COLLECTION, PRODUCTION AND STORAGE OF BLOOD COMPONENTS

Original article

Impact of leukoreduction on the metabolome of ovine packed red blood cells during refrigerated storage

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Background - Blood transfusion is a life-saving intervention for many species of veterinary interest, including sheep. Despite extensive research on the impact of refrigerated storage of packed red blood cells (pRBC) in humans, research on the quality of stored ovine blood is limited and storage guidelines are mostly informed by studies in humans. Human pRBC are currently stored without residual white blood cells, following selective removal of the leukocytes by filtration (leukoreduction). This process delays the onset and mitigates the progression of the storage lesion, a series of molecular changes that RBC undergo as a function of storage duration. However, leukoreduction of ovine pRBC is not routinely performed.

Materials and methods - Here we performed metabolomics analyses of non-leukoreduced (nLR) and LR pRBC from six sheep. Units were stored under standard veterinary blood bank conditions (4°C) for up to 42 days and sterilely sampled weekly for metabolomics analyses of cells and supernatants.

Results - LR-pRBC showed significantly lower levels of mono-, di- and tri-carboxylates in both the cellular and supernatant compartments, and slower accumulation of lactate and immunomodulatory succinate, fumarate and malate. The presence of residual white blood cells in the units accelerated the consumption of glucose from the media, with no increase in detectable high energy phosphate compounds (AMP). nLR showed a higher degree of purine breakdown and deamination products, (hypoxanthine, xanthine and allantoate). Elevated free fatty acids in nLR RBC are consistent with increased lipid peroxidation and lipolysis. Strong sex dimorphism was observed across all samples, independently of storage duration or leukoreduction.

Discussion - Leukoreduction of ovine pRBC delays the onset and mitigates the metabolic storage lesion to central energy and redox metabolism, while almost completely abrogating the accumulation of carboxylates in stored units.

Keywords: *sheep, storage lesions, lipidomic, metabolomic, leukoreduction.*

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INTRODUCTION

Sheep (*Ovis aries*) red blood cells (RBC), like all mammalian erythrocytes, play a central role in oxygen transport and delivery to peripheral tissues. Like the RBC from other mammals

and unlike those from birds, reptiles and amphibians, ovine RBC are biconcave discs that lack a nucleus and organelles, including mitochondria. This evolutionary adaptation has contributed to maximizing hemoglobin content per cell, while enhancing RBC flexibility and deformability to squeeze through capillaries as narrow as 5 µm. Ovine RBC are somewhat comparable to human erythrocytes, with slightly smaller average size (4.5-5.5 µm *vs* 6-8 µm for human RBC), comparable mean cell hemoglobin concentration (34 g/dL, comparable to the human range of 32-36 g/dL) and lifespan (120-150 days for ovine RBC *vs* 120 days for humans)[1-3]. However, sheep have almost double the RBC counts than those in humans (9-15 million/µL *vs* ~5 million/µL)[4]. On top of their relevance *per se*, ovine RBC have historically represented an invaluable model to investigate the impact of genetic heterogeneity on the metabolic characteristics of mammalian RBC, such as the antioxidant capacity to the extent it is regulated by the glutathione system[5].

As in humans, transfusions of packed RBC (pRBC) in sheep are essential life-saving interventions for various clinical scenarios: from acute anemia due to hemorrhage, to hemolysis or chronic anemias. Animals with hemostatic disorders may require repeated transfusions of whole blood, pRBC, plasma or platelets. The use of pRBC is preferred when the goal is to restore oxygen-carrying capacity, since transfusing 10-15 mL/kg of packed RBC raises the ovine recipient's hematocrit by 10%. Refrigerated storage of ovine RBC is not only important for veterinary medicine *per se*, but is also of translational relevance as a potential model of storage and transfusion in a mammal of similar size and anatomy to humans[4]. Compared to human RBC, stored ovine RBC have a similar onset and progression of the so-called storage lesion[4], a series of biochemical and morphological changes that RBC undergo during refrigerated storage for up to 42 days under blood bank conditions[6]. Specifically, compared to human RBC, similar trends were observed with respect to RBC energy metabolism (adenosine triphosphate [ATP] and 2,3-diphosphoglycerate [DPG]), with lower/comparable vesiculation rates in sheep RBC as a function of storage duration, despite higher levels of lipid peroxidation markers such as 12-hydroxyeicosatetraenoic acid (HETE), increased fragility and hemolysis in sheep RBC by the end of storage[4]. Unlike human RBC, sheep RBC are not amenable to laboratory testing for the current gold standards in the determination of post-transfusion performances via radiolabeled ^{51}Cr, as it is rapidly eluted from the ovine bloodstream[7]. Alternative methods are, therefore, necessary to evaluate the quality of stored blood products of ovine origin to test the quality of current practices and inform the development of novel strategies and/or guidelines for improved ovine blood storage. Elegant studies by Simonova and colleagues have shown that the metabolic storage lesion in ovine models seems to be comparable or even mitigated when compared to that of human pRBC stored in standard additive solutions (citrate, phosphate, dextrose saline, adenine, glucose, mannitol [CPD-SAGM]) for up to 42 days, the standard shelf-life of human blood products in most countries[4,8]. Specifically, comparable levels of ATP and lower starting levels of DPG –both declining as a function of storage duration in both species– are observed, with final end-of-storage levels of lactate reported in the 10 mM range[4,8], approximately half to a third of the same levels in stored human RBC[9-11]. Despite these important studies, a comprehensive characterization of the ovine RBC metabolome is currently lacking, which is the focus of the present study.

Over the past two decades, removal of residual white blood cells (WBC) and platelets via filtration –a process known as leukoreduction (LR)– has increasingly become a standard practice in routine processing of human pRBC products almost worldwide[12]. The process of removing WBC and platelets (log4 and 2.5, respectively) from the unit (<5 million WBC per unit) not only decreases the risk of untoward post-transfusion immune and inflammatory reactions, but also removes metabolically active cells from the blood units, thus contributing to the amelioration of the metabolic storage lesion, while also mitigating the accumulation of untoward metabolic end-products from active catabolism in mitochondria-endowed blood cells other than mature mammalian erythrocytes. Such benefits have been observed in humans[13] and multiples species of veterinary interest, such as dogs and horses[14,15]. However, a similar study has not yet been performed in sheep, which is the secondary focus of the present study. Since current veterinary guidelines in Italy allow for non-leukoreduced (nLR) ovine pRBC storage in CPD-SAGM for up to 42 days, here we provide a

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comprehensive characterization of the impact of LR on stored ovine RBC metabolism with the goal of informing, at least in part, novel veterinary guidelines.

MATERIALS AND METHODS

Blood collection, processing and storage

Blood units were obtained from six sheep as part of the donors' program at two University Veterinary Blood Banks equipped with the same instruments and applying the same operating procedures (Department of Veterinary Medicine, University of Perugia, Italy). The subjects fulfilled the requirements set out in Italian Ministry of Health guidelines. The six sheep (*Ovis aries*, pecora *Sarda* breed coming from the same farm, but not inbred), three males and three females (weight ranging from 70 to 90 kg), were submitted to clinical examination and urinalysis, and blood was taken for a complete blood count, serum biochemistry profile, hemostatic profile, and serological testing to evaluate the state of health and exclude hematological infection.

Blood draw, processing and storage

A unit of 450 mL of whole blood was collected from the jugular vein using a commercial closed collection system for human use (IMUFLEX CRC Blood Bag System, Terumo Penpol Ltd, India) consisting in a quadruple bag system with CPD-SAG-M, equipped with a filter for in-process LR. After collection, the whole blood unit was centrifuged at 3,068 RCF for 20 minutes at 4°C in a bag centrifuge (Rotixa 50 RS, Hettich Italia Srl, Milan, Italy). Plasma was removed using a manual plasma extractor (EPM Vasini Strumenti Srl, Ravenna, Italy) and was transferred into a satellite bag. The SAG-M preservative solution contained in a satellite bag was aseptically transferred into the bag containing RBC to obtain the SAG-M + pRBC bag (350 mL). Half of the volume of the parent pRBC unit (about 175 mL) was further processed via a LR filter to obtain a LR-pRBC. The other half remained in the SAG-M + pRBC bag and named nLR-pRBC). A blood bag tube welder (Vasini Strumenti Srl) was used during the procedure to close the tubes and maintain a closed system. A total of six LR-pRBC units and six nLR-pRBC units were obtained, each half the volume of a standard sheep pRBC. All the bags produced were stored in a blood bank refrigerator at 3-4°C (170 ECT-F TOUCH, Fiocchetti, Luzzara, Italy) for 42 days. At weekly intervals –on days 0 (T0), 7 (T1), 14 (T2), 21 (T3), 28 (T4), 35 (T5), and 42 (T6)– samples of 3 mL pRBC were taken aseptically from each bag. One of the LR half-pRBC units (from sheep No. 30) was not sampled due to logistical issues. All pRBC samples were centrifuged (at 1,500 rpm) to separate the RBC and supernatants, both stored at –80°C. Samples were shipped in dry ice within 60 days of collection and analyzed at the University of Colorado Anschutz Medical Campus, as previously described[16,17].

To exclude bacterial contamination, aerobic bacterial cultures were carried out from each sample at T0, T3 and T6, with negative results throughout the storage period through to the end of the shelf life of the units (storage day 42).

Metabolomics analysis

Frozen RBC and supernatant aliquots (50 µL) were extracted 1:10 and 1:25, respectively, in ice cold extraction solution (methanol:acetonitrile:water 5:3:2 *v/v/v*). Samples were vortexed and the insoluble material pelleted. Analyses were performed using a Vanquish UHPLC coupled online to a Q Exactive mass spectrometer (Thermo Fisher, Bremen, Germany). Samples were analyzed using a 1 min and 5 minute gradient-based method, as extensively described in method[16,17] and application papers[14,18]. Peak picking was performed in MAVEN (Princeton University, Princeton, NJ, USA) as described previously[16,17]. Graphs and statistical analyses (including two-way ANOVA, time series plus one factor, linear discriminant analysis) were prepared with GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA), GENE E (Broad Institute, Cambridge, MA, USA), and MetaboAnalyst 5.0 (University of Alberta, Edmonton, AB, Canada)[19].

