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Supplementation with uric and ascorbic acid protects stored red blood cells through enhancement of non-enzymatic antioxidant activity and metabolic rewiring

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ABSTRACT

Redox imbalance and oxidative stress have emerged as generative causes of the structural and functional degradation of red blood cells (RBC) that happens during their hypothermic storage at blood banks. The aim of the present study was to examine whether the antioxidant enhancement of stored RBC units following uric (UA) and/or ascorbic acid (AA) supplementation can improve their storability as well as post-transfusion phenotypes and recovery by using *in vitro* and animal models, respectively. For this purpose, 34 leukoreduced CPD/SAGM RBC units were aseptically split in 4 satellite units each. UA, AA or their mixture were added in the three of them, while the fourth was used as control. Hemolysis as well as redox and metabolic parameters were studied in RBC units throughout storage. The addition of antioxidants maintained the quality parameters of stored RBCs, (e.g., hemolysis, calcium homeostasis) and furthermore, shielded them against oxidative defects by boosting extracellular and intracellular (e.g., reduced glutathione; GSH) antioxidant powers. Higher levels of GSH seemed to be obtained through distinct metabolic rewiring in the modified units: methionine-cysteine metabolism in UA samples and glutamine production in the other two groups. Oxidatively-induced hemolysis, reactive oxygen species accumulation and membrane lipid peroxidation were lower in all modifications compared to controls. Moreover, denatured/oxidized Hb binding to the membrane was minor, especially in the AA and mix treatments during middle storage. The treated RBC were able to cope against pro-oxidant triggers when found in a recipient mimicking environment in vitro, and retain control levels of 24h recovery in mice circulation. The currently presented study provides (a) a detailed picture of the effect of UA/AA administration upon stored RBCs and (b) insight into the differential metabolic rewiring when distinct antioxidant "enhancers" are used.

1. Introduction

Red blood cells (RBCs) stored under blood bank conditions present an array of biochemical and physiological alterations, collectively named storage lesions, known to affect their post-transfusion efficacy. The root of this structural and functional deterioration, which is mainly fueled by the lack of plasma components and hypothermic conditions, is the redox imbalance [1,2]. Indeed, the presence of oxygen in stored

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RBCs leads to the formation of free radicals, which promote damages in structural proteins and enzymes, including energy-related, antioxidant and proteostatic proteins, a phenomenon that further aggravates the redox imbalance [3–5]. This dysregulation in redox and energy metabolism can lead to elevated hemolysis post-transfusion [6,7], a complication which can in turn begin a cascade of adverse events [8,9].

In the last years, there is augmenting research activity in the field of transfusion medicine regarding the effect of specific metabolites and antioxidants upon stored RBCs in the context of ameliorating the detrimental impact of oxidative stress on cell integrity, metabolism and functionality. Recently, through a high-throughput metabolomics platform, the addition of several metabolites was applied to test their influence upon energy and redox metabolism. For example, supplementation of stored RBCs with the antioxidant N-acetylcysteine seemed to beneficially impact the pentose phosphate pathway (PPP), while the addition of glutamine did not alter glutathione (GSH) synthesis, with both results being supported by other studies [10–12]. Incubation of stored RBCs in fruit and vegetable extracts showed that the bio-flavonoid naringin protected them from lysis and externalization of removal signals [13], whereas polyphenol quercetin had a minor impact [14]. Accordingly, use of nanoparticles containing antioxidant enzymes resulted in the improvement of the storability profile of RBCs [15], as also observed in the case of GSH loading [16].

Uric acid (UA), a radical species scavenger, represents the main non-enzymatic antioxidant in human plasma, with beneficial effects upon circulating RBCs [17]. Notably, the extracellular UA appears to be rapidly increased in the first week of storage [18], while stored RBCs donated by individuals with elevated serum UA have proven to cope better with storage stress [19,20]. Nonetheless, to our knowledge, the enhancement of RBC units exclusively with UA has not been studied yet. In the same context, plasma ascorbic acid (AA) is an exceptionally effective antioxidant, able to protect plasma lipids against peroxidation and maintain RBC hemoglobin (Hb) in a reduced state [21]. Its addition to stored RBCs has been found protective against RBC fragility [22] and 2,3-bisphosphoglycerate depletion [23]. In most cases, AA supplementation was examined in combination with other antioxidants, resulting in improved GSH preservation and reduced hemolysis [24,25]. Interestingly, a recent study demonstrated that restoration of normal plasma levels of UA and AA in RBC units achieved a partial prevention of the detrimental metabolic shift observed during RBC storage [26]. Having all of the above in mind, and the fact that UA and AA can act synergistically [27], the aim of the present study was to evaluate the effects of higher-than-physiological concentrations of UA and AA separately, as well as in combination, upon (a) an array of RBC storability parameters, including hemolysis, membrane vesiculation, proteostasis, redox profile, metabolism and protein recruitment to the membrane, and (b) the −unknown to date− post-transfusion RBC physiology and recovery, by using elegant *in vitro* and animal models of transfusion.

2. Materials and methods

2.1. Biological samples and blood unit preparation

Thirty-four leukoreduced RBC units containing citrate-phosphate-dextrose (CPD)/saline-adenine-glucose-mannitol (SAGM) (final volume: 338 ± 27 mL) were prepared from healthy individuals (female/male ratio: 10/24). Each RBC unit was then split under aseptic conditions into four sub-units (resulting in n = 136 smaller units) of equal volume (79 ± 5 mL each). One sub-unit from each donor was used as (untreated) control, while the other three were supplemented with UA (in-bag concentration: 8 mg/dL for male and 7.2 mg/dL for female donors; diluted in 0.01 M NaOH), AA (in-bag concentration: 2.3 mg/dL; diluted in 0.9% NaCl) or their mixture. In this paired analysis, unit splitting contributed to absorbing the well-known donor variation effect upon several redox and physiological parameters of stored RBCs [28–30]. The different levels of UA supplementation in RBC units from male and female donors were chosen on the basis of the known intersex differences in the normal range of serum UA concentration *in vivo* [31], to avoid excessive amounts of uric acid that could potentially harm the stored RBCs from female donors. Since previous studies of our team [19,20] revealed that RBCs from donors with increased serum UA levels demonstrate superior storability profile, we performed preliminary experiments in freshly drawn RBCs to select the optimum concentrations (higher than the normal-range *in vivo* values) with respect to hemolysis, pH and ROS accumulation. In order to prevent immediate contact of the supplementation stock solution with the cells (and thus unwanted effects due to exposure to high solution concentrations) the blood units were left overnight for RBC settling, and the antioxidants were added in the supernatant. All units were stored for 42 days at 4 °C and sampling was aseptically performed after gentle agitation in early (day 7), middle (day 21) and late (day 42) storage. No RBC settling was observed in any of the preparations during the 42-day storage period. All experiments were performed in n = 34 per group, unless otherwise stated. Regarding the experiments with lower number of tested samples, the selection was completely random and not based on the outcome of other experimental procedures. The study was approved by the Ethics Committee of the Department of Biology, School of Science, NKUA. Investigations were carried out upon donor consent, in accordance with the principles of the Declaration of Helsinki.

2.2. Hemolysis Parameters

The levels of spontaneous hemolysis were calculated as extracellular Hb using the spectrophotometric method firstly shown by Harboe [32], along with Allen's correction. In order to evaluate the resistance of RBCs to osmotic lysis, the samples were subjected to ascending NaCl concentrations to finally calculate their mean corpuscular fragility (MCF; % NaCl at 50% hemolysis). Accordingly, to assess the mechanical fragility, the samples were rocked with stainless steel beads for 1h, while non-rocked aliquots were used to subtract spontaneous Hb release. After centrifugation, the levels of extracellular Hb were calculated through Harboe's method [33].

2.3. Redox parameters

The antioxidant capacity of the supernatant (namely, the extracellular compartment of the RBC unit that contains the anticoagulant/preservative solutions and a small volume of the donor plasma) was analyzed by the ferric reducing antioxidant power (FRAP) assay [34], with and without prior treatment with uricase. Oxidative hemolysis was calculated post exposure of the samples to phenylhydrazine (PHZ; 17 mm) for 1h at 37 °C. Afterwards, a centrifugation was performed to measure the released Hb (Harboe's method). Intracellular reactive oxygen species (ROS) accumulation was evaluated by fluorometry (VersaFluor™ Fluorometer System, BIORAD Hercules, CA, United States) at 490 nm excitation and 520 nm emission wavelengths. Small aliquots (~1% hematocrit) of RBCs were treated with 5-(and-6)-chloromethyl-2′, 7′-dichloro-dihydrofluoresceindiacetate, acetyl ester (CM-H_2DCFDA; 10 μmol/L for 30 min at room temperature), a molecule that emits fluorescence when oxidized by ROS. This was also performed for RBCs previously incubated with *tert*-butyl hydroperoxide (tBHP; 100 μM for 45 min at 37 °C). Three quick washes with PBS of 310mOsm at 1000×*g* were performed to remove the excess of tBHP, while after the incubation with DCFDA, the aliquots were washed once and were then incubated for a short recovery time of 10–15 min at room temperature to render the dye responsive to oxidation. The fluorescence units were thereafter normalized to protein concentration (Bradford; Bio-Rad, Hercules, CA). Hemichromes (HMC) were detected spectrophotometrically on isolated RBC membranes by measuring heme absorbance through the following equation: HMC = $-133xAbs_{577}$ $-114xAbs_{630}$ $+233xAbs_{560}$ [35]. Evaluation of membrane lipid peroxidation was based on the formation of a chromogenic complex between malondialdehyde (MDA), a biomarker of

lipid peroxidation, and thiobarbituric acid (TBA). Briefly, the lipid part of RBCs was retrieved after subjection to trichloroacetic acid (TCA; 20%) and was then treated with 0.67% TBA. The chromogenic complex was measured at 532 nm [36].

