Metabolic pathways that correlate with post-transfusion circulation of stored murine red blood cells

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ABSTRACT

Transfusion of red blood cells is a very common inpatient procedure, with more than 1 in 70 people in the USA receiving a red blood cell transfusion annually. However, stored red blood cells are a non-uniform product, based upon donor-to-donor variation in red blood cell storage biology. While thousands of biological parameters change in red blood cells over storage, it has remained unclear which changes correlate with function of the red blood cells, as opposed to being co-incidental changes. In the current report, a murine model of red blood cell storage/transfusion is applied across 13 genetically distinct mouse strains and combined with high resolution metabolomics to identify metabolic changes that correlated with red blood cell circulation post storage. Oxidation in general, and peroxidation of lipids in particular, emerged as changes that correlated with extreme statistical significance, including generation of dicarboxylic acids and monohydroxy fatty acids. In addition, differences in anti-oxidant pathways known to regulate oxidative stress on lipid membranes were identified. Finally, metabolites were identified that differed at the time the blood was harvested, and predict how the red blood cells perform after storage, allowing the potential to screen donors at time of collection. Together, these findings map out a new landscape in understanding metabolic changes during red blood cell storage as they relate to red blood cell circulation.

Introduction

Transfusion of stored red blood cells (RBCs) is amongst the most frequent inpatient therapies; for example, in the United States, approximately 1 out of every 70 people are transfused each year. However, while the process of RBC collection, storage, and transfusion is well-controlled, there remains substantial variability in the quality of RBC units, presumably as a result of varying donor characteristics.[1] It is understood that some donors' RBCs consistently store poorly, and there are currently no methodologies to identify such donors (other than autologous ^{51}-Cr survival studies).[1] Thus, measuring and standardizing the quality of stored RBCs remains elusive.

The biological changes that occur during RBC storage, collectively called the "storage lesion" consist of myriad cellular and biochemical alterations.[2,3] While the catalog of changes known to occur with RBC storage continues to grow into the thousands (with the application of omics technologies), it remains unclear which changes correlate to clinical performance of transfused RBCs and which are coincidental. Both historical and more recent data have demonstrated that RBCs change

during storage and the underlying metabolism of RBCs are both heritable traits in humans.[4-9]

Much attention has been paid in recent years to the concern that transfusion of longer stored RBC units may result in worse medical outcomes. These concerns are largely fueled by numerous retrospective studies reporting such an effect.[10] Recently, several randomized controlled trials, in particular in clinical settings, have been completed, and no difference was observed between groups.[11-13] There remains considerable debate surrounding this issue;[14] however, this is unrelated to the goal of providing the best RBC units available, and to storing RBCs so as to generate the most efficacious product. The goal of the current study is to elucidate donor biology that affects RBC storage, and the ability of the RBC to circulate post transfusion.

To identify biochemical components of the storage lesion that are correlated with RBC performance, in particular the ability of stored RBCs to circulate post transfusion, we analyzed 13 commonly available inbred strains of mice. Herein, we report significant variation amongst strains, both with respect to post-transfusion recovery of stored RBCs, and also the basic metabolomics of stored RBCs. Moreover, significant correlations between biochemical pathways and RBC storage are identified. In particular, lipid metabolism and oxidation (and underlying anti-oxidant pathways), emerged as a dominant theme in differences in RBC storage from genetically distinct murine donors. Together, these findings help to distinguish biochemical components of the storage lesion that correlate with RBC function in a mouse model. These studies provide mechanistic insight into the biology of RBC storage, define the landscape of murine specifics for ongoing basic research, and also highlight novel hypotheses to provide a rational basis for subsequent human studies.

Methods

Mice

The following strains of mice were purchased from Jackson Labs (Bar Harbor, ME, USA): KK/HlJ, LG/J, AKR/J, FVB/NJ, C3H/HeJ, DBA/2J, NOD/ShiLtJ, 129X1/SvJ, 129S1/SvImJ, A/J, BTBR/ T+ tf/J, Balb/cByJ, C57Bl/6J). All were female and used for blood donation at 12-15 weeks of age. UbiC-GFP male mice, which are on a C57BL/6 background, were bred to FVB/NJ females in the Bloodworks NW Research Institute (BWNWRI) Vivarium and offspring were used as RBC recipients at 24-28 weeks of age. HOD mice, used as a fresh tracer population for transfused RBCs, were likewise bred in the BWNWRI Vivarium. The HOD mouse was first described on an FVB background,[15] but has now been backcrossed onto C57BL6J for greater than 20 generations. All mice were maintained on standard rodent chow and water in a temperature- and light-controlled environment. All experiments were performed according to approved Institutional Animal Care and Use Committee (IACUC) procedures.