RESULTS

Storage, leukoreduction and individual subjects have a significant effect on the metabolome of stored ovine red blood cells and supernatants

Metabolomics analyses were performed on RBC and supernatants from LR and nLR ovine pRBC (No.=6) (**Figure 1A**). Results are reported extensively in tabulated form in *Online Supplementary* **Table SI**. A summary overview of the data is provided in the form of heat maps and significance plots from linear discriminant analyses of RBC (**Figure 1B**, **C**) and supernatants (**Figure 1D**, **E**), as a function of storage duration (**Figure 1**), LR (**Figure 2**) and subject (*Online Supplementary* **Figure S1**). The top 100 metabolites from these analyses are shown for RBC and

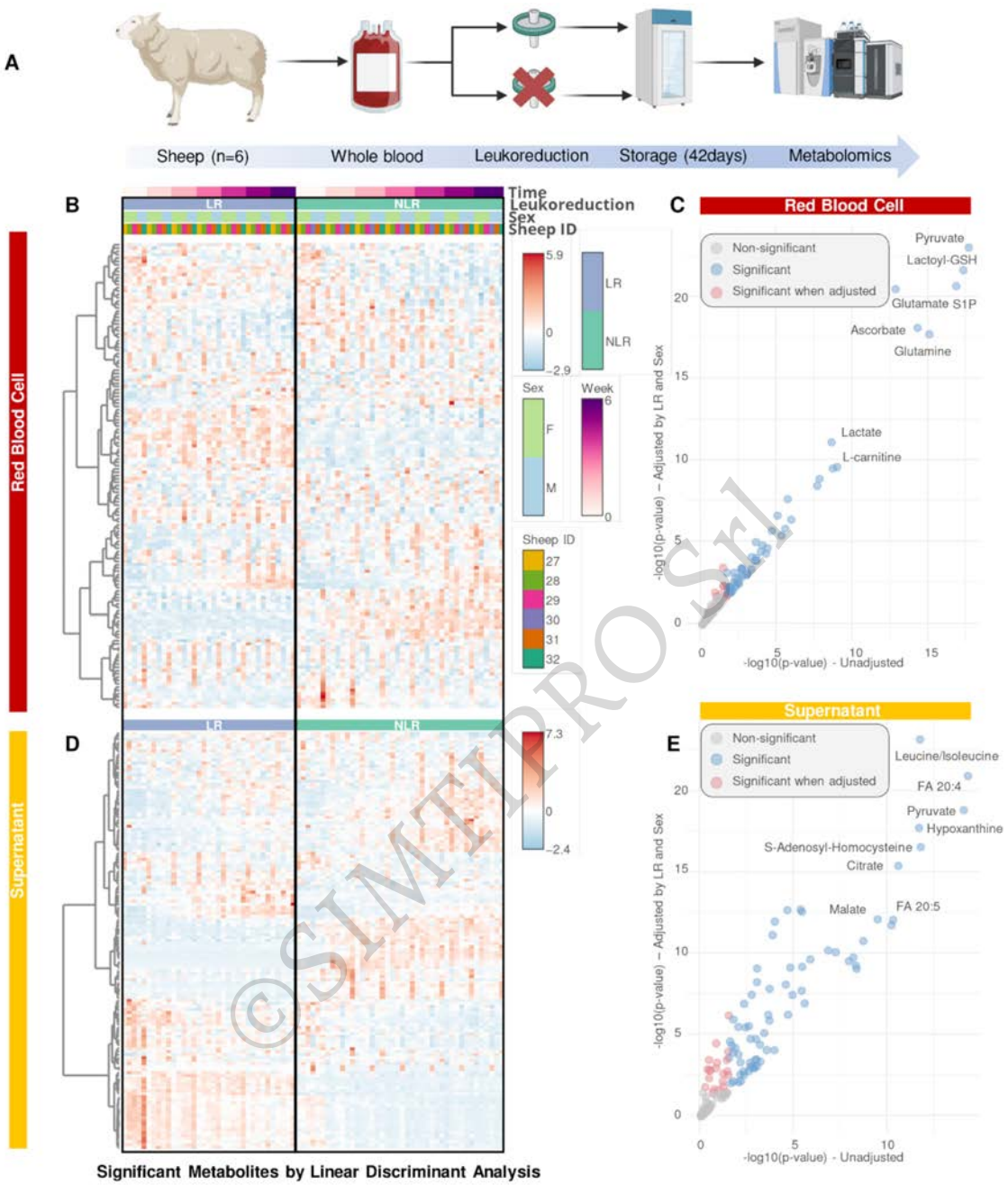

Figure 1 - Metabolomics of stored ovine red blood cells
(**A**) Overview of the experimental design. (**B**, **C**) and (**D**, **E**) Heat map and linear discriminant analysis summary of the significant metabolic changes in stored ovine red blood cells and supernatants, respectively.

supernatants in *Online Supplementary* **Figures S2** and **S3**, respectively. Altogether, these analyses clearly demonstrate a strong impact of storage duration and leukoreduction on glycolysis (pyruvate, lactate; **Figure 1C**), glutaminolysis and glutathione metabolism (glutamine, glutamate, lactoyl-GSH; **Figure 1C**) and purine homeostasis (hypoxanthine, S-adenosyl-homocysteine; **Figure 1D**; adenine, adenosine; **Figure 2A**), carboxylic acid (lactate, citrate, malate; **Figure 1C**, **D**; succinate, fumarate, malate, lactate; **Figure 2A**) and fatty acid metabolism (FA 20:4; FA 20:5; **Figure 1D**; FA 10:0 and 12:0; **Figure 2B**), which will be explored by pathway in detail below.

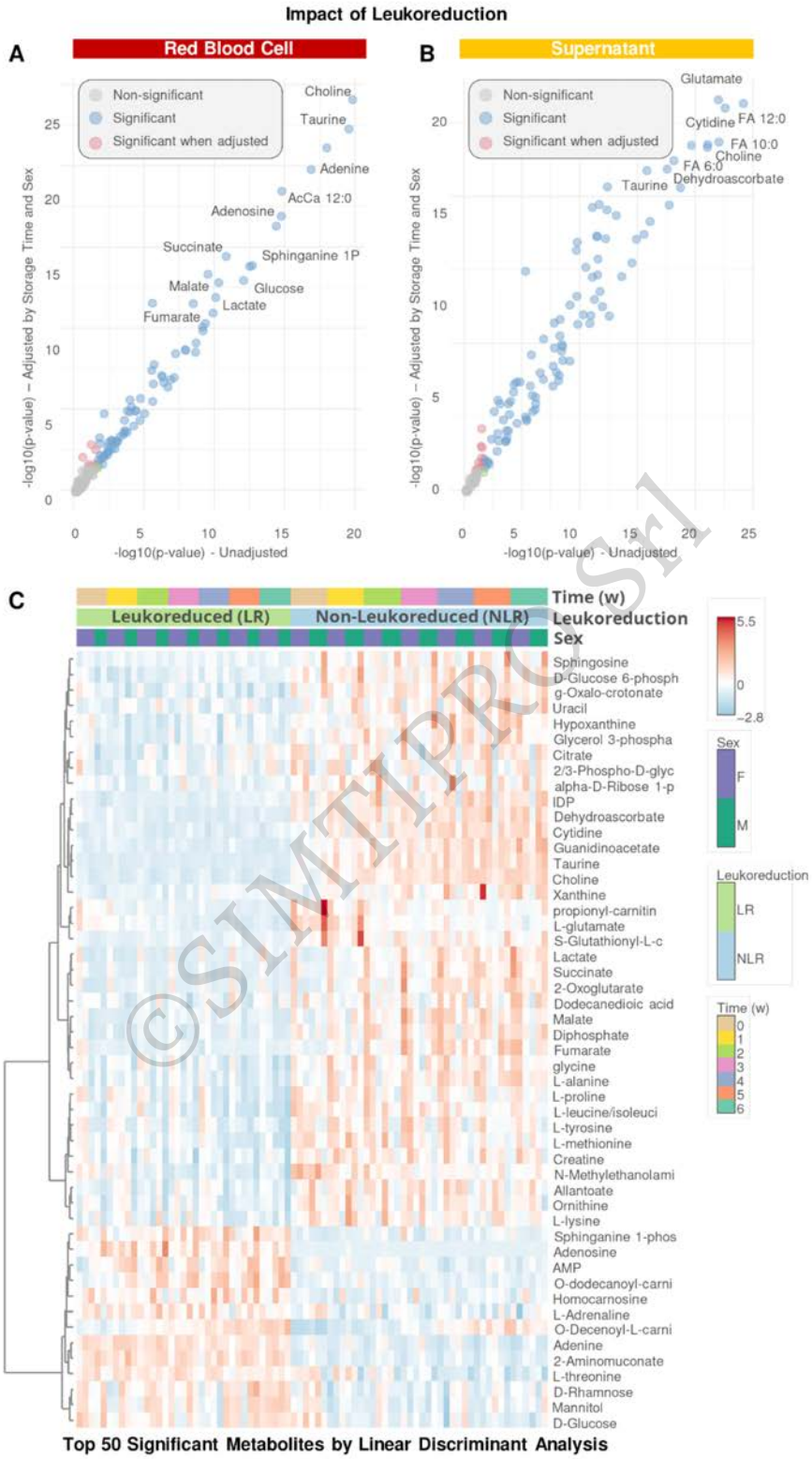

Figure 2 - Impact of leukoreduction on stored ovine red blood cells
(**A**, **B**) Linear discriminant analysis of metabolites in red blood cells and supernatants, respectively, as a function of leukoreduction, adjusted by storage duration. (**C**) Heat map of the top 50 significant metabolites based on the linear discriminant analysis.