2.4. Membrane and vesicle isolation and immunoblotting

Hypotonic lysis was performed to isolate RBC membranes (n = 6 per group), as previously extensively described [37]. Briefly, packed RBCs (~1.6 mL after centrifugation at 1000×*g*/10 min and supernatant disposal) were exposed for 45 min (1:20 ratio for membrane isolation; 1:10 for cytosol isolation) to hypotonic sodium phosphate buffer (5 mmol/L, pH 8.0) supplemented with protease inhibitors (0.3 mM phenyl-methyl-sulfonyl fluoride; PMSF). A centrifugation was then performed at 19,000×*g* for 20 min, after which cytosols were collected and stored, while the precipitated membranes were washed under the same conditions to remove the excess of Hb, until full discoloration of the pellet. Samples of supernatant from late-stored RBCs (starting volume of 8 mL) were ultra-centrifuged at 37,000×*g* for 1 h, after passing through sterile 0.8 μm nitrocellulose filters (Millipore, Carrigtwohill, County Cork, Ireland), to isolate extracellular vesicles (EV; n = 6 per group). The EV pellet was resuspended in PBS and was ultracentrifuged twice under the same conditions. Finally, the isolated EVs were resuspended in PBS along with protease and phosphatase inhibitors (1 mM PMSF, 1/100 protease inhibitor cocktail, 2 μL/mg phosphatase inhibitor cocktail; Sigma, St. Louis, MO) and the protein concentration was determined (by the Bradford method) to calculate total vesicular protein per RBC unit volume [38].

Equal amount of isolated membranes (30 μg) or EVs (40 μg) were loaded in 10% Laemmli gels and transferred to nitrocellulose membranes for immunoblotting. Primary antibodies against 4.1R (kindly provided by Prof. J. Delaunay, Laboratoire d' Hématologie, d' Immunologie et de Cytogénétique, Hopital de Bicetre, Le Kremlin-Bicetre, France), Band 3 (Sigma-Aldrich, Munich, Germany), Hb (Europa Bioproducts, Wicken, UK), stomatin (kindly provided by Prof. R. Prohaska, Institute of Medical Biochemistry, University of Vienna, Austria), Caspase-3, DJ-1, CD9 (Cell Signaling Technology, Danvers, MA, USA), calpain-1, heat shock protein 70 (HSP70; Santa Cruz Biotechnology, Santa Cruz, CA, USA), peroxiredoxin-2 (prdx2; Acris, Luzern, Switzerland) and human IgGs (Sigma Aldrich, St. Louis, MO, United States) were used. The immunoblots were developed through chemiluminescence, thus species-specific HRP-conjugated secondary antibodies were also used. The evaluation of the developed bands was assessed by scanning densitometry (Gel Analyzer v.1.0, Athens, Greece). In order to estimate the carbonylation of membrane proteins, the Oxyblot kit was used, as per manufacturer's specifications (Oxyblot, Millipore, Chemicon, Temecula, CA, USA).

2.5. Intracellular calcium, procoagulant EVs and RBC morphology

The accumulation of Ca^{2+} in the cytosol was analyzed through fluorometry using Fluo-4 AM (2 μmol/L final concentration for 40 min at 37 °C; Invitrogen, Molecular Probes, Eugene, OR), an ester that emits fluorescence when interacting with calcium cations [39]. The measured fluorescence units were normalized to protein concentration (Bradford assay). The procoagulant activity of EVs was evaluated using a functional ELISA kit (Zymuphen MPactivity, Hyphen BioMed, Neuvillesur-Oise, France). The principal on which this assay is based is the conversion of prothrombin to thrombin in the presence of phosphatidylserine on EVs, a phenomenon that is captured by using a chromogenic thrombin substrate (absorbance at 405 nm). Morphological evaluation of the late-stored RBCs (n = 3) was performed by confocal laser scanning microscopy (CSLM; Digital Eclipse C1, Nikon, NY) following labeling with the lipophilic dye D-383 (1,1′-Didodecyl-,3,3′,3′-Tetramethylindocarbocyanine Perchlorate), as per manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The fluorescence excitation and emission maxima for membrane-bound dye were 549 nm and 565 nm, respectively. The percentage of discocytes in each sample was evaluated in duplicate assays after counting at least ~600 RBCs per group in randomly chosen fields.

2.6. Proteasome activity

The activity of the proteasome machinery was measured in both cytosol and membrane fractions (n = 6 per group). To determine the three distinct proteasome activities, the samples were incubated with the fluorogenic substrates Suc-Leu-Leu-Val-Tyr-aminomethylcoumarin (AMC) for chymotrypsin-like (CH-like), z-Leu-Leu-Glu-AMC for caspase-like (CASP-like), and Boc-Leu-Arg-Arg-AMC for trypsin-like (TR-like) activity for 1.30 h (CH-like) or 3 h (CASP- and TR-like) at 37 °C [30]. The same procedure was followed in the presence of inhibitors (10–20 μM bortezomib for the CH- and CASP-like activities, 200 μM MG-132 for the CH-like activity, and 100 μM lactacystin for the TR-like activity) showing high levels of inhibition (93–98%). All substrates and inhibitors were procured from Enzo Life Sciences (New York, NY, United States). Fluorescent units (after subtraction of the unspecific levels) were normalized to protein concentration (Bradford assay).

2.7. Metabolomics analysis

Each sample (n = 6 per group) was added to 1000 μl of a chloroform/methanol/water (1:3:1 ratio) solvent mixture stored at −20 °C. The tubes were mixed for 30 min and subsequently centrifuged at 1000×*g* for 1 min at 4 °C, before being transferred to −20 °C for 2–8 h. The solutions were then centrifuged for 15 min at 15,000×*g* and dried to obtain visible pellets. Finally, the dried samples were resuspended in 0.1 mL of water, 5% formic acid and transferred to glass autosampler vials for LC/MS analysis. Twenty-microliter of extracted supernatant samples was injected into an ultra-high-performance liquid chromatography (UHPLC) system (Ultimate 3000, Thermo) and run in positive ion mode. A Reprosil C18 column (2.0 mm × 150 mm, 2.5 μm - Dr Maisch, Germany) was used for metabolite separation. Chromatographic separations were achieved at a column temperature of 30 °C and flow rate of 0.2 mL/min. A 0–100% linear gradient of solvent A (ddH_2O, 0.1% formic acid) to B (acetonitrile, 0.1% formic acid) was employed over 20 min, returning to 100% A in 2 min and a 1-min post-time solvent A hold. The UHPLC system was coupled online with a mass spectrometer Q-Exactive (Thermo) scanning in full MS mode (2 μscans) at 70,000 resolution in the 60–1000 *m/z* range, target of 1 × 106 ions and a maximum ion injection time (IT) of 35 ms. Source ionization parameters were: spray voltage, 3.8 kV; capillary temperature, 300 °C; sheath gas, 40; auxiliary gas, 25; S-Lens level, 45. Calibration was performed before each analysis against positive ion mode calibration mixes (Piercenet, Thermo Fisher, Rockford, IL) to ensure sub ppm error of the intact mass. Data files were processed by MAVEN.8.1 (http://genomics-pubs.princeton.edu/mzroll/) upon conversion of raw files into mzXML format through MassMatrix (Cleveland, OH).

2.8. In vitro Model of Transfusion

An *in vitro* model of transfusion was used to evaluate the impact of recipient plasma and body temperature on stored RBCs, as previously extensively described [40]. For this purpose, fresh blood was drawn by ten healthy potential recipients into citrate vacutainer tubes. Then, packed RBCs of early and late storage (n = 10 per group) were mixed with these plasma samples. A portion of the stored unit's supernatant was added in the fresh recipient plasma to reach a ratio that mimics the administration of two blood units per transfusion recipient, resulting in 32–34% final hematocrit. The samples were incubated at body temperature (simulation of the temperature environment of the recipient) for 24h (the standard time point of transfusion evaluation). Hemolysis and oxidative stress-related parameters were then assessed.

2.9. Animal model of transfusion

All the experiments regarding the xenobiotic model of transfusion were performed in the Biomedical Research Foundation of the Academy of Athens (BRFAA), and the study protocol was approved by the Department of Agriculture and Veterinary Service of the Prefecture of Athens (Permit Number: 534915, July 23, 2020). For the evaluation of 24 h post-transfusion recovery, a total of 24 wild type C57BL/6J male mice, 8–12 weeks old, were used, as previously described [40]. Briefly, RBCs of early and late storage (n = 6 per group) were labeled with the lipophilic dye D-383, as per manufacturer's instructions, washed 3 times, and then infused into recipient-mice by intravenous injection in the tail vein. The volume infused (200 μL at ~55% hematocrit with sterile PBS of 310 mOsm) is respective to the transfusion of two blood units. To assess the 24h recovery of the transfused RBC through flow cytometry (FACSAria IIu/Diva software, BD Pharmingen, San Jose, CA, USA), blood sampling was performed (~50–70 μL) via the facial vein after 20 min (100% recovery) and 24 h post-transfusion. For the flow cytometry analysis, the blood samples were diluted (1:200) in a PBS 310 mOsm buffer containing 5% glucose and 1% BSA, and were then filtered for debris removal. The fluorescence excitation and emission maxima for membrane-bound dye were 549 nm and 565 nm, respectively. A minimum of 1,000,000 events per sample was counted and the recovery was calculated as the % ratio of the fluorescence levels of the 24h time point to the respective levels of the 20 min measurement. The animals were evaluated weekly regarding their well-being (weight, mobility, food and water consumption, social behavior).

