be the result of inactivation, relocation of catalytically active components to the membrane, and release by RBCs.

Indeed, we show for the first time that storage is associated with increased binding of active proteasome components to the membrane. Thus, both proteomic and activity profiles expand the notion of a membrane-centered regulation of homeostasis in RBCs[38] in which active cytoplasmic proteins (proteasomes, "repair or destroy" proteins,[7,39] etc.) co-translocate to the membrane as part of cell responses to oxidative stress and protein damage[40]. Such heterocomplexes have been previously detected in the membrane of s28 RBCs[41], suggesting a cross-talk between anti-oxidant/chaperoning activities and protein breakdown. Chaperones have a protective role against the oxidative inactivation of proteasome[42] and a promoting role in in the proteasome-dependent degradation of oxidised targets[43].

The trigger behind the membrane targeting of proteasomes in stored RBCs might be the repeatedly reported accumulation of ROS and calcium[24,44], as recently reviewed in detail[45]. These stresses may lead

to proteolysis, oxidative defects, oligomerisation, unfolding and, commonly, translocation of denatured/oxidised cytosolic proteins to the membrane[46,47]. Oxidative reactions, including carbonylation[48] and cysteine redox status[49] are both storage time dependent and compartment dependent[37]. For instance, more RBC antioxidant enzymes were found carbonylated at mid-storage compared to early or late storage periods[48]. The energy and redox challenged G6PD$^-$ RBCs are expected to be more susceptible to storage-induced proteasome modifications because they are intrinsically more susceptible to oxidative and denaturation damages[50]. To support this, hyperoxidised Prxd2 exposes hydrophobic patches that drive oligomerisation[51], similar to those we detected in the G6PD$^-$ membrane (*Online Supplementary* **Figure S2**). Failure to prevent or repair this damage would result in increased vesiculation and/or degradation[52] by ATP/ubiquitin-dependent[16,53,54] or -independent[6,55,56] ways. The defected proteins are likely susceptible to proteasome activity at early/mid-storage, but later, extensive covalent crosslinking may render them progressively resistant to proteolysis, and the proteasome less efficient in the degradation of its clients (**Figure 6**). Proteasomal activity in stored RBCs likely varies according to both the