Increased glucose consumption and lactate generation in non-leukoreduced ovine blood units

In all samples across all conditions, storage duration promoted glucose consumption, depletion of early-stage glycolytic intermediates and accumulation of the end-products pyruvate and lactate in cells and supernatants (**Figure 3**), consistent with the literature in humans and other species[18,20,21]. Leukoreduction had a significant effect on RBC glycolysis, as noted by a significant increase in the consumption of glucose in the supernatants (significant after week 3) and accumulation of intra- and extra-cellular lactate in nLR units throughout storage (**Figure 3**). These differences were not attributable to glucose intracellular availability, which was higher in the LR group, but rather to a decreased synthesis of hexose phosphate (isomers) and fructose bisphosphate, lower in the LR group (**Figure 3**). Accumulation of alanine, a transamination product of pyruvate[22], was observed as a function of storage duration, but it was significantly higher in the nLR group (**Figure 3**). Accumulation of lactate and pyruvate was highest in female sheep (*Online Supplementary* **Figure S4**).

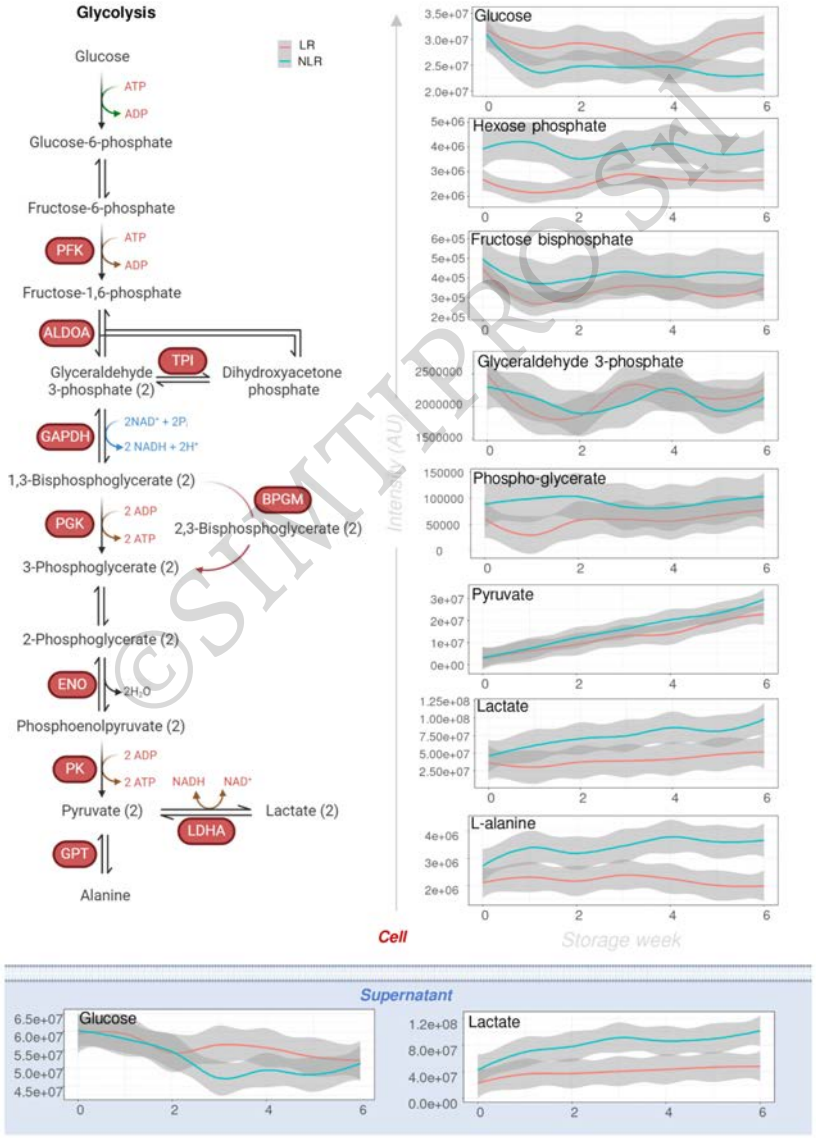

Figure 3 - Impact of leukoreduction on red blood cell glycolysis
Line plots show median ± quartile range for leukoreduced (light red [LR]) and non-LR (light blue [NLR]) red blood cells. x axes indicate storage week and y axes indicate relative metabolite abundance (integrated peak areas, arbitrary units).

Consistent with the literature in humans[23-25] and other species[26,27], storage duration promoted the consumption of glutathione pools (both reduced and oxidized glutathione), glutathione utilization *via* the glyoxylate pathway (resulting in the generation of lactoyl-glutathione) and the accumulation of the end-product of the gamma-glutamyl cycle, 5-oxoproline (**Figure 4**). Unlike leukoreduction in other species of veterinary interest, such as dogs and horses, leukoreduction of ovine pRBC did not promote significant alterations in the overall glutathione pool (reduced glutathione and oxidized, glutathione disulfide, **Figure 4**). Indeed, increased oxidant stress has been described in nLR pRBC units from other mammals, including humans[28]. This observation is in part reconciled by the appreciation of significantly higher levels of oxidized vitamin C (dehydroascorbate) in stored ovine pRBC, despite concomitant storage-dependent and LR-independent elevation in ascorbate (**Figure 4**). This species-specific observation is consistent with the appreciation of the fact that ruminants can synthesize vitamin C endogenously, while humans and other non-rodent mammals (except guinea pigs[20]) rely on its uptake and/or exogenous supplementation to preserve the reduced vitamin C pools[16,29-31].

In addition, nLR pRBC were characterized by higher levels of ribose phosphate (pentose phosphate isomers, throughout storage) and sedoheptulose phosphate (until week 3), suggestive of a potential role of compensatory

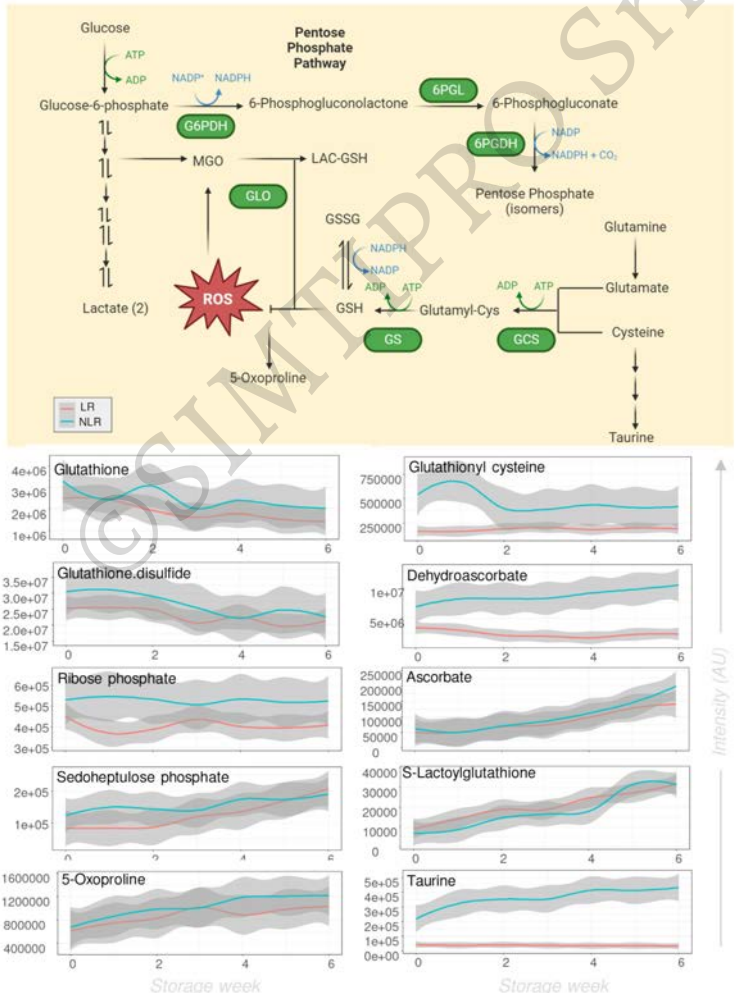

Figure 4 - Impact of leukoreduction on red blood cell glutathione metabolism and the pentose phosphate pathway
Line plots show median ± quartile range for leukoreduced (light red [LR]) and non-LR (light blue [NLR]) red blood cells. x axes indicate storage week and y axes indicate relative metabolite abundance (integrated peak areas, arbitrary units).

activation of the pentose phosphate pathway to generate NADPH, which is required to convert oxidized glutathione back to its reduced form (**Figure 4**).

Finally, elevation in taurine, a potent antioxidant that is six times more abundant than any other amino acids in platelets[32], was observed in the nLR RBC (**Figure 4**); in this view, it is worth noting that taurine concentration in platelets, lymphocytes and neutrophils is significantly higher than that in RBC, whereby exogenous supplementation of taurine through dietary intervention boosts energy and antioxidant metabolism[33]. Taurine levels are particularly altered in platelets during storage under refrigerated conditions, to the extent that taurine supplementation has been proposed as an additive to currently licensed solutions for platelet cold storage[34].

Elevation of carboxylates of potential mitochondrial origin is observed in non-leukoreduced packed red blood cells as a function of storage duration

Since RBC are devoid of mitochondria, while functional mitochondria are present in platelets and WBC, it did not come as a surprise that storage of nLR pRBC –but not LR pRBC– was accompanied by the intracellular and supernatant accumulation of di- and tri-carboxylic acid metabolites, especially, 2-oxoglutarate, succinate, fumarate and malate (**Figure 5**).

Despite comparable arginine levels between nLR and LR pRBC throughout storage, higher ornithine and guanidinoacetate levels and lower citrulline levels are consistent with ongoing arginine catabolism in the nLR group, the full urea cycle only occurs in mitochondria, with significant up-regulation of arginase activity as inferred from ornithine/arginine ratios at steady state (**Figure 5**).