2.10. Statistical analysis

All experiments were performed in duplicate. The statistical package SPSS Version 22.0 (IBM Hellas, Athens, Greece, administered by NKUA) was used for the statistical evaluation of the results. All parameters were tested for normal distribution and the presence of outliers (Shapiro-Wilk test and detrended normal Q–Q plots). In order to present as reliable as possible results regarding the differences between the selected

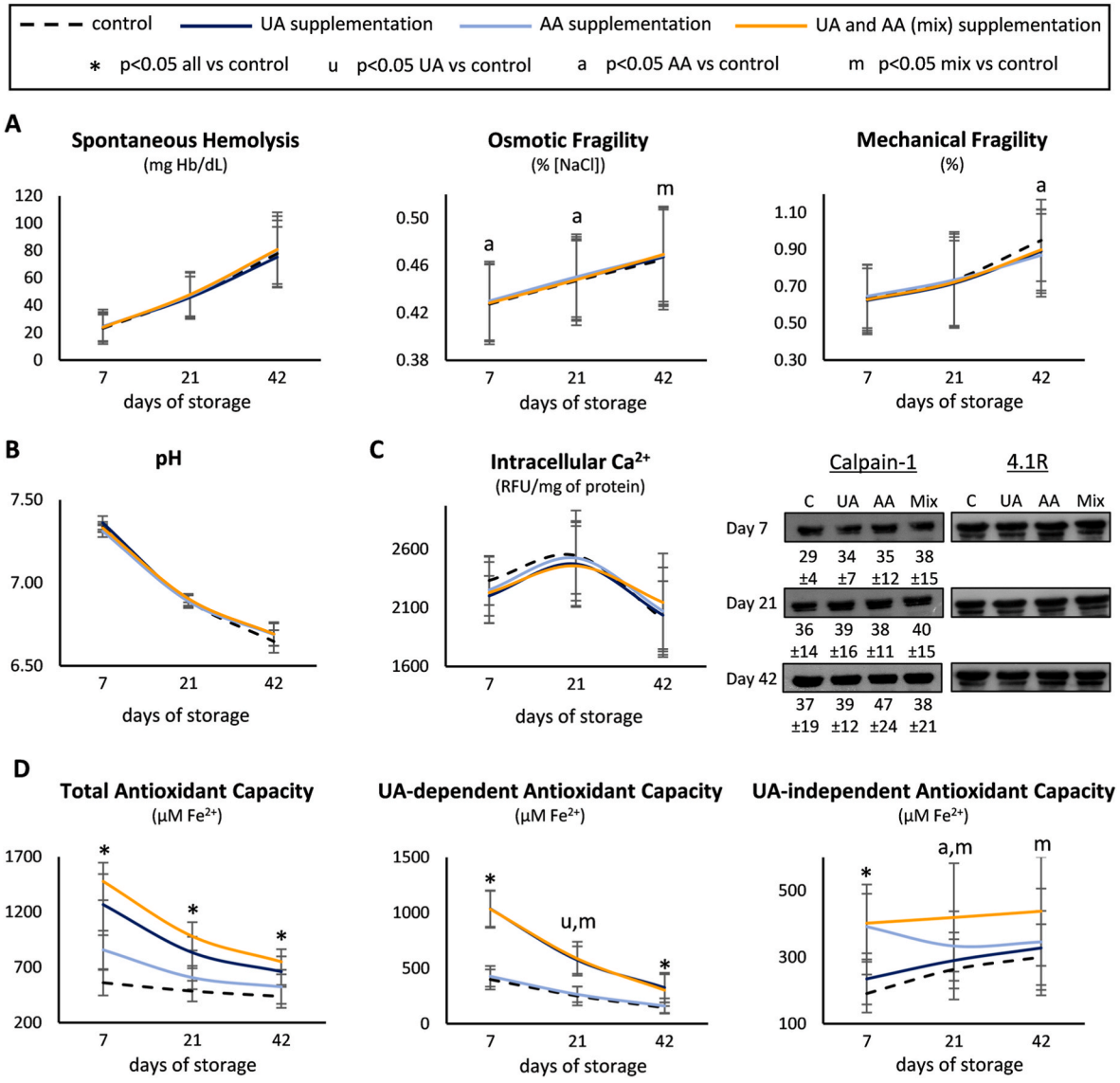

Fig. 1. Antioxidants supplementation effects upon qualitative parameters of stored red blood cells. Hemolysis parameters (A), pH (B), calcium accumulation along with calpain-1 recruitment to the membrane (C) and extracellular antioxidant capacity (D) during storage of red blood cells under standard conditions or upon supplementation with uric acid (UA) and/or ascorbic acid (AA). Representative immunoblots are shown (n = 6). 4.1R protein was used as internal loading control. Data are presented as mean ± SD. C: control samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

treatments the following strategy was followed: in the presence of extreme outliers the respective values were excluded from the analysis and the statistical test of choice was repeated. If the statistical outcome was not affected by the presence of the outlier, its value was considered "valid" and was re-entered to the data set. In the opposite case, the outlier was removed. Two-way repeated measures ANOVA with Bonferroni-like adjustment for multiple comparisons was used for the determination of the between groups differences throughout the storage period. Significance was accepted at $p < 0.05$. In the case of metabolomics analyses, and in order to identify which metabolic pathways were mostly affected in the treated samples compared to the control groups, the MetPA (Metabolomic Pathway Analysis) bioinformatic tool included in the MetaboAnalyst 5.0 Software package, which combines the results from the pathway enrichment analysis with the pathway topology analysis, was employed. False discovery rate (FDR) was used for controlling multiple testing (q-value threshold was ≤ 0.05). Accepted metabolites were verified manually using HMDB, KEGG, and PubChem databases. A Human library was used for pathway analysis (KEGG). Global test was the selected pathway enrichment analysis method, whereas the node importance measure for topological analysis was the relative betweenness centrality.