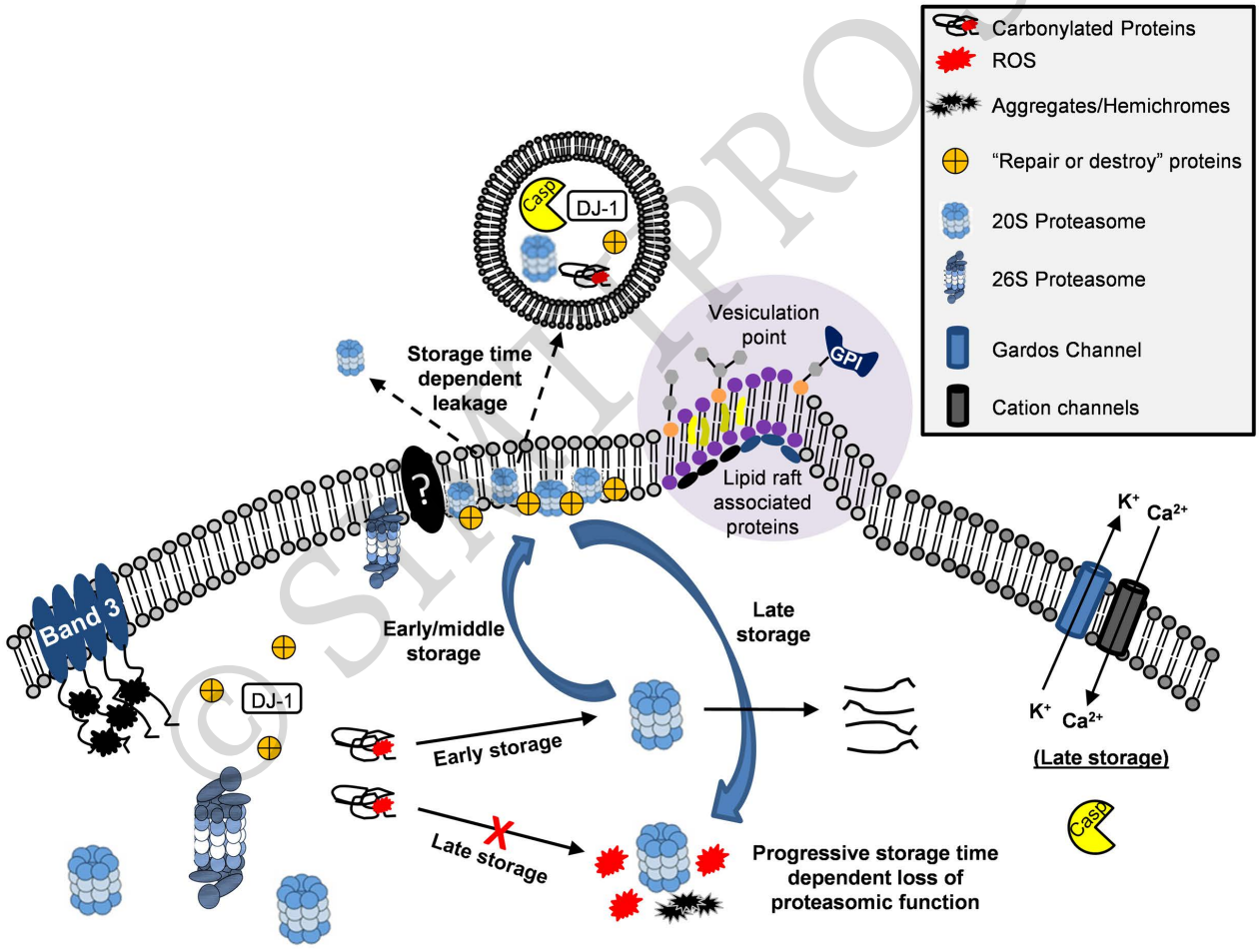

Figure 6 - Proposed model for the regulated distribution and activity of red blood cell (RBC) proteasomes during storage
20S and (fewer) 26S proteasomes work in the RBC cytosol throughout storage. Active cytosolic particles/subunits along with "repair-or-destroy" proteins involved in responses to oxidative stress and protein damage may migrate from the cytosol to the membrane, as a function of storage- and donor-associated factors, including storage time, proteome stress and glucose 6-phosphate dehydrogenase (G6PD) deficiency. Partial unfolding, denaturation and oxidation of the abundant haemoglobin (Hb) and other potential membrane-associated proteins make them susceptible to proteasome activity at early/middle storage. Later on, extensive covalent crosslinking renders them progressively resistant to proteolysis. Proteasome probably returns to cytosol in a less active form, less efficient to degrade cytosolic clients. Storage induces release of active proteasomes by RBCs in soluble form and inside extracellular vehicles (EVs).

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structural/functional integrity of the machinery per se (i.e., it is also subjected to storage lesions) as well as to variations in the amount of oxidised proteins it recognises[48].

The long-term impact of storage on RBC proteasomes can be assessed by examining their performance post transfusion. In transfusion-mimicking conditions *in vitro*, the storage-induced ROS accumulation was mitigated, verifying the antioxidant[57] and cytoprotective[58] effects of plasma. In a similar way, some of the storage-induced effects on proteasome activities (e.g., CASP- and TR-like cytosolic activities) seemed to be reversible in transfusion-mimicking conditions. However, CH- and CASP-like activities of the stored RBCs were not restored in the cytosol or the membrane, respectively, upon reconstitution with plasma at body temperature, suggesting a novel irreversible storage lesion, further associated with membrane accumulation of ubiquitinated proteins and stress markers (**Figure 4C**). This modification in proteasome activities may have functional consequences to the reconstituted RBCs, because the levels are substantially lower than those of fresh (non-stored) RBCs (**Figure 2**). It is especially interesting that, in the metabolically challenged G6PD$^-$ RBCs, donor variability in CASP-like activity showed repetitive correlations with variation in metabolic biomarkers of storage quality and the *in vivo* efficacy of RBC transfusion (including phthalates and hypoxanthine, a marker of poor recovery in mice and humans[59]), and on the other hand, the G6PD$^-$ specific metabolic profile of the donor was mostly reflected in the CASP-like activity levels of the stored RBCs throughout storage. These data suggest that proteasome modifications constitute another piece in the storage biology of G6PD$^-$ RBCs that undermine their post-transfusion recovery *in vivo*, as recently reported[26].