Leukoreduction mitigated purine breakdown and deamination

While previous studies have reported on comparable storage-induced consumption of ATP between ovine and human RBC[4,8], limited information is available about ATP breakdown to lower energy adenosine phosphate compounds (AMP) and its deamination and phosphorolysis to hypoxanthine, a hallmark of the RBC metabolic lesion[25] and a predictor of post-transfusion performances in mice[35] and humans[36]. Here we show that storage induced accumulation of xanthine, rather than hypoxanthine, in stored ovine RBC (**Figure 5**). However, significantly higher levels of AMP, adenosine, and adenine, and significantly lower levels of hypoxnthine, xanthine, allantoate (end products of the pathway) were detected in LR pRBC, suggestive of a protective effect on purine oxidation by leukoreduction, in keeping with literature in humans and other species[13-15].

Higher levels of choline and methionine, but lower levels of S-adenosyl-hoomcysteine were detected in nLR pRBC throughout storage, with the former increasing as a function of storage duration only in the nLR group, and the latter two metabolites decreasing in both groups over time (*Online Supplementary* **Figure S5**).

Leukoreduction prevents accumulation of free fatty acids in supernatants, while sex dimorphism, but not leukoreduction, influences acyl-carnitine pools

Storage duration was accompanied by the accumulation of free fatty acids in storage supernatants across all conditions (**Figure 6**). However, while LR RBC showed the highest levels of short and medium chain saturated fatty acids, supernatants of nLR pRBC were characterized by the highest levels of long, very-long poly and highly-unsaturated free fatty acids (from 14:0 to 22:6, **Figure 6**). While no difference was observed with respect to free L-carnitine and acyl-carnitine pools as a function of leukoreduction, a decline in total levels of acyl-carnitines was observed over storage (*Online Supplementary* **Figure S6**), consistent with the literature in humans[36]. Interestingly, sex dimorphism was observed across subjects, with male ovine samples showing the highest levels of all acyl-carnitines irrespective of storage duration and leukoreduction status (**Figure 6**, *Online Supplementary* **Figure S5**), a male dominant dimorphic effect that appears to be even stronger than that observed in human fresh and stored RBC[37].

Storage-dependent elevations in sphingosine 1-phosphate (S1P) were comparable between the two groups, although significantly higher levels were dosed in nLR pRBC at storage week 5 (*Online Supplementary* **Figure S7**). Lower levels of the S1P precursor sphinganine 1-phosphate were instead observed in LR RBC, which also showed significantly lower levels of free lipid heads (glycerol 3-phosphate, methanolamine phosphate), consistent with a lower degree of lipolysis and release of free fatty acids from phospholipids upon leukoreduction (*Online Supplementary* **Figure S8**).

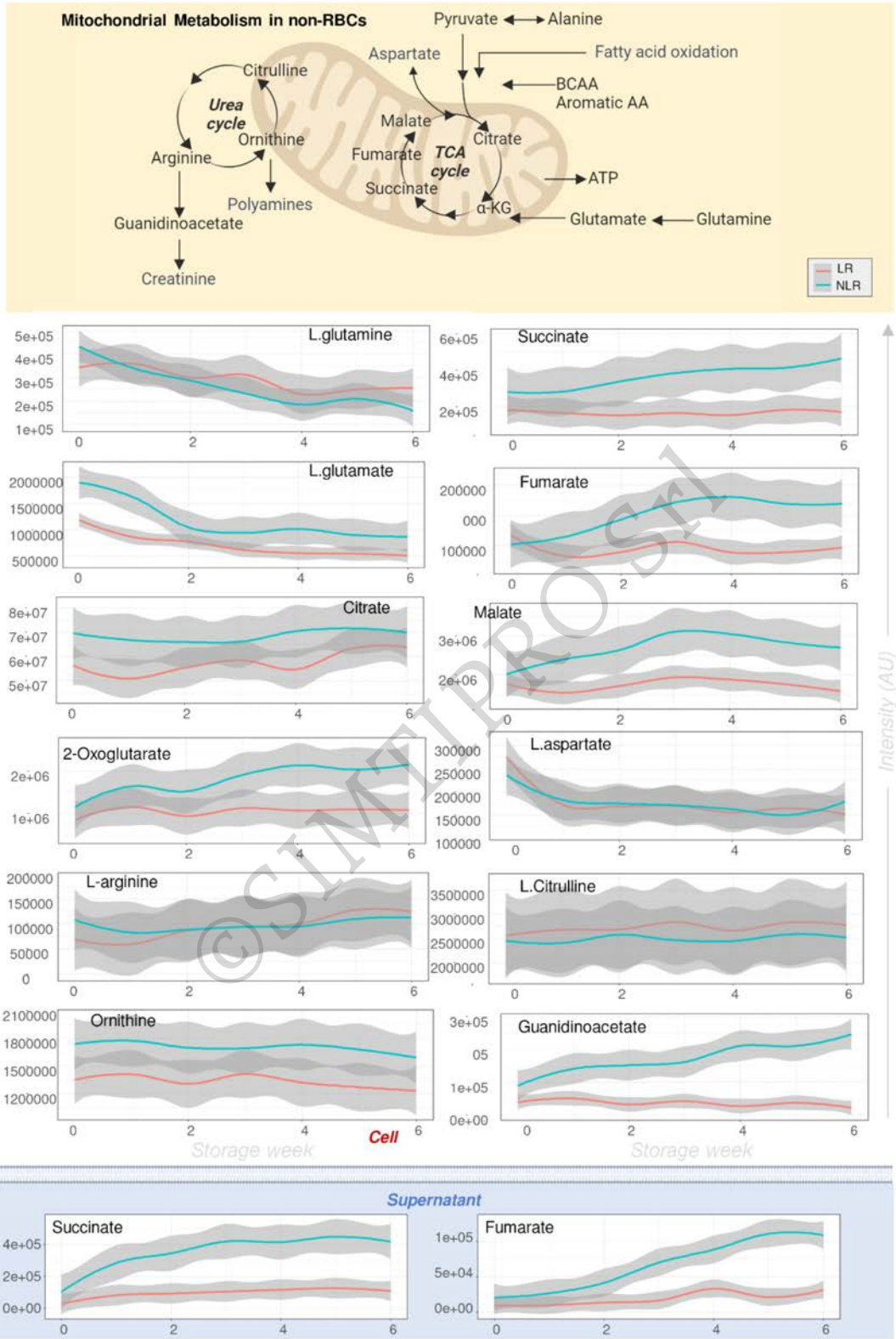

Figure 5 - Impact of leukoreduction on red blood cell carboxylic acid metabolism
Line plots show median ± quartile range for leukoreduced (light red [LR]) and non-LR (light blue [NLR]) red blood cells. x axes indicate storage week and y axes indicate relative metabolite abundance (integrated peak areas, arbitrary units).

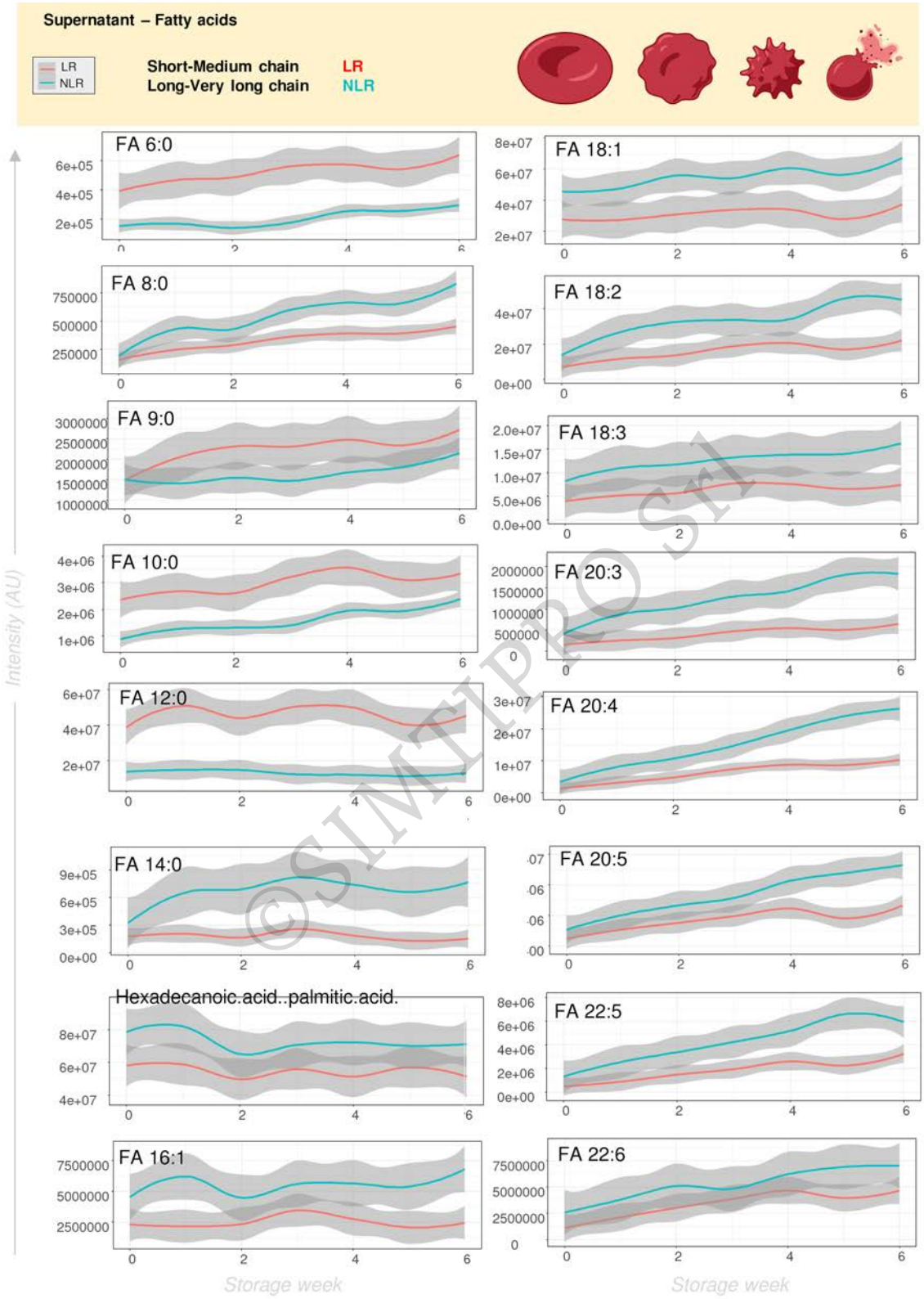

Figure 6 - Impact of leukoreduction on supernatant free fatty acids
Line plots show median ± quartile range for leukoreduced (light red [LR]) and non-LR (light blue [NLR]) red blood cells. x axes indicate storage week and y axes indicate relative metabolite abundance (integrated peak areas, arbitrary units).