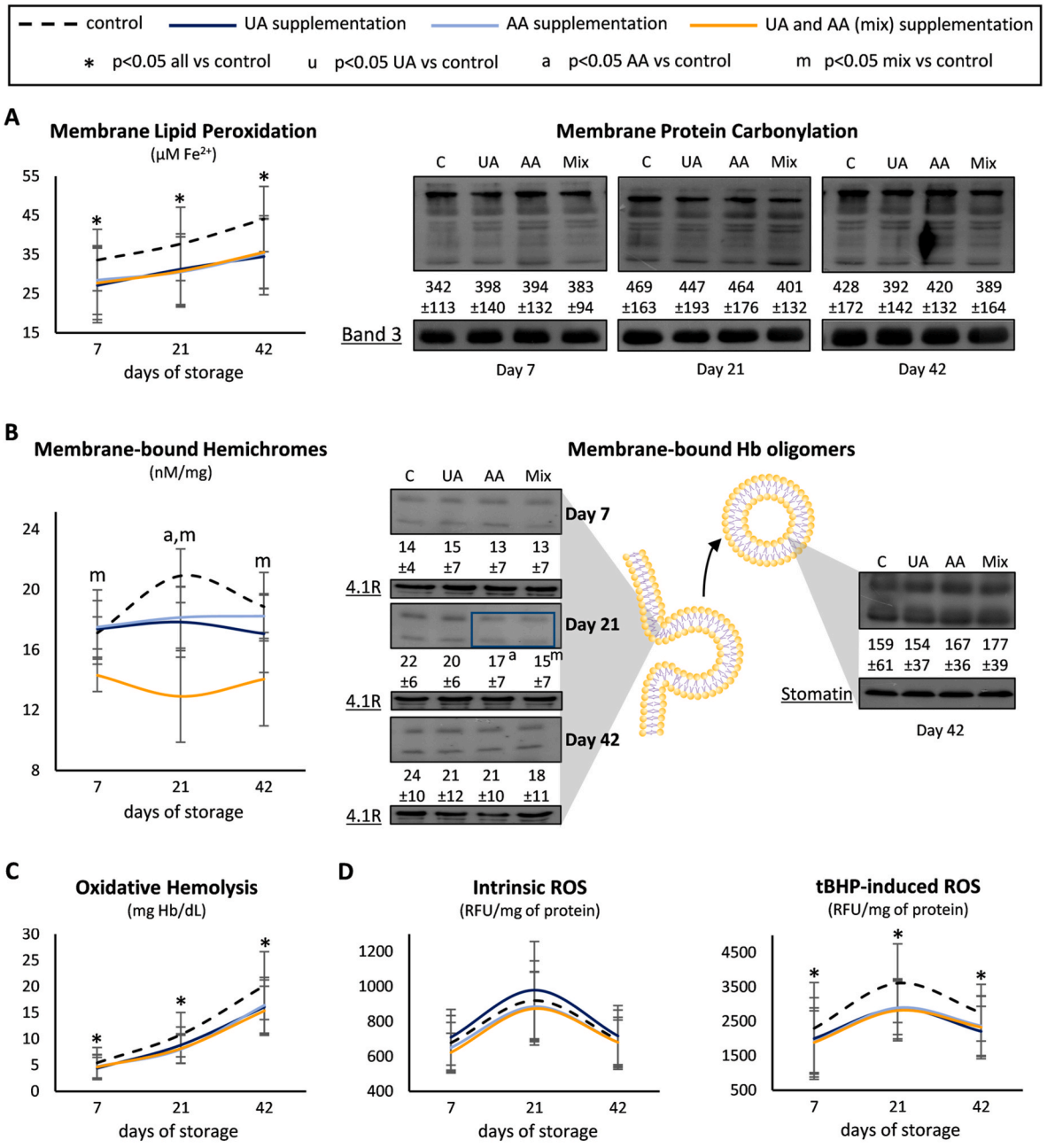

Fig. 2. Antioxidants supplementation effects upon the oxidative stress of stored red blood cells. Membrane lipid oxidation and protein carbonylation (A), membrane-bound hemichromes and hemoglobin (Hb) oligomers (B), oxidatively-induced hemolysis (C) and intracellular ROS accumulation (D) during storage of red blood cells under standard conditions or upon supplementation with uric acid (UA) and/or ascorbic acid (AA). Representative immunoblots are shown (n = 6). Band 3 and 4.1R proteins were used as internal loading controls for membrane samples, while stomatin served the same role in extracellular vesicles. Blue rectangular: statistically significant reduction ($p<0.05$). Data are presented as mean ± SD. C: control samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Physiological, redox and proteostasis parameters

The addition of UA, AA or their combination did not alter the levels of released Hb, while the presence of AA slightly increased the RBC osmotic fragility during storage (e.g., ~0.5% elevation during early storage), but decreased the mechanical fragility of long-stored cells (Fig. 1A). The supplementations tested did not affect extracellular pH (Fig. 1B), nor the intracellular accumulation of calcium and the recruitment of the calcium-dependent protease calpain-1 to the membrane (Fig. 1C). The number of discocytes in the modified units of late storage was also similar to the control (Supplementary Fig. 1). On the contrary and as expected, the total antioxidant capacity (TAC) of the supernatant was increased in all cases in comparison to control, while its UA-dependent and UA-independent aspects were mostly elevated in the presence of UA or AA, respectively (Fig. 1D).

Regarding oxidative-stress related phenotypes, while the levels of membrane lipid peroxidation were lower in the modified units when compared to control, membrane protein carbonylation did not differ (Fig. 2A). In the same context, the spectrophotometric measurement of membrane-bound hemichromes, that represent denatured and oxidized Hb species, revealed lower levels in the samples supplemented with both antioxidants during storage, a pattern also observed in AA-treated RBCs of middle-storage (Fig. 2B). Through a different approach, namely, immunodetection of Hb oligomers in isolated RBC membranes, a similar result was retrieved, with AA- and mix-supplemented units presenting decreased Hb oligomers in day 21. However, this difference did not appear between isolated EVs of late-stored RBCs (Fig. 2B). PHZ-induced hemolysis demonstrated a universal reduction in the modified units in

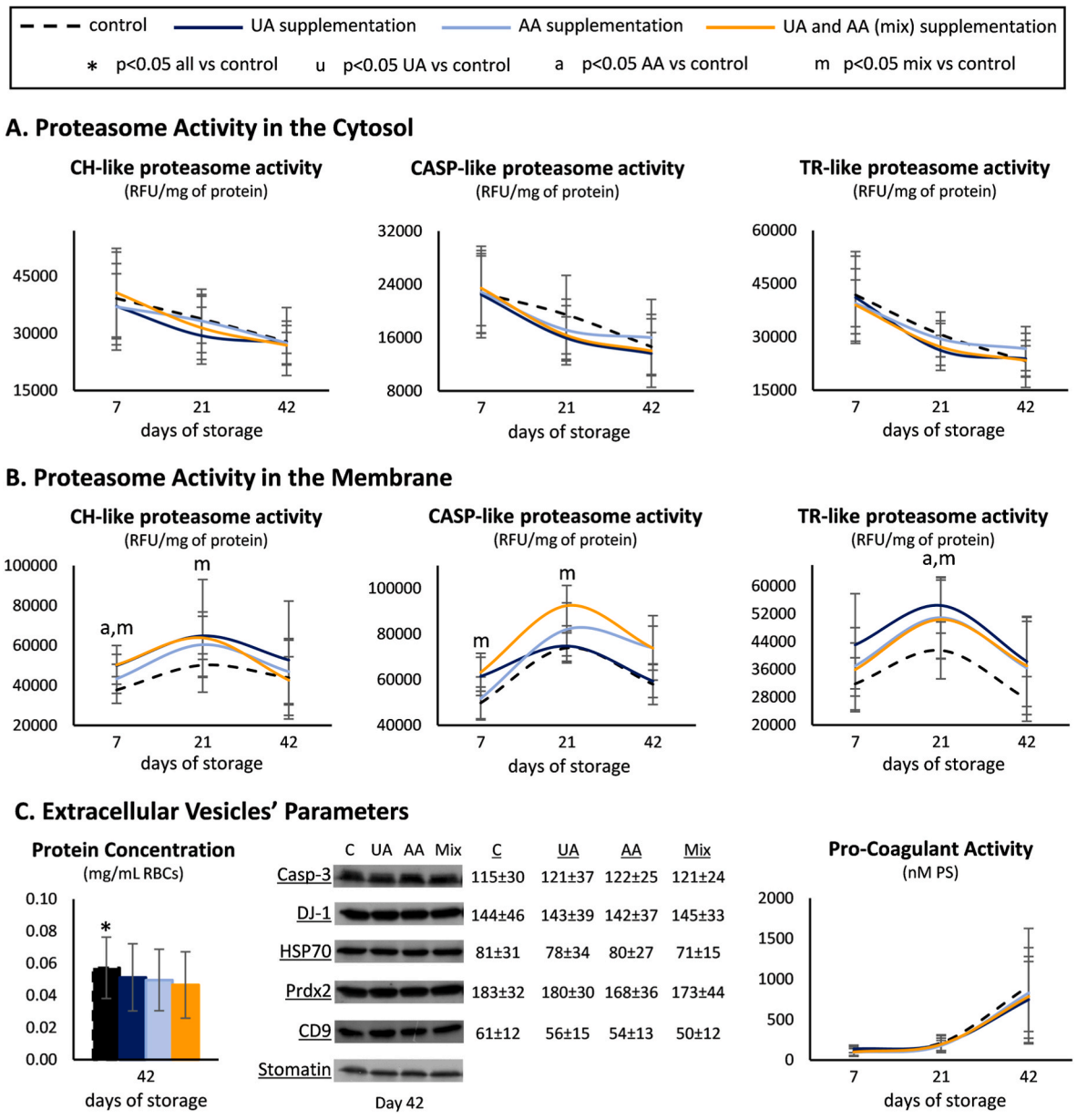

Fig. 3. Antioxidants supplementation effects upon the proteasomal activity and extracellular vesicle's (EVs) parameters of stored red blood cells. Proteasomal activities in the cytosol (A) and at the membrane (B), and EV protein concentration, procoagulant activity and specific proteins (C) during storage of red blood cells under standard conditions or upon supplementation with uric acid (UA) and/or ascorbic acid (AA). Representative immunoblots are shown (n = 6). Stomatin was used as internal loading control. Data are presented as mean ± SD. C: control samples; Prdx2: peroxiredoxin-2; Casp-3: caspase-3. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

comparison to controls (Fig. 2C), whereas ROS accumulation was decreased only in the case of tBHP-triggered RBCs and not in non-stimulated samples (Fig. 2D). Prdx2 binding to the membrane was similar between all sub-groups, while its oligomerization presented non-significant trends for lower levels in UA and AA enhancements (e.g., day 21: 6.1 ± 2.4, 5.4 ± 1.9 and 8.1 ± 3.7 vs 7.6 ± 2.2 densitometry A.U., UA-, AA- and mix-supplemented vs control units, $0.05 < p < 0.10$ for UA, AA vs control).

To examine if proteostasis mechanisms were affected in stored RBCs, the three distinct proteasomal activities were assessed in fractionated samples of RBC membranes and cytosols. While the proteasome activity levels of the supplemented RBCs did not vary from the respective control values in the cytosol (Fig. 3A), the addition of AA −alone or combined with UA− lead to sporadically increased levels of the three activities in the membrane during early and middle storage (Fig. 3B). At the same time, translocation of HSP70 to the membrane was mostly uniform across modified and control units with the exception of a not statistically significant trend for higher levels in AA middle-aged units (67 ± 11, 74 ± 22 and 70 ± 23 vs 64 ± 17 densitometry A.U., UA-, AA- and mix-supplemented vs control units, $0.05 < p < 0.10$ for AA vs control). The total protein concentration of EVs collected from the supernatant of modified long-stored units was lower than that of controls (Fig. 3C). Nonetheless, non-significant between-group differences in the protein composition were detected for an array of typical EV proteins. It should be noted that the exosome-marker CD9 was also detected in the isolated EVs of all units. Finally, control levels of pro-coagulant activity were detected in EV samples collected from the supplemented units throughout storage (Fig. 3C).