Collection and storage of blood

Whole blood (600 µl) was collected *via* cardiac puncture into 84 µl CPDA-1 (12.3%) in a sterile 1.7 mL snapcap microcentrifuge tube. Hematocrits were adjusted to approximately 75% (by removal of supernatant) and samples were stored in Eppendorf tubes for seven days at 4°C. After storage, 50 µl stored RBCs were resuspended in 510 µl PBS and 5 µl of fresh HOD packed RBCs were added to the suspension as an internal control. The mixture of RBCs was then directly transfused into FVB/NJ x UbiC-GFP recipients by intravenous tail vein injection. The remaining sample of stored RBCs was snap frozen in liquid nitrogen for future metabolomics analysis. Ratios of donor blood to HOD tracer RBCs was enumerated, both at baseline in the cells to be transfused (pre-transfusion mixture) and also in peripheral blood acquired from recipients 24 h after transfusion (post-transfusion samples collected into ACD). Pre-transfusion and post-transfusion RBCs were washed three times with PBS, and stained for 30 min with 0.5 µg anti-Fy3 (clone MIMA29) in 50 µl PBS. Stained cells were then washed three times with PBS and incubated with 0.2 µg APC goat-anti-mouse Igs (BD cat. 550826) in 50 µl PBS for 30 min, which stains RBCs bound with MIMA-29, and thus identifies HOD tracer RBCs. Cells were then washed three times, re-suspended in PBS, and analyzed by flow cytometry; 500 HOD+ events were counted for each sample. This approach utilizes MIMA-29 to stain HOD tracer RBCs with a color that is different than the GFP RBCs, which fluoresce spontaneously. Forward and side scatter were used to exclude fragmented or lysed RBC fragments, such that counts reflected intact RBCs. Final RBC survival was calculated by the formula:

(Circulating Stored RBC/HOD RBC of post-transfusion sample) /(Stored RBC/HOD pre-transfusion sample)

For experiments studying "fresh RBCS", the RBCs were collected and processed identically to stored RBCs, with the only difference being they were used after collection and without further storage.

Ultrahigh performance liquid chromatography-tandem mass spectroscopy and gas chromatography-mass spectroscopy

Details of ultrahigh performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) and gas chromatography-mass spectroscopy (GC-MS) are available in the *Online Supplementary Appendix*. Mass Spec was carried out by a commercial vendor (Metabolon Inc., NC, USA).

Statistical analysis

Comparisons of recoveries between strains were performed separately on each experiment using one-way ANOVA followed by Tukey's multiple comparisons test, with a single pooled variance. In the case of stored RBCs, the data were first $\log_{10}$-transformed to approximate a normal distribution; the data from the fresh RBCs required no data transformation. Analyses were performed using Graphpad PRISM 6 software. Linear correlations were summarized by Pearson's coefficient with *P* values by F-tests and q-values to account for multiple testing in the evaluation of statistical significance.

Results

Strain dependent variation in post-transfusion recoveries of stored red blood cells

To test the genetic variation in RBC storage phenotype amongst mice, a panel of inbred strains was analyzed. These strains were chosen due to commercial availability, well characterized biology and resolved genetic sequence. Consideration was also given to sampling different phylogenetic arms (Figure 1A). A well-characterized murine model of RBC storage was utilized[17] with minor modifications (see *Methods*). RBCs from each of the indicated test strains were collected, processed, and either transfused as "fresh" RBCs or stored for seven days. The post-transfusion circulation of test RBCs, 24 h after transfusion (24-h

recovery) was determined by transfusing test RBCs into GFP-F1 recipients and enumerating GFP negative populations in peripheral blood 24 h post transfusion. This approach allows analysis of RBC recovery without having to risk altering the RBCs through any labeling procedure. To isolate donor biology as a variable, a single common transfusion recipient was utilized for all donor strains; in particular, an F1 cross between UbiC-GFP and FVB mice (GFP-F1). RBCs from UbiC-GFP mice express high levels of green fluorescent protein (GFP) in RBCs and are on a C57BL/6 background. Thus, the GFP-F1 mice are heterozygous at all loci between B6 and FVB mice and have a GFP transgene.