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DISCUSSION

In the present report we present the results of an observational study on the impact of leukoreduction on stored sheep pRBC metabolism. Given their larger size compared to rodent model organisms, sheep models have been increasingly adopted in biomedical research[38-40], from immunology to cardiovascular diseases and other areas indirectly relevant to transfusion medicine. Understanding RBC storage biology in sheep is immediately relevant for veterinary applications, especially in regions such as Italy, in which sheep and goat farming contributes to a combined economic value of about €1.5 billion annually, with sheep farming being the larger contributor. In the present study, our results indicate that storage duration has similar effects on ovine RBC and supernatants as those in other mammalian species, including humans, non-human primates (macaques, baboons), rodents (mice, rats, guinea pigs), dogs, equines (horses, donkeys) or cattle (cows)[18,20,21]. Specifically, as storage progresses glucose is consumed, early glycolysis slows down and late glycolysis is fed by the breakdown of DPG (as reported by Simonova et al.[4,8]) to ultimately promote the accumulation of lactate and pyruvate. Our data add to this by showing a mitigating effect on the fluxes in this pathway by leukoreduction, perhaps as a function of the removal of mitochondria-containing WBC and platelets. Indeed, nLR pRBC were here found to accumulate dicarboxylic acids in cells and supernatants, especially pro-inflammatory and immunomodulatory succinate, which can stabilize hypoxia inducible factor 1α and stimulate interleukin 1β synthesis[41] upon transfusion in the recipient, thus potentially mediating untoward inflammatory sequelae to blood transfusion, such as neutrophil infiltration and acute respiratory distress syndrome (or acute lung injury)[42]. Of all the leukoreduction-specific signatures, the presence of excess metabolites of mitochondrial origin represents a key finding of this study, one that would support the standard implementation of leukoreduction not just of ovine blood units, but also canine and equine products[14,15], in keeping with previous studies in humans[13]. While stored ovine RBC have been previously noted to be more fragile than stored human counterparts[4], our data here suggest that leukoreduction attenuates the lipolysis burden and free fatty acid accumulation in supernatants, especially poly and highly-unsaturated fatty acids that are substrates for lipid peroxidation, a process that mechanistically underlies the increased propensity of stored RBC to undergo extravascular hemolysis[43] via splenic sequestration and erythrophagocytosis upon transfusion[44].

Of note, unique leukoreduction and species-specific signatures were observed with respect to antioxidant pathways, such as sulfur metabolites beyond glutathione homeostasis. Namely, nLR ovine pRBC were characterized by the residual presence of taurine-rich platelets and WBC in nLR units. Interpretation of these signatures should also account for the capacity of sheep to synthesize their own ascorbate endogenously, a characteristic they share with cattle and some rodents, such as guinea pigs[20].

The present study has several limitations. First of all, direct comparison of paired LR and NLR pRBC units was based on splitting a parent standard sheep pRBC unit in half, so consideration should be given to the potential impact of altered surface to volume ratios, although any such effects would extend to both the LR and nLR units tested herein. The focus on the impact of leukoreduction on ovine pRBC over storage and the use of relative quantitation approaches does not allow for cross-species comparison against the literature. Nevertheless, the appreciation of species-specific convergent (e.g., glycolysis, carboxylic acid, free fatty acid metabolism) and divergent pathways (e.g., ascorbate metabolism) can inform future storage strategies/additives tailored towards the optimization of storage conditions for ovine RBC, or rather informs on caveats in the interpretation of future blood transfusion studies that will leverage sheep as a tractable animal model. Another limitation pertains to sample collection and processing. Given the lability of the high energy phosphate compounds ATP and DPG, special protocols are usually applied to ensure the preservation of these compounds via rapid sample extraction, while minimizing transport. Here, internationally shipped samples, combined with logistics associated with the veterinary blood bank setting have introduced additional untoward variables that contributed to the sub-optimal characterization of these two specific metabolites. Nevertheless, relevant information on deamination and breakdown of purine phosphate pools was obtained from this study, which corroborated evidence from studies in humans and other species on the beneficial effects

of leukoreduction on the preservation of adenylate pools[13-15]. The present study was limited to metabolomics analyses of RBC and supernatants, while orthogonal characterization of morphology and other relevant biochemical features (e.g., protein levels and oxidation) and biology (e.g., vesiculation) will be pursued in follow-up studies.

At net of the limitations noted above, the present study clearly highlights a beneficial effect of leukoreduction on the metabolic quality of ovine pRBC (onset, progression and severity of the metabolic storage lesion). This study also identifies ovine-specific metabolic trends over storage duration and offers orthogonal information complementary to previous elegant blood transfusion studies that leveraged sheep as a relevant model[4,8]. It is worth mentioning that there are many different breeds of sheep, which, in the future, might yield interesting results based on genetics, similar to what has been found in mice and other animal models. Finally, the extensive metabolic characterization of these ovine samples revealed, for the first time, a sexually dimorphic signature in acyl-carnitine metabolism, which is relevant in light of the role of this pathway (and related interventions via exogenous carnitine supplementation) in other species, including mice and humans[37].

ETHICAL STATEMENT

The experimental protocols were approved by the Italian Ministry of Health, as part of the following project: "*Analisi lipidomica, proteomica e metabolomica delle unità di eritrociti concentrate stoccati di cane, gatto, cavallo, asino, bovino e ovino nell'ambito della valutazione delle lesioni da stoccaggio*" (which translates to "Lipidomics, proteomics, and metabolomics analysis of stored packed red blood cells from dogs, cats, horses, donkeys, cattle and sheep for the evaluation of storage lesions").

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AUTHORS' CONTRIBUTIONS

AM, MDT and FR generated the samples. JAR performed the metabolomics analyses. AD'A analyzed the data and prepared the figures. AD'A and AM wrote the first version of the manuscript. All the Authors contributed to and approved the final version of the manuscript.

The Authors declare no conflicts of interest.

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ONLINE SUPPLEMENTARY CONTENT

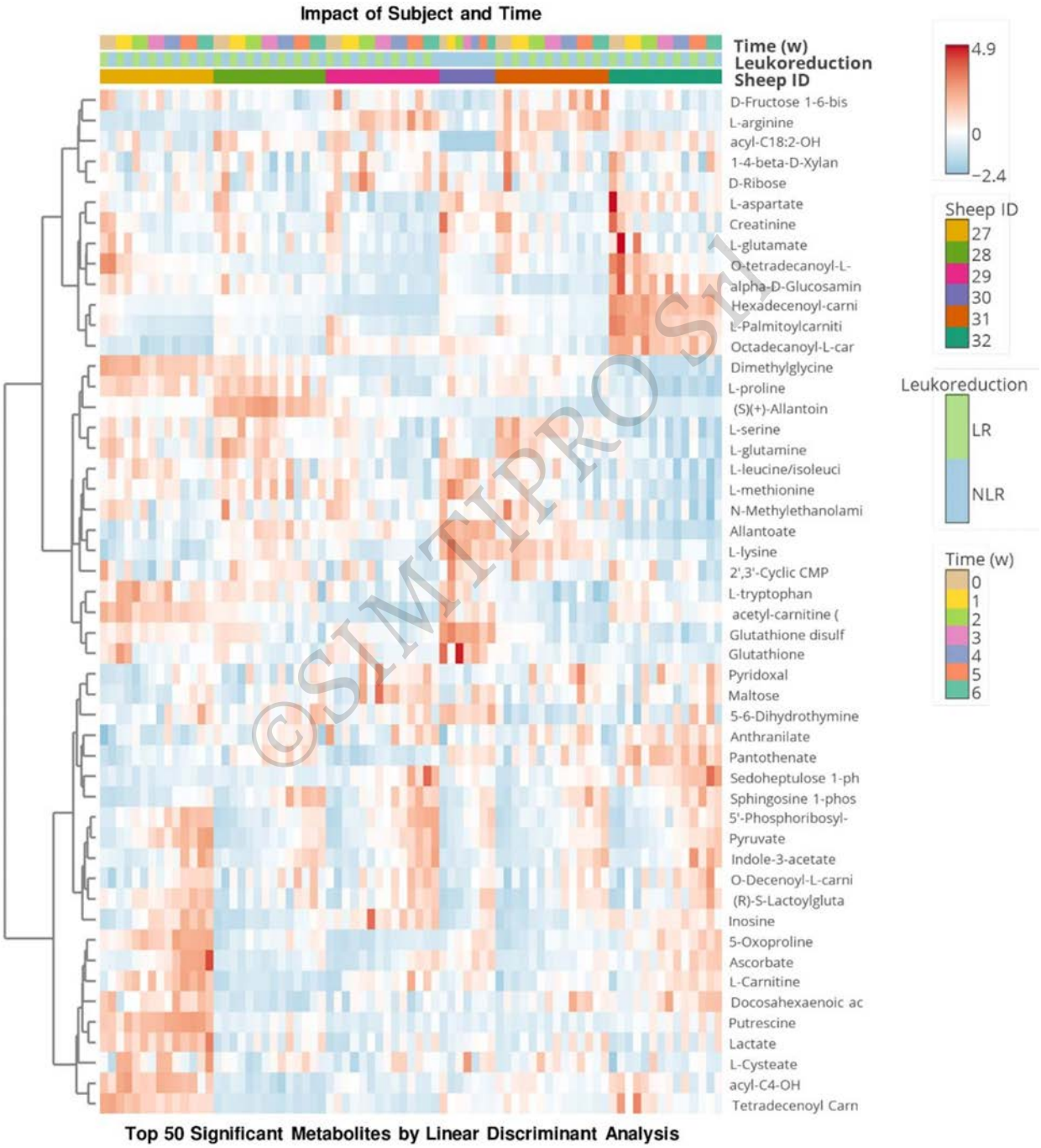

Figure S1 - Top 50 metabolites by linear discriminant analysis as a function of storage duration, leukoreduction, organized by subject (sheep: No. =6)