3.2. Metabolomics analysis

The results of the combined pathway enrichment and topology analyses (Supplementary Fig. 2) highlighted that energy metabolism (e.g., glycolysis, pyruvate metabolism or tricarboxylic acid (TCA) metabolites) was significantly perturbed during early storage in all modifications compared to controls, an observation that was mainly maintained in AA-treated blood units throughout storage. Pentose phosphate pathway (PPP) was affected in mix-treated samples regardless of storage time, whereas, along with GSH metabolism, they were altered during middle storage in every condition tested. Interestingly, purine and glutamate/glutamine metabolism arose as key affected pathways in the UA- and mix-supplemented RBC units. Folate metabolism of early stored and methionine/cysteine metabolism of early and late stored UA-treated samples were also impressively perturbed (Supplementary Fig. 2).

In light of the well-known fluctuations in the metabolic processes and fluxes during the storage period [41] and since the aim of the present study was the evaluation of differences between the modified RBC units and their respective controls, all metabolic data are presented in the graphs as fold change vs control. Starting from energy metabolism, glycolysis metabolites were mostly decreased in all tested supplementations versus the respective controls during early storage, a difference that was gradually reversed, especially in the AA-supplemented units, that were characterized by higher pyruvate levels from middle storage onwards (Fig. 4). Of note, pyruvate was also higher in early stored mix-treated RBCs, while lactate was lower in their older counterparts. Concerning TCA metabolism some differences were observed in metabolites such as fumarate, malate or succinate, especially in early storage of supplemented RBCs. Interestingly, α-ketoglutarate (a metabolite related to GSH synthesis via glutamate production) was upregulated in both AA- and mix-treated RBCs until the middle of the storage period, a time-point in which AA supplementation resulted in significant upregulation of the majority of TCA metabolites. PPP was unaffected in day 7, however metabolites of its early oxidative phase, such as 6-phospho-D-gluconate were increased in middle storage in all treatments, along with D-gluconic acid (only in the case of mix supplementation; 1.43, 1.06 and 1.52 fold change, UA-, AA- and mix-supplemented vs control, $p<0.05$ only for mix). The non-oxidative part of PPP started to present increased values in day 21 of mix-treated samples, but it was mainly altered (sporadically) in late-stored RBCs of all modified units versus controls (Fig. 4).

GSH synthesis during storage was affected by different metabolic routes in the distinct supplementations (Fig. 5). In the case of early-stored AA units, there was a slight (trend or statistically significant) increase in metabolites such as aspartate and alanine (along with the abovementioned α-ketoglutarate), known to be implicated in GSH synthesis through glutamate formation. Two weeks later, the effect was more evident in both AA- and mix-treated RBCs, with significant fold changes in all implicated metabolites, along with GSH itself. Notably, glutathione disulfide (GSSG) was simultaneously decreased in comparison to controls. This pathway ceased to be altered in late storage of AA samples, but lower levels of some of the initial metabolites, along with higher levels of GSSG were observed in mix samples. It should not be omitted that the levels of intracellular ascorbate were higher in both shortly- and long-stored AA-supplemented RBCs compared to controls. Regarding the addition of UA, it presented an impact upon folate (Supplementary Fig. 3) and methionine cycle during the first week of RBC storage. More specifically, all metabolites involved in these cycles were upregulated in UA-treated RBCs, including 5-methyltetrahydrofolate that acts as a methyl donor in the remethylation of homocysteine to methionine. In the next two time-points tested, increases arose in metabolites implicated in the production of GSH through methionine and cysteine (e.g., cysteine, cys-gly) leading to elevated levels of GSH itself (Fig. 5).

The addition of UA −alone or in combination with AA− also influenced purine metabolism (Fig. 6). During early storage, intracellular UA was increased in both UA and mix supplementations, while the general pathway was moderately altered in the case of mix samples (e.g., increased IMP). During middle storage, the mix-supplemented RBCs presented elevation in all implicated metabolites, while the UA-supplemented ones had more minor modifications, with trends for increased UA and xanthine and significantly elevated xanthosine (10.36, 9.29 and 8.64 fold change, UA-, AA- and mix-supplemented vs control, $p<0.05$) and AMP. In the last day of storage all metabolites downstream of hypoxanthine until uric acid were increased in both modifications enriched with UA when compared to controls. Nonetheless, the final oxidation products of UA, namely allantoate (Fig. 6) and allantoin (0.979, 0.823 and 0.950 fold change, UA-, AA- and mix-supplemented vs control, $p>0.05$), exhibited control levels.

3.2. Post-transfusion phenotypes and recovery

To take a step further and study the effect of the antioxidant supplementation beyond storage, and more specifically, in the final link of the transfusion chain, both *in vitro* and animal models of transfusion were used. Exposure of supplemented stored RBCs to recipient plasma at body temperature did not affect their hemolysis phenotypes and intrinsic ROS production, but ultimately decreased the levels of oxidatively-induced hemolysis and ROS accumulation, in comparison to controls (Fig. 7A). The membranes of stored RBCs of early (for all supplementations) or late (for the combination of the two antioxidants) storage presented decreased attachment of immunoglobulins (IgGs) (Fig. 7B). However, this minor opsonization was not reflected in terms of differential 24h survival post-transfusion in animal models, since no differences were observed (Fig. 7C). In addition, the transfused mice were characterized by equal levels of post-transfusion intravascular hemolysis (e.g., 20min post-transfusion of late-stored RBCs: 75.32 ± 29.92, 86.67 ± 31.12 and 89.93 ± 36.20 vs 81.11 ± 30.08 mg/dL, UA-, AA- and mix-supplemented vs control units, $p>0.05$) and hematuria (e.g., 20min post-transfusion of late-stored RBCs: 52.66 ± 23.12, 59.32 ± 30.18 and 61.20 ± 25.82 vs 55.73 ± 21.38 mg/dL, UA-, AA- and mix-supplemented vs control units, $p>0.05$), as well as physiological mobility, social behavior and food/water consumption.

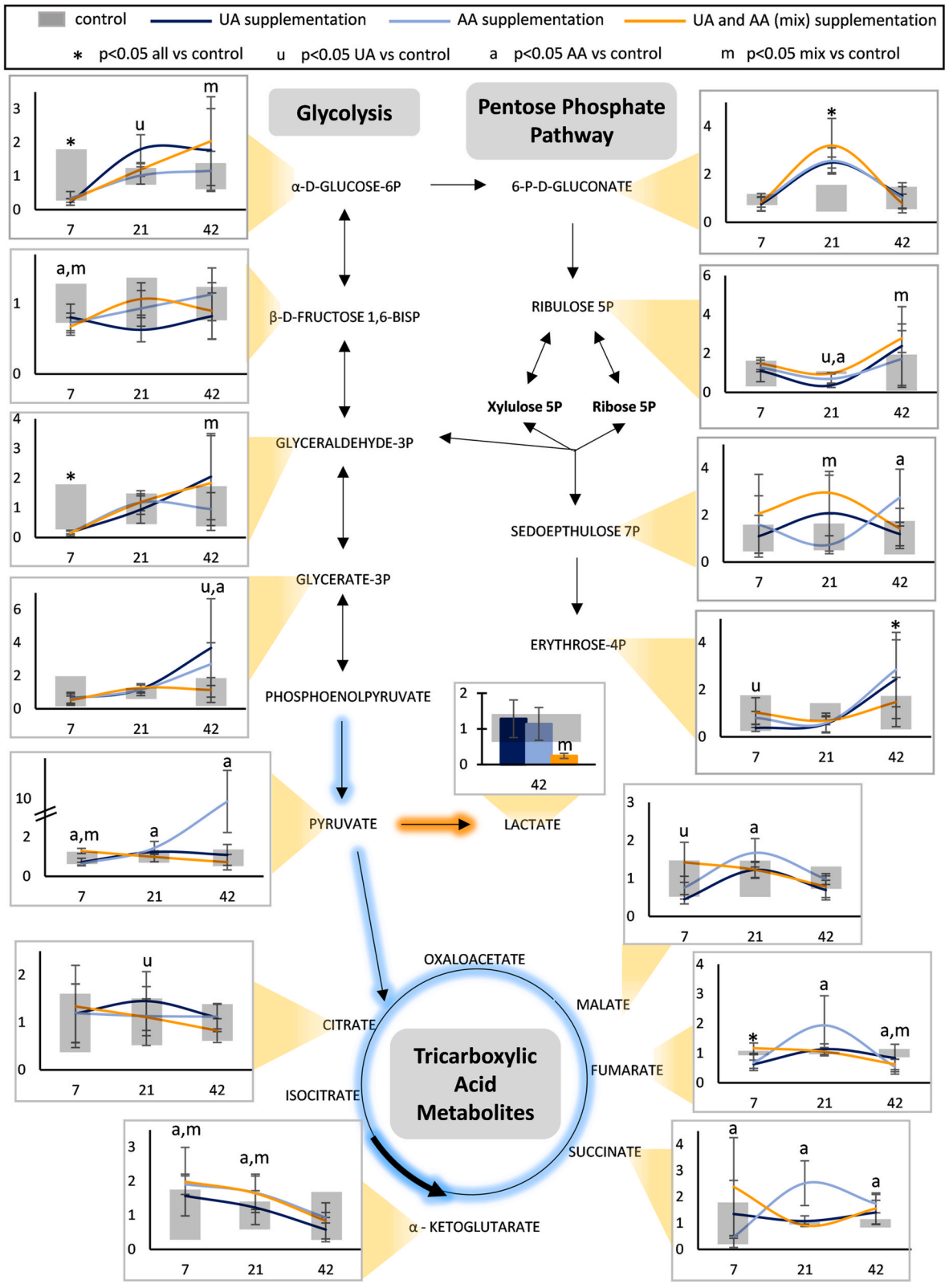

Fig. 4. Antioxidants supplementation effects upon glucose metabolism. Fold change of selected metabolites during storage (x axis: storage days) after red blood cell supplementation with uric acid (UA) and/or ascorbic acid (AA). Y axis: fold change versus control. Transparent gray bands' thickness is representative of the mean ± SD of control samples. Highlighted pathways: significant divergence in specific supplementations (color defined). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