To control for differences in transfusion volume and phlebotomy, and also to allow enumeration on a cell by cell basis, a "fresh tracer" control RBC population was added to each test RBC population prior to transfusion (Figure 1B, left panel). The tracer population consisted of HOD RBCs, which express an easily detectable transgene on RBCs. Essentially no GFP negative events are observed in untransfused recipient mice (Figure 1B, upper right panel). The ratio of test RBCs to tracer RBCs 24 h after transfusion (Figure 1B, lower right panel) was then corrected to the pre-transfusion ratio. Recovery was evaluated for each strain for both seven days of storage and in freshly isolated RBCs.

Three independent experiments were performed for stored RBCs from each of the indicated strains, and the results of each experiment are shown (Figure 1C). Whereas there was relative consistency in a given strain across experiments, there was substantial strain-to-strain variability in RBC recoveries after storage; no statistically significant differences in 24-h recovery were observed from freshly isolated samples from the 13 different strains (Figure 1D). Extensive ANOVA comparisons between each strain, for both stored and fresh RBCs, were performed and multiple differences of statistical significance were noted only for stored RBCs (*Online Supplementary Table S1*).

General metabolomic analysis of strain variation in red blood cell storage

For each experiment, prior to transfusion, a sample of the RBCs was snap-frozen. Samples were subsequently subjected to analysis of metabolites by LC-MS/MS, resulting in the resolution and relative quantification of 520 compounds of known identity. Principal component analysis (PCA) showed a clear distinction between fresh and stored RBC samples within each of the 13 mouse strains, as a function of the RBC metabolome (Figure 2). In addition, fresh samples from the C57Bl/6J strain demonstrated limited segregation from the general sample groupings and suggest that this strain may exhibit a base-line metabolic profile that further differentiates it from other strains. However, there was otherwise no clear distinction in general metabolomes between strains as a function of storage.