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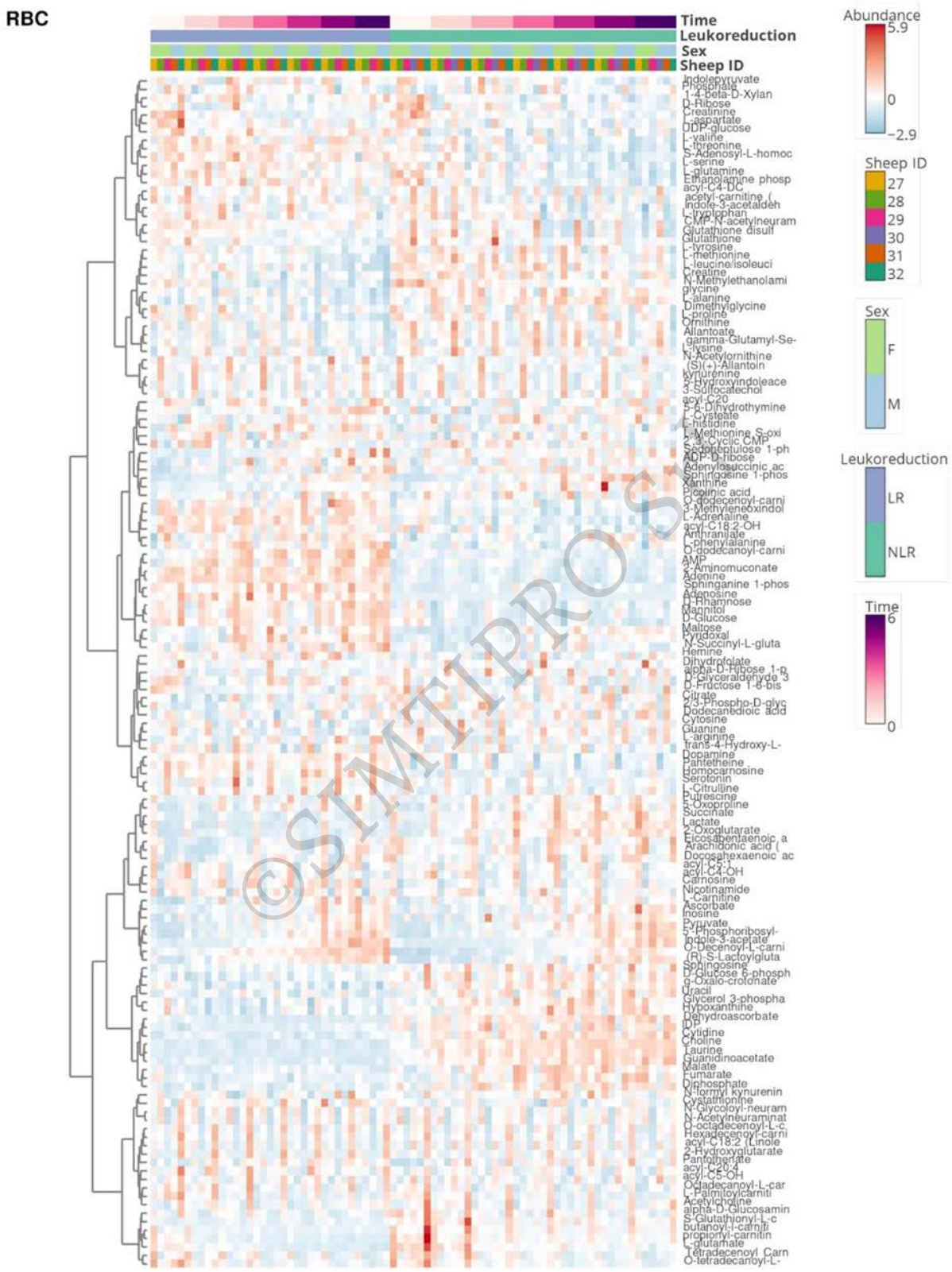

Figure S2 - Top 100 RBC metabolites by linear discriminant analysis as a function of storage duration and leukoreduction

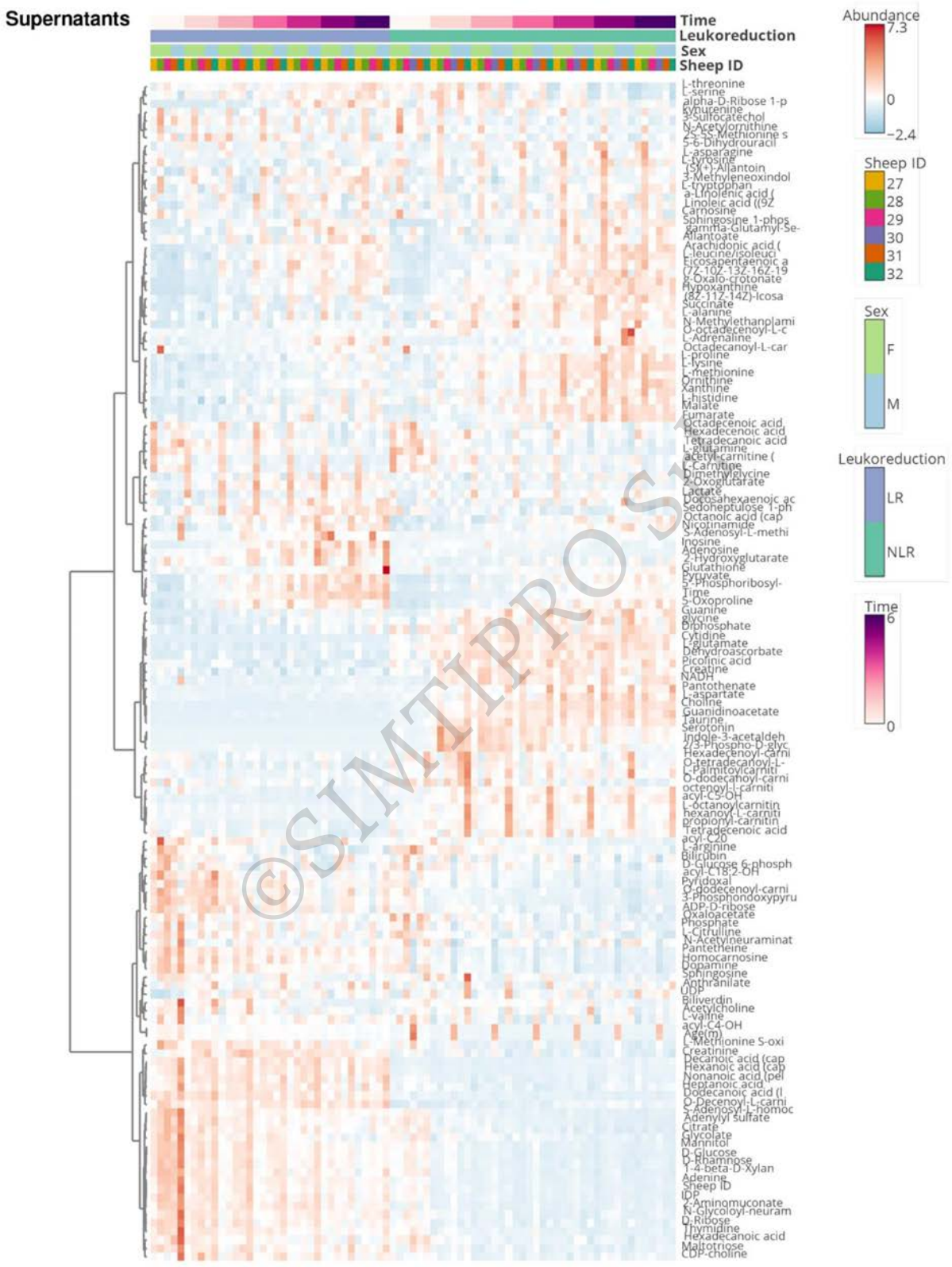

Figure S3 - Top 100 supernatant metabolites by linear discriminant analysis as a function of storage duration and leukoreduction