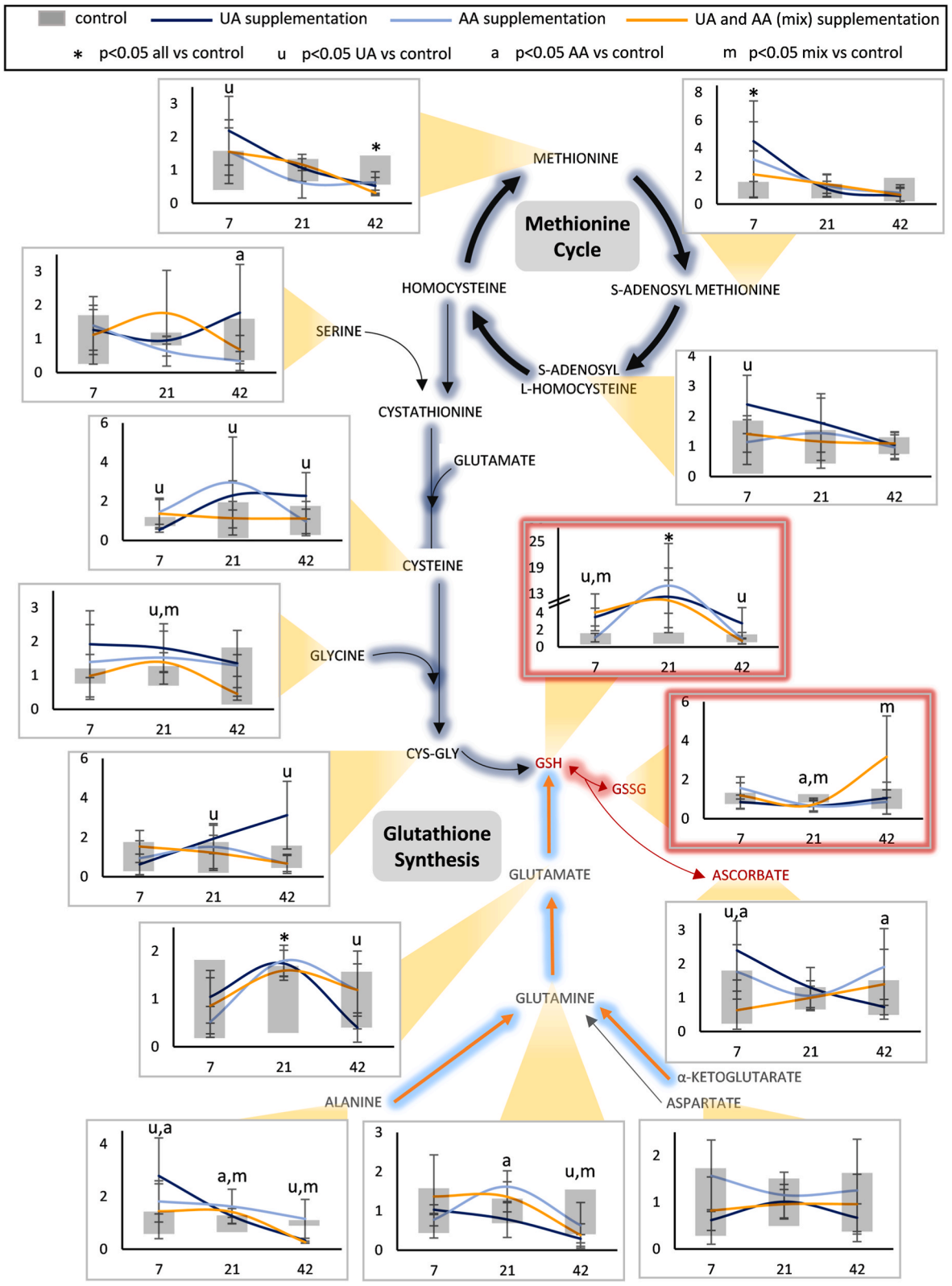

Fig. 5. Antioxidants supplementation effects upon methionine and glutathione (GSH) metabolism. Fold change of selected metabolites during storage (x axis: storage days) after red blood cell supplementation with uric acid (UA) and/or ascorbic acid (AA). Y axis: fold change versus control. Transparent gray bands' thickness is representative of the mean ± SD of control samples. GSSG: glutathione disulfide. Highlighted pathways: significant divergence in specific supplementations (color defined). Red highlight: final product of both pathways shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

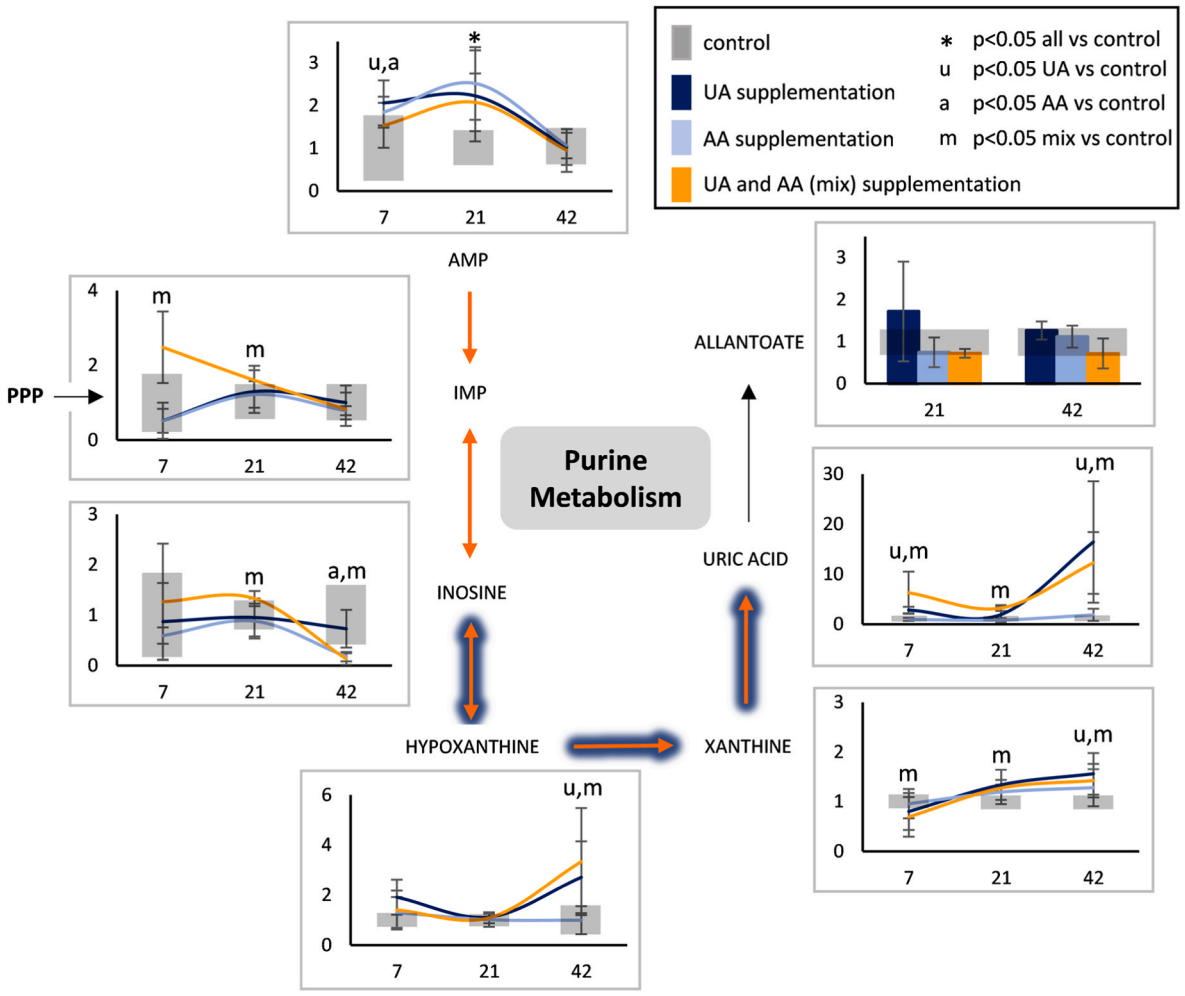

Fig. 6. Antioxidants supplementation effects upon purine metabolism. Fold change of selected metabolites during storage (x axis: storage days) after red blood cell supplementation with uric acid (UA) and/or ascorbic acid (AA). Y axis: fold change versus control. Transparent gray bands' thickness is representative of the mean ± SD of control samples. Highlighted pathways: significant divergence in specific supplementations (color defined). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

There is concrete evidence linking the storage lesion of RBCs in hypothermic conditions, which is mainly based on oxidative stress and metabolism decline, with their post-transfusion efficacy and effects. Thus, development of novel storage strategies able to improve these storability features would be potentially beneficial for the transfusion therapy *per se*. This study revealed that the addition of natural antioxidants, namely uric acid (UA) and ascorbic acid (AA), to the preservative solution of stored RBCs maintains their quality parameters, such as hemolysis, and furthermore, it protects them from oxidative defects through enhancement of both extracellular (e.g., TAC) and intracellular (e.g., GSH) antioxidant powers. Moreover, through *in vitro* and animal models of transfusion, it was hinted that this supplementation predisposes transfused RBCs with the capacity to confront pro-oxidant triggers in the recipient environment, while retaining at the same time control 24h recovery in the circulation.