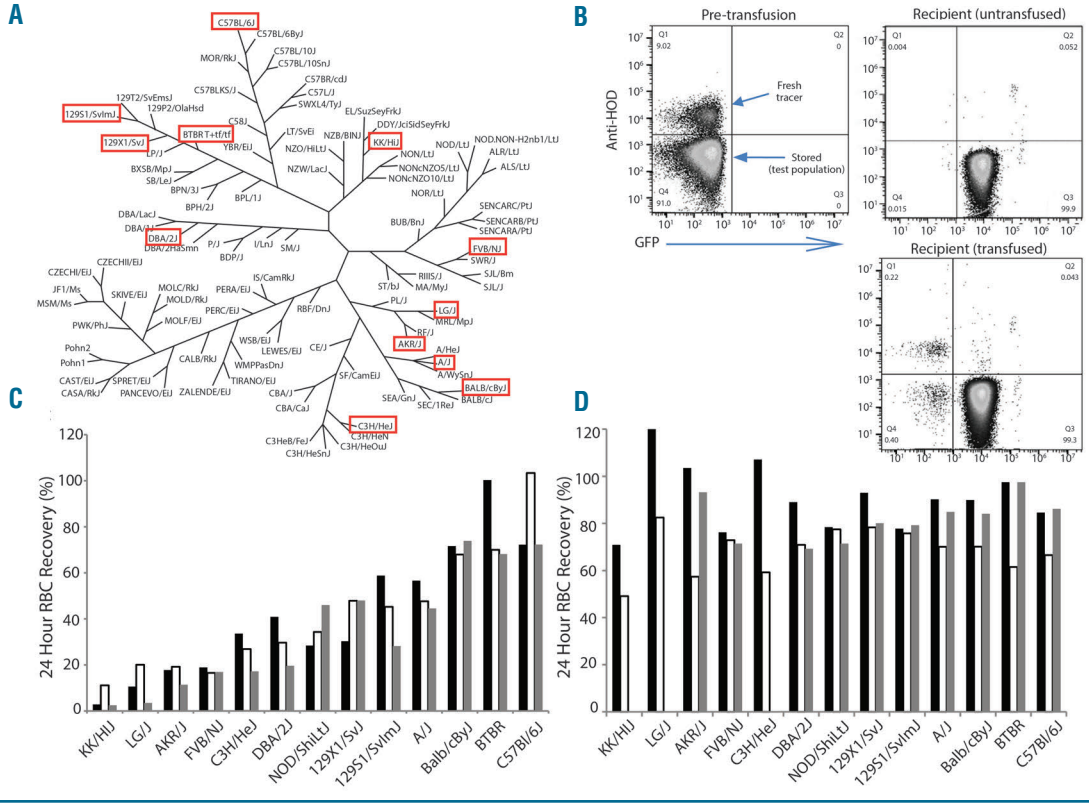

Figure 1. Twenty-four hour recovery of fresh and stored red blood cells (RBCs) across 13 inbred strains of mice. (A) Phylogenetic distribution of mouse strains used for this study (reproduced from Genome Res 2004:14:1806-11 with kind permission of Dr. Petkov and Genome Research[16]). (B) Representative flow cytometry plots are shown to indicate the enumeration of fresh tracer and test RBCs in a pre-transfusion sample (left panel). Recipient GFP$^+$ RBCs are shown, including empty gates where fresh tracer and test RBCs appear (upper right panel). The final mixture in a recipient mouse 24 h after transfusion is shown (lower right panel). (C) Individual results of three out of three experiments of stored RBCs is shown. (D) Individual results of three out of three experiments of fresh RBCs is shown (only 2 replicates were obtained for fresh RBCs for KK/HiJ, LG/J and the C3H/HeJ mouse strains).

Glucose metabolism of red blood cell storage across inbred mouse strains

Analysis of RBC storage has traditionally focused on generation of ATP as a necessary energy source for maintenance of RBC physiology. In addition, much attention has been paid to generation and maintenance of 2,3-DPG, due to its ability to regulate oxygen affinity by hemoglobin. Analysis of glycolytic metabolites across strains demonstrated decreases in glucose during storage in all cases (Figure 3A). Likewise, in all strains, 2,3-DPG dropped substantially at seven days storage (Figure 3B). The end point of glycolysis (lactate) had a similar increase in stored RBCs from each strain (Figure 3C). ATP was not detected as a metabolite by this approach, and thus was not assessed in the current study.

Metabolites and pathways that correlate with red blood cell storage

To investigate which metabolites (and metabolic pathways) may be associated with the ability of RBCs to survive post transfusion, metabolites in stored RBC units were chosen based upon the following criteria (correlation greater than 0.5 or less than -0.5, $P<0.05$, q value <0.01; see *Online Supplementary Table S2* for a full list of compounds). Using these criteria, 11 metabolites had a positive correlation with RBC storage (*Online Supplementary Table S2*, stored RBCs, positive correlation), 9 of the 11 metabolites identified were lipids, although various lipid subspecies were identified, including free fatty acids (polyunsaturated, and monohydroxy), lysolipids, and glycerol species. Also noted was vitamin E (alpha-tocopherol), a common cellular anti-oxidant most involved with oxidative stress in the lipid compartment. 12-HETE is an arachidonic acid (AA) metabolite that has biological properties. Finally, tryptophan was noted. As examples of the typical distributions, both of the fatty acids docosapentaenoate (DPA) and docosahexaenoate (DHA) showed a pattern (in general) of decrease over storage in strains that stored poorly and increase in storage of strains that stored well (Figure 4A and C) resulting in a positive correlation with final levels and 24-h RBC recovery (Figure 4B and D).

A total of 49 metabolites had a negative correlation with stored RBCs that fit the above criteria, the majority of which were lipid species (*Online Supplementary Table S2*, stored RBCs, negative correlation). Of these, 19 were either dicarboxylic acids (DCAs) or monohydroxy fatty acids (MHAs), known to be associated with lipid oxidation and peroxidation, 10 of which had inverse correlations greater than 0.80 with both P values and q values less than 0.0001. In all of these cases, levels were low at time of collection and increased over storage, to a greater extent in strains that stored poorly. Representative box plots of the relative amounts of a DCA (dodecanedioate) and a MHA (16-hydroxypalmitate) are shown (Figure 4E and G) along with the correlation plots between these analytes and 24-h RBC recovery (Figure 4F and H). Of note, among the MHAs identified are products with known biological function, including 13-HODE, 9-HODE and the dihydroxy fatty acid (9,10-DiHOME). In addition, 4-hydroxy-2-nonenal fit these criteria, and is a well-known product and mediator of lipid peroxidation.[18] 5-HETE, which is an eicosanoid, was also observed to have a similar pattern (Figure 4I and J). Of note, a related species (12-HETE) showed an opposite correlation (Figures 4K and L), although to a less dramatic effect. Two lysolipids and a monoacylglycerol were also observed (*Online Supplementary Table S2*). In addition to lipid species, negative correlates also included metabolites involved in glutathione metabolism (4-hydroxy-nonenal-glutathione and methionine sulfoxide).