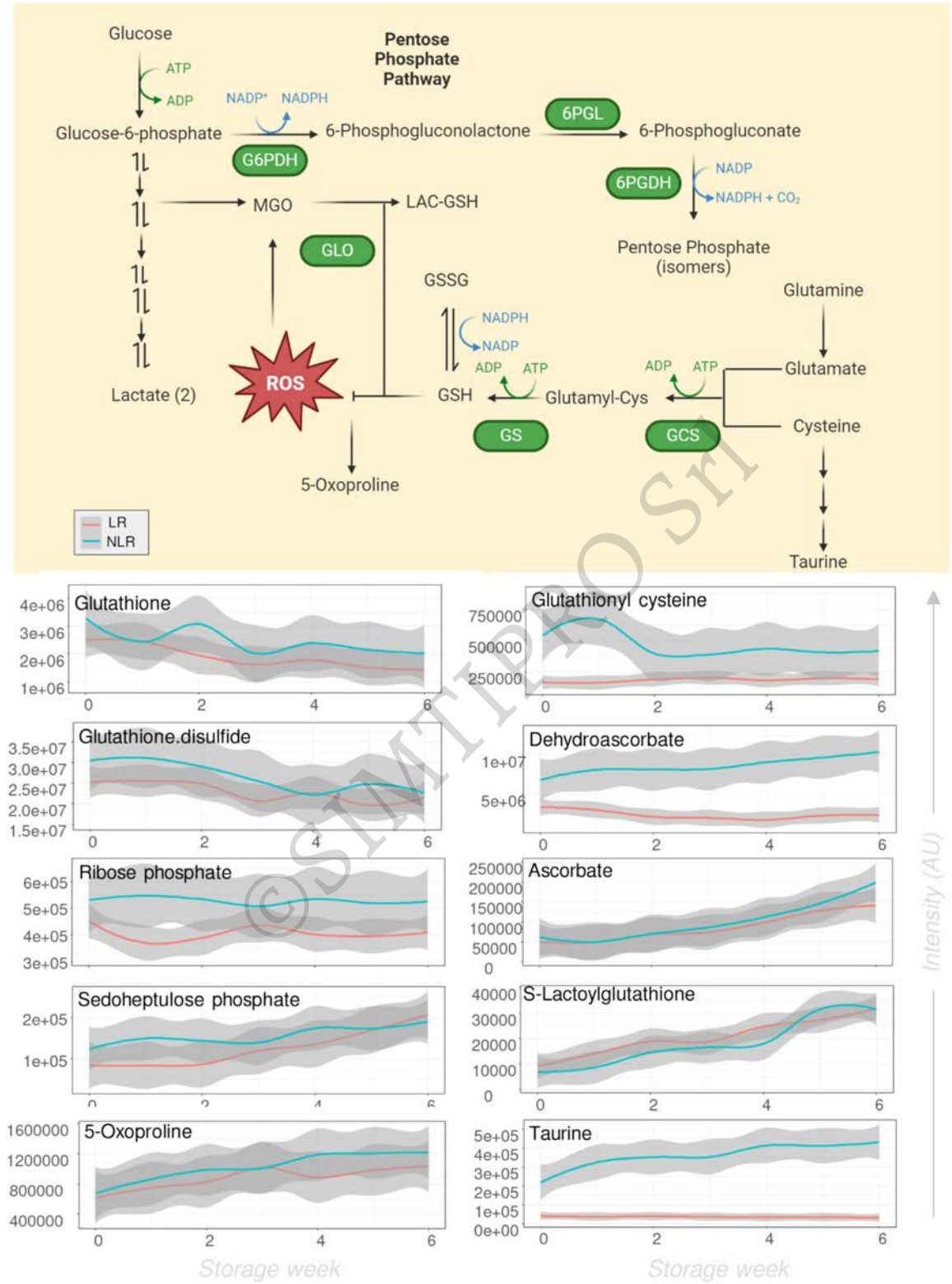

Figure S4 - Impact of sheep sex on stored ovine RBCs metabolism
In **A**-**B**, linear discriminant analysis of metabolites in RBCs and supernatants, respectively, as a function of sex, storage duration and leukoreduction. In **C**, heat map of the top 50 significant metabolites based on the LDA analysis.

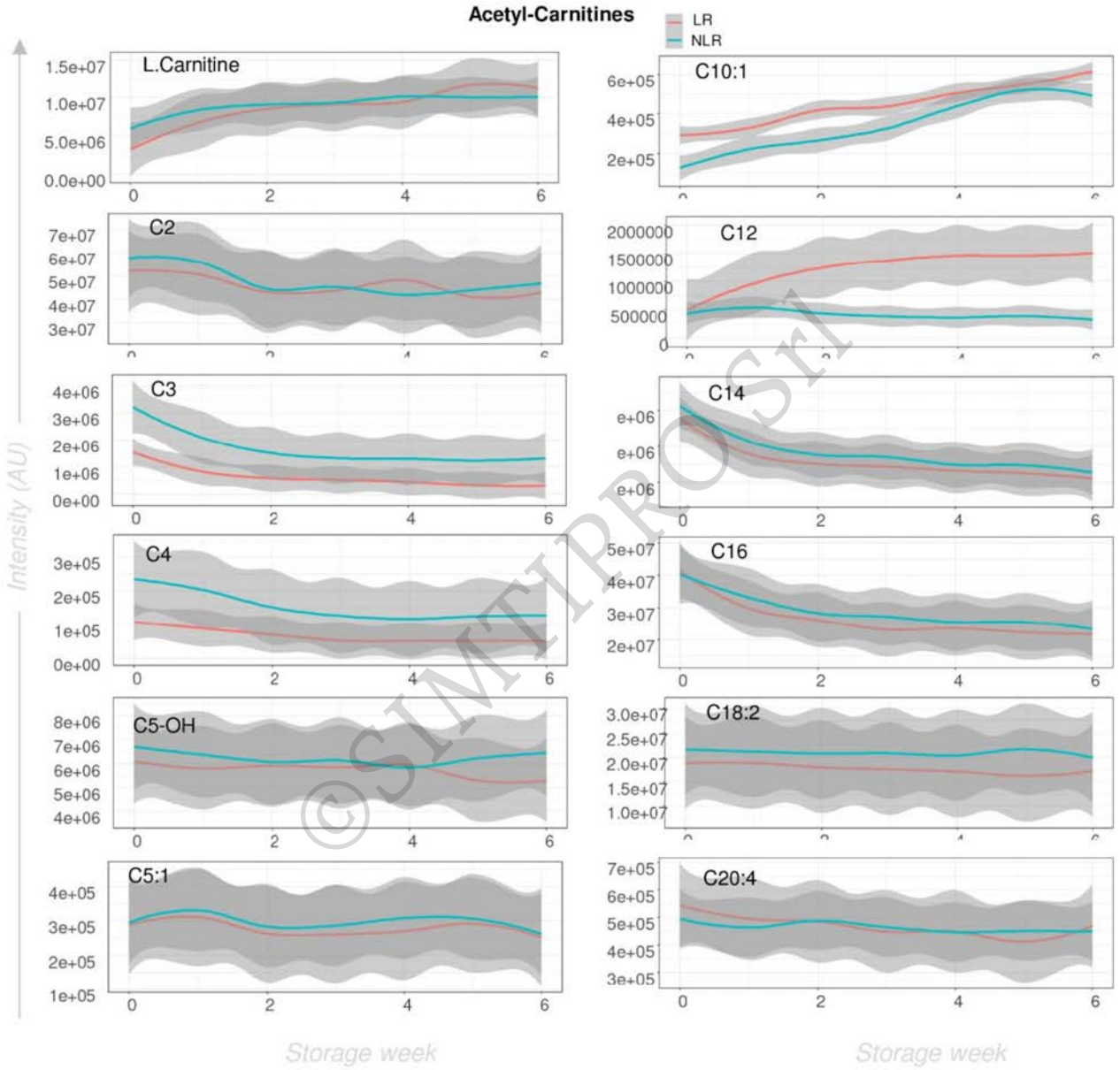

Figure S5 - Line plots of Acyl-carnitines (CN:n – indicates the fatty acyl-carnitine chain length [N] and degree of unsaturation [n])
In the light blue: non-Leukoreduced RBCs; in red: leukoreduced RBCs; X axis indicate storage weeks; Y axes indicate metabolite levels (peak areas, arbitrary units).

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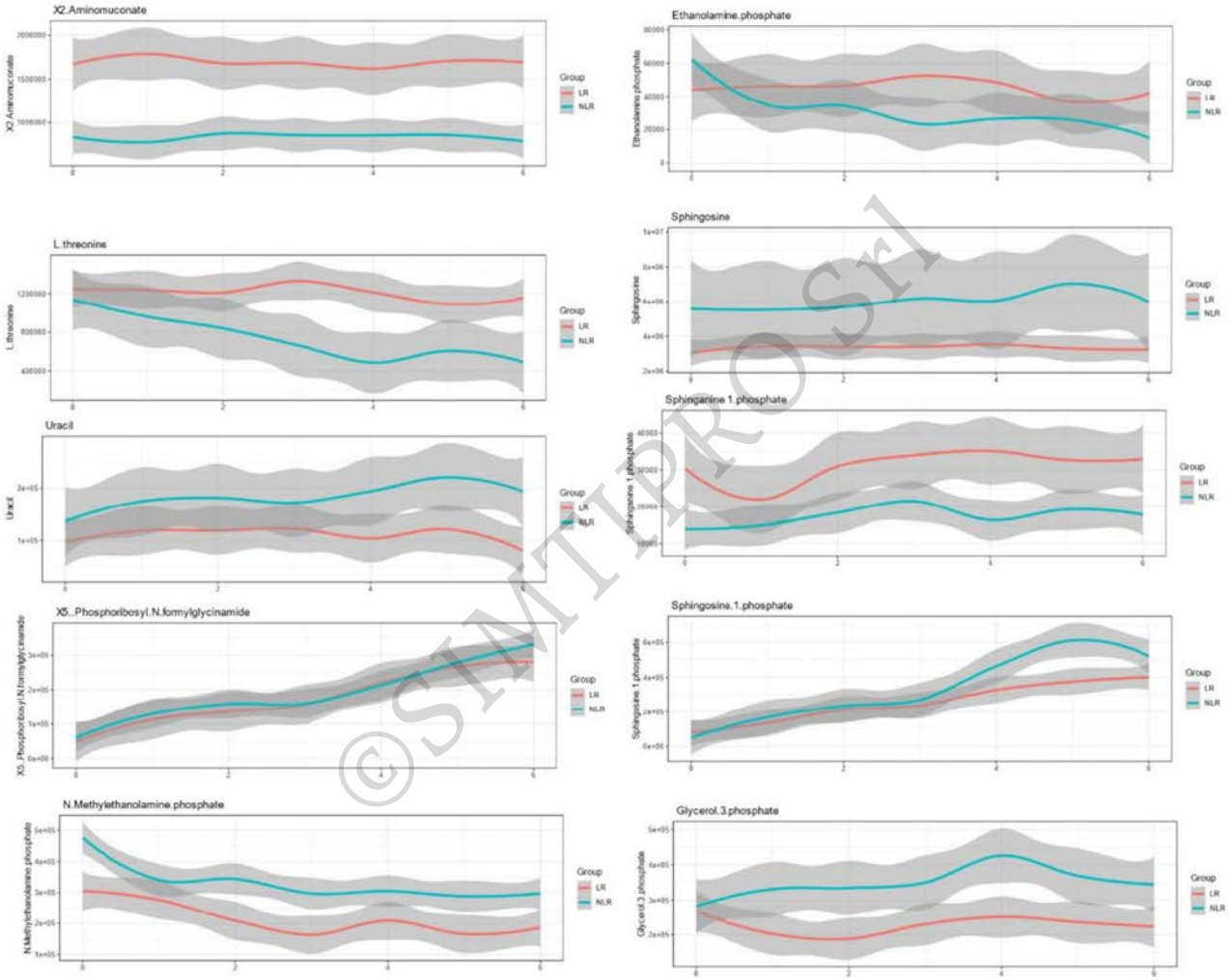