Extracellular proteasomes in the red blood cell units

Low-activity proteasomes, mainly consisting of 20S complexes[60], are found in human plasma. They are considered to have special biological functions, as their concentration increases significantly in inflammatory, autoimmune and neoplastic diseases[61,62]. Regarding storage, there is evidence for release of 20S proteasomes and proteasome-associated components during haemolysis[63] or through membrane vesiculation. Taking into consideration the fact that there was no difference in haemolysis but membrane vesiculation was greater in the G6PD$^-$ *vs* control RBCs[24], the finding of greater activity in the supernatant of G6PD$^-$ units likely reflects the high intracellular levels, but it may also suggest increased release by EVs (**Figure 3**).

While proteasome proteins have not been detected in plasma microvesicles *in vivo*[13], storage[11] and several physiological disturbances (such as G6PD deficiency) seem to promote their release. Despite the fact that proteasome subunits do not represent a major component of the storage EVs, the finding of greater proteasomal activity inside EVs compared to the soluble form verifies the protective effect of EVs on their loads. Release of proteasomes and associated proteins in transfusates may be clinically relevant to the recipient. To support this, the immunogenic activity of EVs released by endothelial cells is regulated by their active 20S proteasomes[64], while the soluble ubiquitin of the stored RBC units exhibits immunomodulatory effects *in vitro* on peripheral blood mononuclear cells[65].

As expected[12,24], the EVs are enriched in stress protein markers compared to the membrane of origin. DJ-1 protein, which is reported for the first time in storage EVs, is a redox-activated chaperone with several physiological roles in erythroid cells, including antioxidant defence[66]. It is also a dual regulator of 20S proteasome to which it physically binds and inhibits its activity, rescuing partially unfolded proteins from degradation, while under oxidative stress up-regulates it[67]. Oxidised DJ-1 was found at higher levels in the RBCs of unmedicated patients with Parkinson's disease, in physical interaction with Hsp90 and 20S proteasomes[68]. Of note, DJ-1 (and Prdx2) is subjected to reversible cysteine oxidations in stored RBCs as opposed to catalase that is targeted by irreversible oxidations[49].

CONCLUSIONS

Storage reversibly or irreversibly affects the topology and the activity of RBC proteasomes. There is a membrane shift of active proteasomes in stored RBCs in which the CASP-like catalytic activity likely plays a critical role. G6PD deficiency is associated with augmented proteasome activity and release. Variability in the CASP-like activity levels among G6PD$^-$ deficient donors correlates with metabolites, such as hypoxanthine and phthalates, which characterize the quality of the RBC unit and the *in vivo* efficacy of transfusion.

The probable clinical impact of the storage-induced modifications in the concentration, activity and subcellular/extracellular topology of RBC proteasomes is of great interest. The time dependence of proteasome modulation in stored RBCs (both during the storage period and the first 24-h in a recipient-like environment) may be translated to a different pattern of RBC performance and intercellular communication *in vivo*, especially in recipients with haematologic cancers treated by proteasome inhibitors. In the transfusion setting, pre-treatment of platelets with proteasome inhibitors reduces thrombosis in thrombocytopenic animals post transfusion[69], while bortezomib administration in animal recipients reduces RBC alloimmunisation[70]. As an emerging part of RBC physiology and storage lesion, and as a modulator of transfusion outcomes, RBC proteasomes deserve further thorough investigation by transfusion specialists.

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AUTHORSHIP CONTRIBUTIONS

MHA, AGK and ADA conceived the idea and designed the study. MD performed the proteomics analysis. VLT, ATA and DGK performed the physiological experiments. VLT and ATA performed the statistics and designed the graphs. MHA, ADA, VLT and ATA analysed the data. MHA and ADA wrote the paper. AV, PS, KS and AGK contributed to donor recruitment, medical support, blood processing and the final draft of the paper. ISP critically contributed to the final draft of the paper. All Authors read and approved the final manuscript.

The Authors declare no conflicts of interest.

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