To test the hypothesis that base-line metabolite levels,

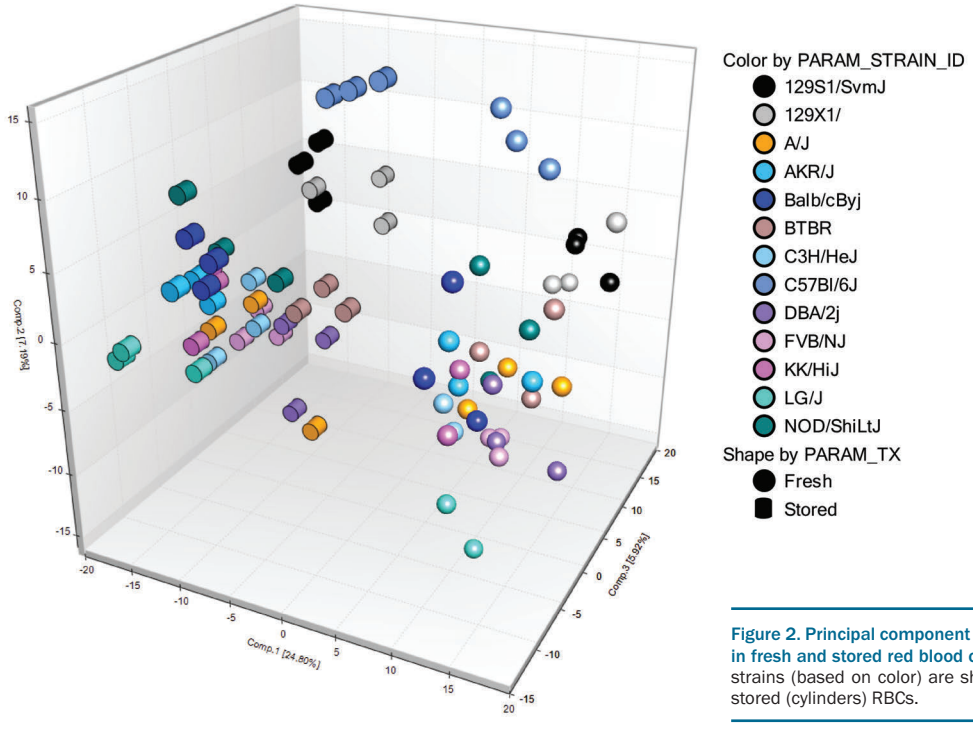

Figure 2. Principal component analysis of metabolites measured in fresh and stored red blood cells (RBCs). PCA for the indicated strains (based on color) are shown for both fresh (spheres) and stored (cylinders) RBCs.

present at time of blood collection, would predict quality of RBC storage over time, correlations were calculated between metabolite levels in freshly collected RBCs and post-transfusion recovery after storage *(Online Supplementary Table S2*, fresh RBCs). The same cut-off values of significance were used as above (correlation >0.5 or <-0.5, $P<0.05$, q value <0.01). Fourteen metabolites met these criteria with regards to positive correlation, in a variety of pathways, including amino acid metabolism, pyrimidine metabolism, and the urea cycle (*Online Supplementary Table S2*, fresh RBCs, positive correlation). Aspartate is presented as an example of a positively correlating analyte (Figure 4M). Twenty-four metabolites met the criteria with a negative correlation (*Online Supplementary Table S2*, fresh RBCs, negative correlation). Like the negative correlation of metabolites after storage, a substantial clustering of fatty acid metabolites was observed, including 9 long chain fatty acids of different composition, polyunsaturated fatty acids (linoleate and derivatives), and monoacylglycerols. As an example, palmitate (16:1) is shown (Figure 4N). In addition, tocopherol, 4-hydroxy-nonenal-glutathione, and other amino acid metabolites were observed, which is expanded on in the discussion of anti-oxidant pathways below. Finally, correlations were analyzed for the fold change of a given analyte over storage by calculating the ratio in fresh RBCs compared to seven days of storage (*Online Supplementary Table S2*, ratio of stored to fresh). The analysis of fold change reveals the same general patterns as observed by analyzing data from seven days of storage, with 17 ratios having a positive correlation and 33 ratios having a negative correlation, which fit the same significance criteria as above. In general, the same classes of compounds emerged, with a few notable differences, discussed below.