4.1. Supplementation did not alter hemolysis parameters and calcium homeostasis of stored RBCs

Hemolysis is the gold standard of storage quality, and it is critical, for clinical reasons, to remain at low levels throughout storage. Spontaneous hemolysis was unaltered in the currently presented modifications. In previous studies, elevated levels of serum UA in RBC donors were neutral towards storage hemolysis [19,20]. On the other hand, AA has been shown to either have a neutral effect [24,42] or lead to lower [22] or even higher susceptibility to lysis [25,43], depending, among other, on its concentration. Its anti-hemolytic effects are mainly observed when in combination with other antioxidants, like vitamin E or N-acetylcysteine [24,25], but not with UA, as previously evidenced in a small study [26] and currently confirmed in the present one. Despite the −not clinically significant− slight increase in the osmotic fragility of AA samples, the same supplementation resulted in resistance to mechanical lysis, a finding consistent with the bibliography [22]. Mechanical fragility reflects the existence of sublethal injuries in stored cells that render them prone to removal post-transfusion, as recently shown by using microfluidic devices [44]. The unaltered Ca^{2+} homeostasis and control levels of calpain recruitment to the membrane are in line with the (mostly) unchanged mechanical stability and the control levels of discocytes in the treated sub-groups, at least in late storage. Calcium is involved in pathways implicated to membrane integrity [45], including the activation of calpain, that degrades cytoskeletal components impairing RBC deformability as previously shown in knockout mice [46].

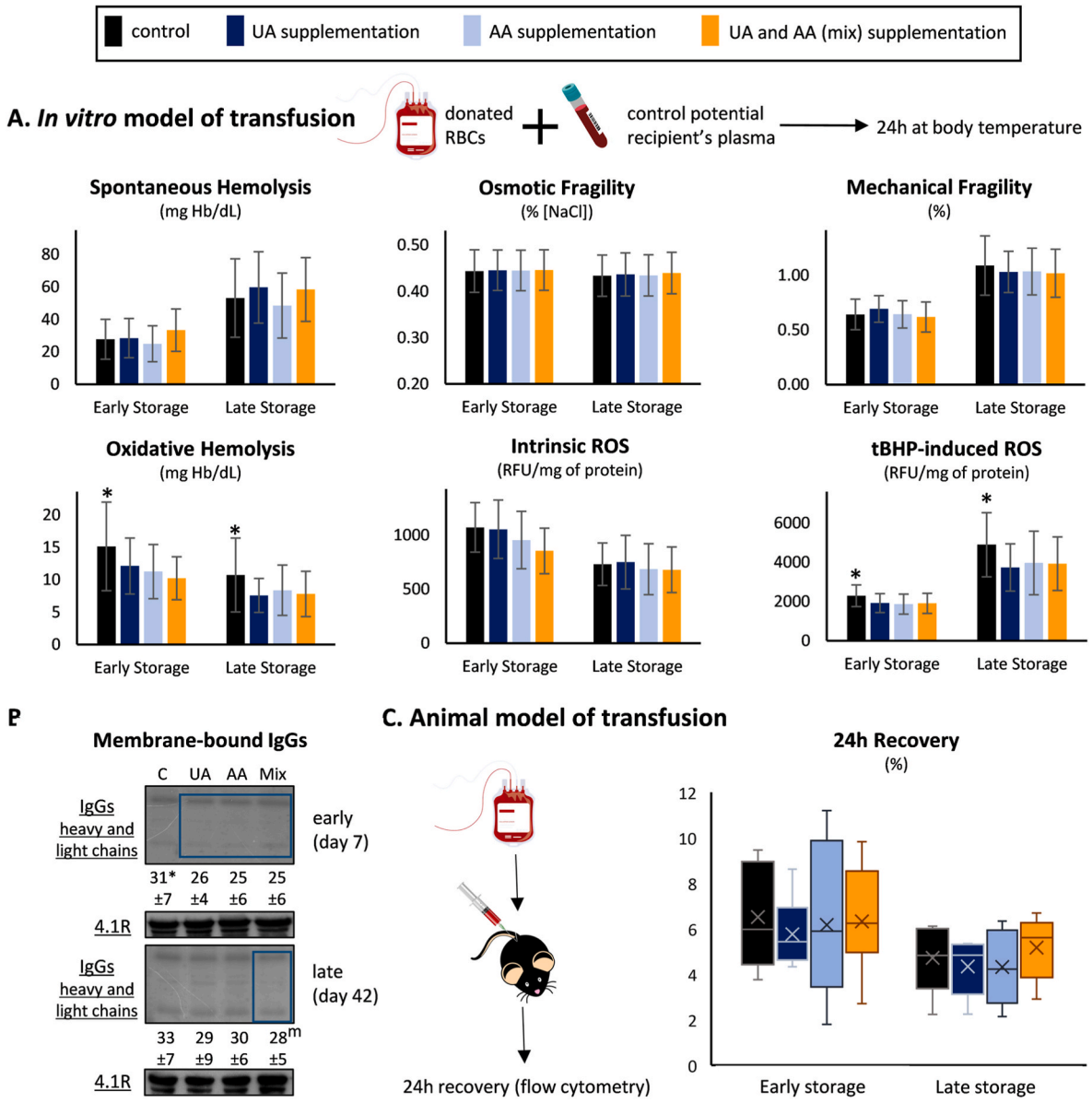

Fig. 7. Post-storage physiology and post-transfusion recovery of stored red blood cells supplemented with antioxidants. (A) Hemolysis and redox parameters after 24h incubation in conditions mimicking a transfusion recipient environment (n=10 for each group). (B) Representative immunoblots of membrane-bound IgGs in early and late storage (n=6 for each group; 4.1R protein: internal loading control). Blue rectangular: statistically significant reduction ($p<0.05$). Data are presented as mean ± SD. (C) 24h RBC recovery post-transfusion in a xenobiotic animal model (n=6 for each group; X: mean). C: control, UA: uric acid, AA: ascorbic acid. (*) $p<0.05$ control vs. all treatments, (m) $p<0.05$ mix vs. control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4.2. Supplementation of RBC units with UA and AA enhanced their extracellular antioxidant power and (partly) the intracellular levels of GSH

As expected, addition of UA and AA to RBC units boosted their extracellular antioxidant capacity without affecting the pH. The antioxidant capacity of UA lies in its ability to act as an electron donor and a scavenger of reactive oxygen and nitrogen species [34]. Additionally, it inhibits iron-mediated oxidation reactions [47] and delays oxidatively-induced hemolysis [27]. Similarly, AA is a radical scavenger, implicated in one-electron-transfer reactions. The produced ascorbate free radical is highly reactive to radical species, so it also emerges as an important antioxidant [48]. Nonetheless, UA and AA can also act as pro-oxidants [49,50].

As suggested many years ago, UA potentially enters RBCs by glycolysis-dependent and ATP-activated facilitated transport [51,52] through membrane transporters, such as URAT1 and ABCG2 [53]. UA transport seems to be accelerated with decreasing pH [52]. It has been accordingly shown at storage conditions that the membrane permeability for UA greatly increases from middle period onwards due to the drop of pH that follows the accumulation of lactate [54]. This could be a plausible explanation for the currently observed radical UA elevation at late storage (Fig. 6). On the other hand, dehydroascorbate (DHA), the fully oxidized form of AA, enters RBCs through glucose transporter GLUT1 [55]. DHA is an unstable molecule that can be rapidly degraded irreversibly both inside and outside cells [56], a conversion that is avoided through the activity of reducing agents such as GSH [57], glutathione S-transferases [58] and the duodenal isoform of cytochrome *b*561 [59]. Interestingly, a recent study reported a model regarding the adaptation of RBCs to carry out recycling of intracellular and

extracellular AA [60].

The antioxidant enhancement of RBC units affected distinct metabolic pathways in the treated cells. A large consumption of glycolysis substrates was evident in all modifications by the first week of storage, leading to increased levels of pyruvate in the case of mix- and later on in AA-samples too. Of note, end-of-storage lactate, one of the most classic markers of metabolic age in stored RBC [61], was lower in mix samples compared to control. If transaminated to alanine and converted to malate, pyruvate can fuel tricarboxylic acid interconversions, leading to α-ketoglutarate production [12]. This metabolite is a co-substrate for 2-oxoglutarate-dependent deoxygenases that perform hydroxylations on several molecules including proteins and lipids [62,63]. AA aids in this process by recycling Fe^{3+}/Fe^{4+} to Fe^{2+} and restoring the activity of deoxygenases [64]. The addition of AA (either alone or with UA) seemed to affect α-ketoglutarate production early on, leading to increased levels of tricarboxylic acid metabolites at later storage periods, compared to controls. This pathway is probably activated to participate in the recycling of reduced equivalents (NADPH, NADH) [12]. In the same context, PPP is partially activated in these samples to counteract oxidative stress and maintain redox balance, in line with a previous finding of Bardyn *et al.* in stored RBC units supplemented with physiological concentration of UA/AA [26].