Figure S6 - Line plots for selected metabolites impacted by storage duration or leukoreduction
In the light blue: non-Leukoreduced RBCs; in red: leukoreduced RBCs; X axis indicate storage weeks; Y axes indicate metabolite levels (peak areas, arbitrary units).

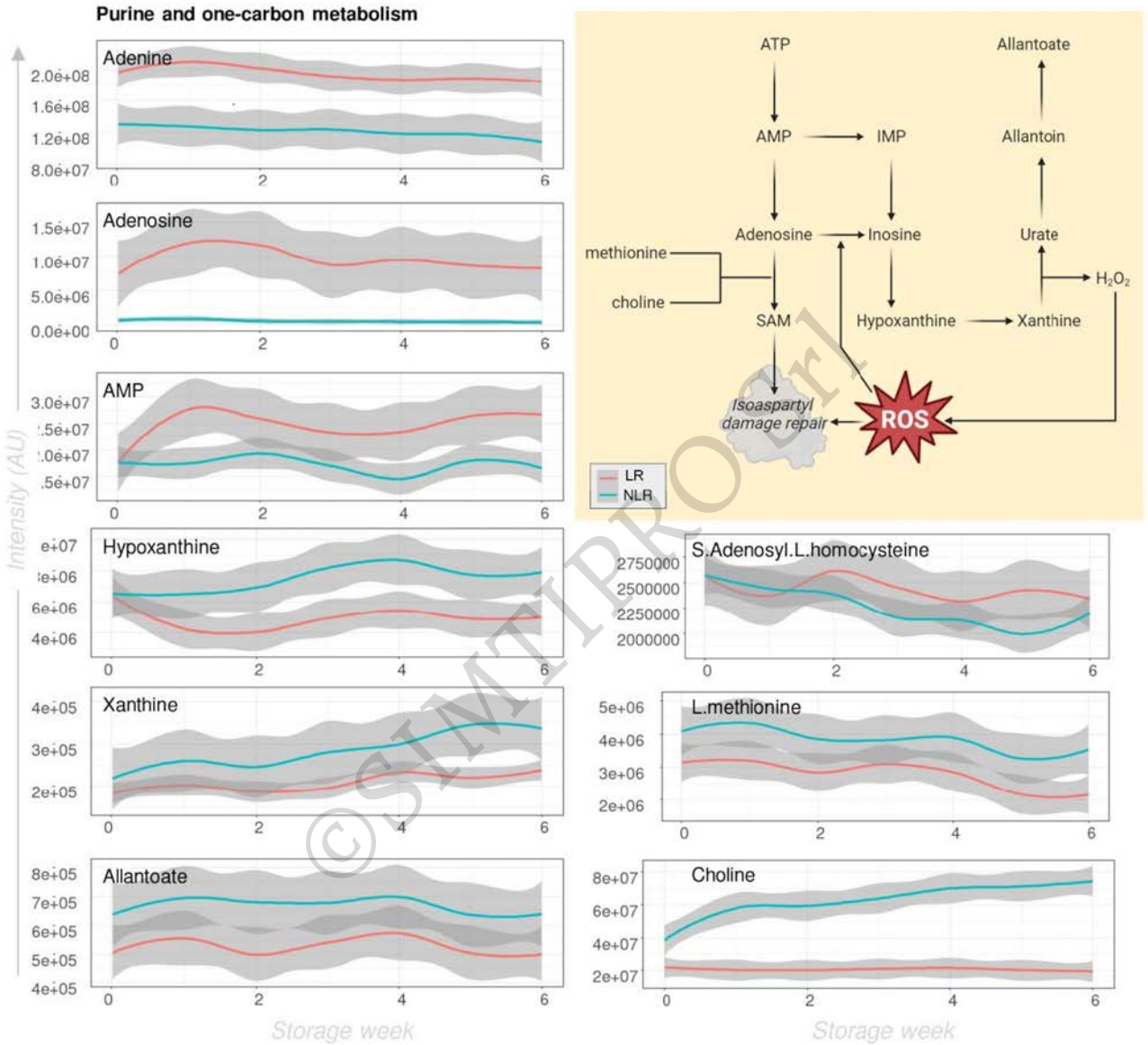

Figure S7 - Impact of leukoreduction on RBC purine metabolism and oxidation
Line plots show median + quartile range for leukoreduced (light red [LR]) and non-LR (light blue [nLR]) RBCs. X axes indicate storage week and Y axes indicate relative metabolite abundance (integrated peak areas – arbitrary units).

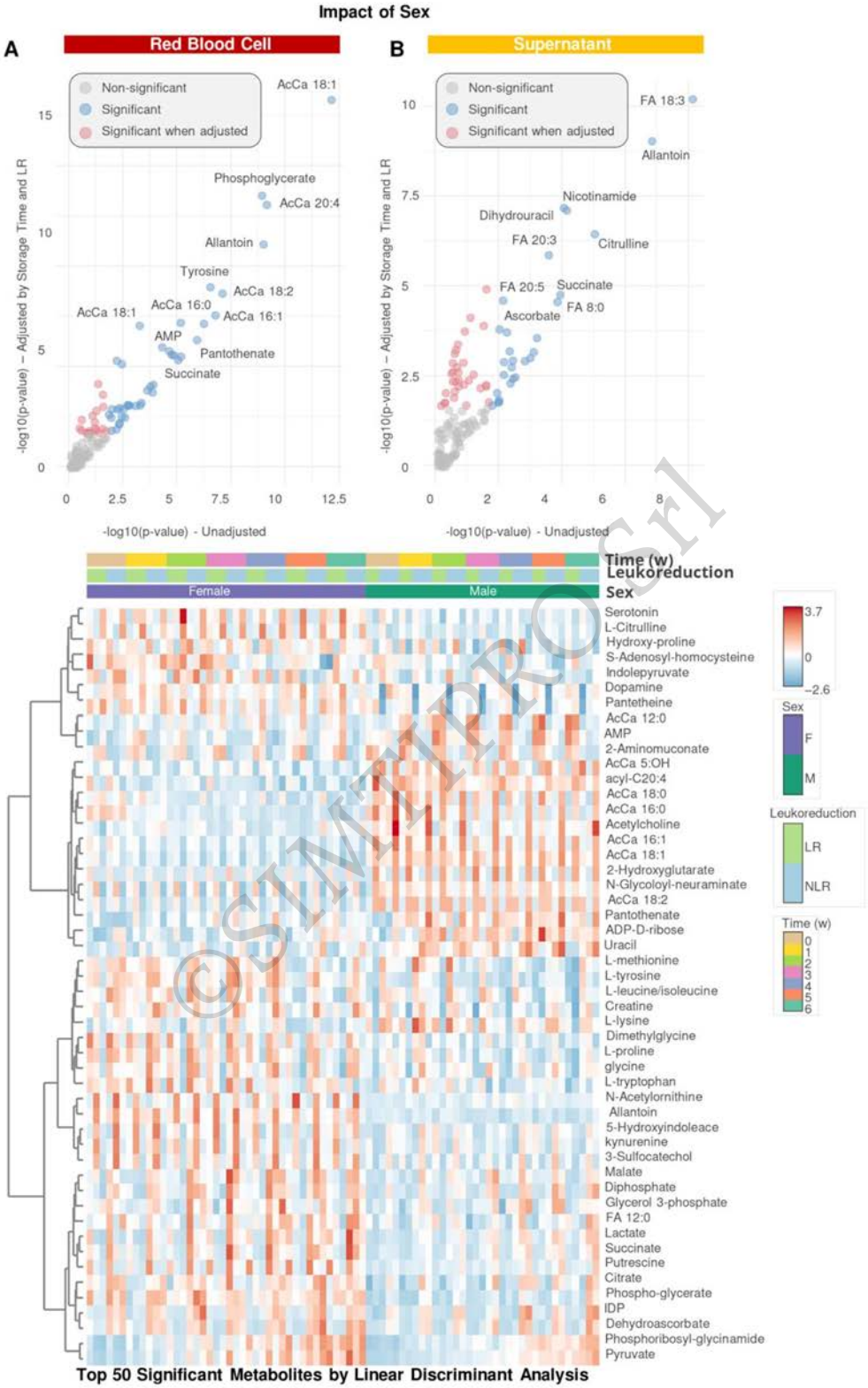

Figure S8 - Impact of sex, storage duration and leukoreduction on stored ovine RBCs and supernatants
In A and B, linear discriminant analysis (LDA) of RBCs and supernatants, respectively, either unadjusted (X axis) or adjusted by storage duration and leukoreduction (LR - Y axis). In C, heat map of the top 50 significant features based on the analysis in A.