One of the most significant results of this study was the impressive elevation of GSH levels in all treatments when compared to control during the middle storage period. GSH synthesis rates decrease under hypothermic storage, leading to insufficient levels of this non-enzymatic antioxidant, and thus, inferior antioxidant defense [65]. At the same time, GSH serves as a co-factor for glutathione peroxidases, antioxidant enzymes linked to beneficial RBC storability [66]. It has been proposed that addition of the amino acids necessary for its biosynthesis in the storage solution could improve this lesion [67], thus the boosted production of GSH observed in this study is important. It is intriguing that this beneficial outcome was achieved through differential metabolic rewiring between UA blood units and the other two groups. In AA and mix units, production of GSH was fueled by that of glutamate (by α-ketoglutarate, aspartate, alanine and glutamine) [68,69], while in UA units GSH production can be attributed to higher fluxes through the cysteine route [70]. Indeed, metabolites implicated in methionine and folate cycles, which are intertwined through 5-methyltetrahydrofolate and homocysteine, presented increased levels in UA samples compared to controls at early storage. This upregulation progressively fuels downstream metabolites involved in GSH synthesis, such as cysteine and cys-gly, in consistency with previous attempts of antioxidant enhancement of RBC units [24]. Finally, supplementation with UA upregulated a significant part of the purine oxidation/deamination pathways in UA and mix blood units (vs controls), including xanthosine and uric acid, but not hypoxanthine or allantoate, until the middle of the storage period. It seems that until this time point UA acts on xanthosine rather than classic stress-biomarkers (hypoxanthine, allantoin; [71,72]) to prevent oxidative stress.

4.3. Antioxidant enhancement seemed to protect Hb and membrane lipids against oxidative defects

Taken together, the beneficial effects of the supplementations on the extracellular and intra-cellular antioxidants provide a protection against the storage-related oxidative insults to RBCs. Well characterized phenotypes of storage lesion, including membrane lipid peroxidation [73], GSSG generation [74], oxidatively-induced lysis, and defected Hb binding to the membrane [5,75,76], were ameliorated in the presence of antioxidants. Both AA and UA protect lipids from detectable peroxidative damage and quench ROS, either alone [21,77,78] or in combination [79]. Additionally, the role of GSH in lipid safeguarding becomes clear when considering that its depletion results in a lethal accumulation of lipid peroxides during ferroptosis [80]. With respect to Hb, it was impressive that antioxidant supplementation (mainly AA; alone or mixed with UA) prevents its oxidation/denaturation and minimizes its translocation to the inner side of the membrane. In fact, both antioxidants, along with GSH, are known for their ability to maintain Hb in functional, non-oxidized states [81]. Moreover, ascorbate can reduce the formation of ferryl Hb, while GSH shields heme against excessive oxidation, forming together a synergistic antioxidant system to protect Hb [82]. It is noteworthy that Hb oligomerization and hemichrome binding to the membrane was found decreased in the modifications that presented elevated membrane proteasomal activity versus control. These findings are intertwined because proteasome is implicated in the degradation of oxidized Hb [83]. The fact that RBC-derived EVs from all treatments presented control levels of Hb oligomers supports the hypothesis that the enhanced blood units deal better with Hb oxidation, since the differential Hb-binding to the membrane is not attributed to increased waste-removal through vesiculation. Moreover, the lower EV protein concentration, combined with the equal levels of protein stress-markers sorted in these EVs, could be informative regarding the extent of vesiculation in supplemented blood units. It is tempting to hypothesize that the minor Hb oxidation drives a lower microvesiculation rate [76] in the modified units. However, the lack of absolute enumeration, a limitation of the present study, cannot allow drawing broad conclusions about it.

Most of the abovementioned improvements presented maximum deviation from control levels at mid-storage when upregulation of GSH was also maximized. Of note, loading of RBC units with GSH results in prevention of free radical injury [16]. Middle storage stands as a crucial time point for three distinct reasons. First of all, a metabolic shift regarding energy metabolism, as well as GSH synthesis, is observed [41,61]. Additionally, at this time period stored RBCs present high (or the highest) levels of intracellular ROS and membrane oxidative defects [5]. Finally, in terms of clinical relevance, middle-aged stored RBCs represent the standard-issue blood units for RBC transfusions [84,85]. Consequently, improvement of these units might prove of great importance for maintaining RBC redox equilibrium and physiology in respect to their clinical performance.

4.4. Attenuation of the antioxidant protection during late storage resulted in the emergence of stress markers, especially in mix-treated samples

It should not be overlooked that heading towards the last days of storage the extent of antioxidant improvement was notably reduced (e.g., extracellular antioxidant capacity) or totally lost (e.g., intracellular GSH). This finding is consistent with the stabilization of the divergence observed between the modified and control units with respect to oxidative stress markers. It seems that the beneficial effect of the antioxidant enhancement upon metabolism, and especially upon GSH biosynthesis, is attenuated in prolonged storage. This becomes more evident through the emergence of increased levels of stress-related metabolites of the purine oxidation pathway in UA and mix samples in comparison to controls. For instance, hypoxanthine, a metabolite that has been suggested to be related with poor post-transfusion survival [71], is significantly elevated. Especially in the case of mix-treated samples, the end-of-storage markers of oxidative stress include not only purine oxidation metabolites, but also GSSG. At this point, it should be noted that the only incidents of outlier hemolysis values (nonetheless very lower than the 0.8% threshold set for RBC transfusion) were observed in 3 out of 34 mix-treated RBC units. We can hypothesize that use of higher-than-normal serum levels of both antioxidants, perhaps have led to some kind of "burnout" during late storage, especially since the currently observed stress-markers were not induced upon restoration of UA and AA physiological values [26]. Indeed, high-dose administration of vitamin C increases generation of hydrogen peroxide in RBCs [86]. In the same context, patients with hyperuricemia (e.g., in chronic kidney disease and metabolic syndrome) are characterized by increased levels of oxidative stress [87], therefore a blood product with high levels of extracellular UA may not represent an optimum therapy for them. In

these cases, a washing step of stored supplemented RBCs could be performed before transfusion to prevent the elevation of extracellular UA in such cohorts. Finally, it would be interesting to test supplementation of RBC units with UA and AA at intermediate levels, between the restoration and the currently used concentrations to reach the antioxidant merit, while at the same time minimizing the pro-oxidative effects.

4.5. RBCs supplemented with UA and/or AA were better equipped to cope with specific redox-related stresses in the recipient's environment while showing control survival in animal models of transfusion

The fact that RBCs from the modified units presented the same physiological profile when found in a recipient mimicking environment supports that the antioxidant enhancement equipped these RBCs during storage in blood bank conditions and beyond. Thus, RBCs taken from the modified units were not in danger of post-mixing hemolysis while at the same time they retained the benefit of coping with additional oxidative stress against their control counterparts. Notably, intracellular ascorbate and GSH of stored RBCs anticorrelate with ROS accumulation and oxidative lysis after exposure to recipient plasma [40]. This profile might prove to be clinically beneficial for patients under redox-reactive medication or with distinct redox status, who need regular [88] or sporadic transfusions [89]. Although the improved phenotypes were not translated to superior post-transfusion RBC recovery in mice circulation, the decreased membrane binding of IgGs in stored RBCs from the enhanced units could be relevant to post-transfusion RBC survival in a human context. To support this, supplementation of stored murine RBCs with AA resulted in both reduced alloimmunization and increased post-transfusion recovery in mice recipients [90]. Despite the usefulness of xenobiotic transfusions in studying post-transfusion parameters [40, 91] it should be noted that some differences might be "masked". Having this limitation in mind, perhaps the use of humanized mice could provide more clear results before proceeding to a human clinical setting.

5. Conclusions

By using a wide array of different experimental approaches, ranging from physiology and biochemistry to metabolism and *in vitro/in vivo* models of transfusion, the currently presented study achieved to provide (a) a detailed picture of the effect of UA and AA administration upon RBCs stored under blood bank conditions and (b) insight into the differential metabolic rewiring that corresponds to the supplementation with distinct antioxidant "enhancers". The observed improvement of the redox equilibrium in a context of well-preserved quality characteristics may offer the basis for future experimentation regarding the antioxidant enhancement of stored RBCs through elegant techniques, including high throughput redox proteomics and carbonylomics.

Author Contribution

VT and MA designed the study. VT, ATA and V-ZA performed the physiological experiments. VL, GF and SR performed the metabolomic analyses. ECP, ACA, EB and NK were responsible for the animal model of transfusion. KS and AK were responsible for the sample acquisition and the preparation of RBC units. VT, ATA, VL, GF and SR analyzed the results and prepared the figures. VT, ATA, and MA wrote the first draft of the manuscript. V-ZA, VL, GF, ECP, ACA, EB, NK, EGP, IP, KS, AK and SR drafted the manuscript and contributed to the final version. All authors contributed to the article and approved the submitted version.

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102477.

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