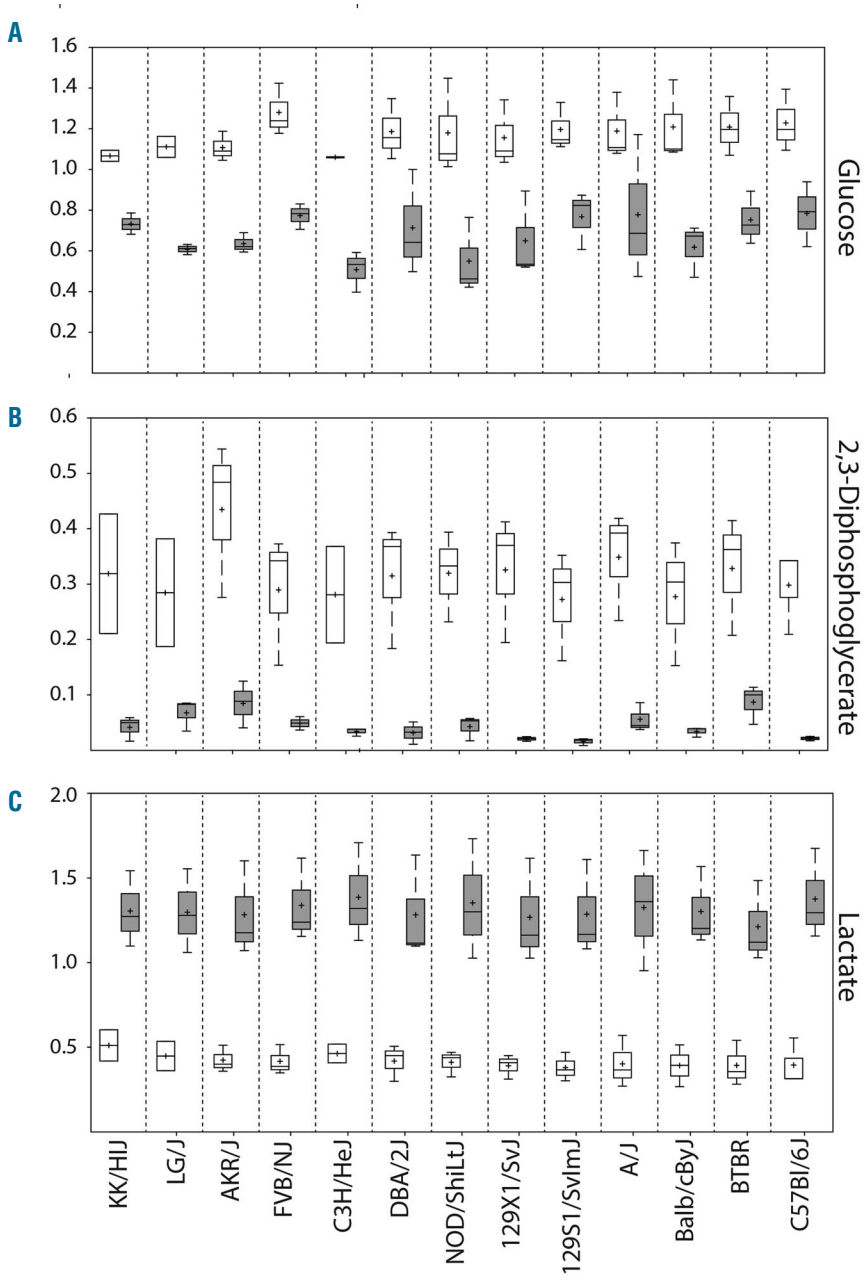

Figure 3. Products of glycolysis are common across strains and do not correlate with 24-h recovery. (A) Glucose levels were equivalent in fresh red blood cells (RBCs) from each strain and decreased commonly after storage. (B) 2,3-DPG was equivalent across strains and uniformly decreased after seven days of storage. (C) Lactate was uniformly low in fresh RBCs and increased equivalently, over storage, for all strains analyzed. Open boxes represent levels at time of collection whereas gray boxes indicate levels after storage. All levels of metabolites are relative concentrations based upon areas under the peaks and are averages for all three experiments shown in Figure 1.

Analysis of common anti-oxidant pathways

Due to the preponderance of lipid peroxidation products, we analyzed differences in common anti-oxidant pathways. Alpha-tocopherol (vitamin E) is a major hydrophobic anti-oxidant that mainly exerts its effects in lipid membranes. Levels of alpha-tocopherol in fresh RBCs negatively correlated with recoveries of stored RBCs (Figure 5A and F, top panel). At the same time, levels of alpha-tocopherol in stored RBCs positively correlated with recoveries (Figure 5F, middle panel). In other words,

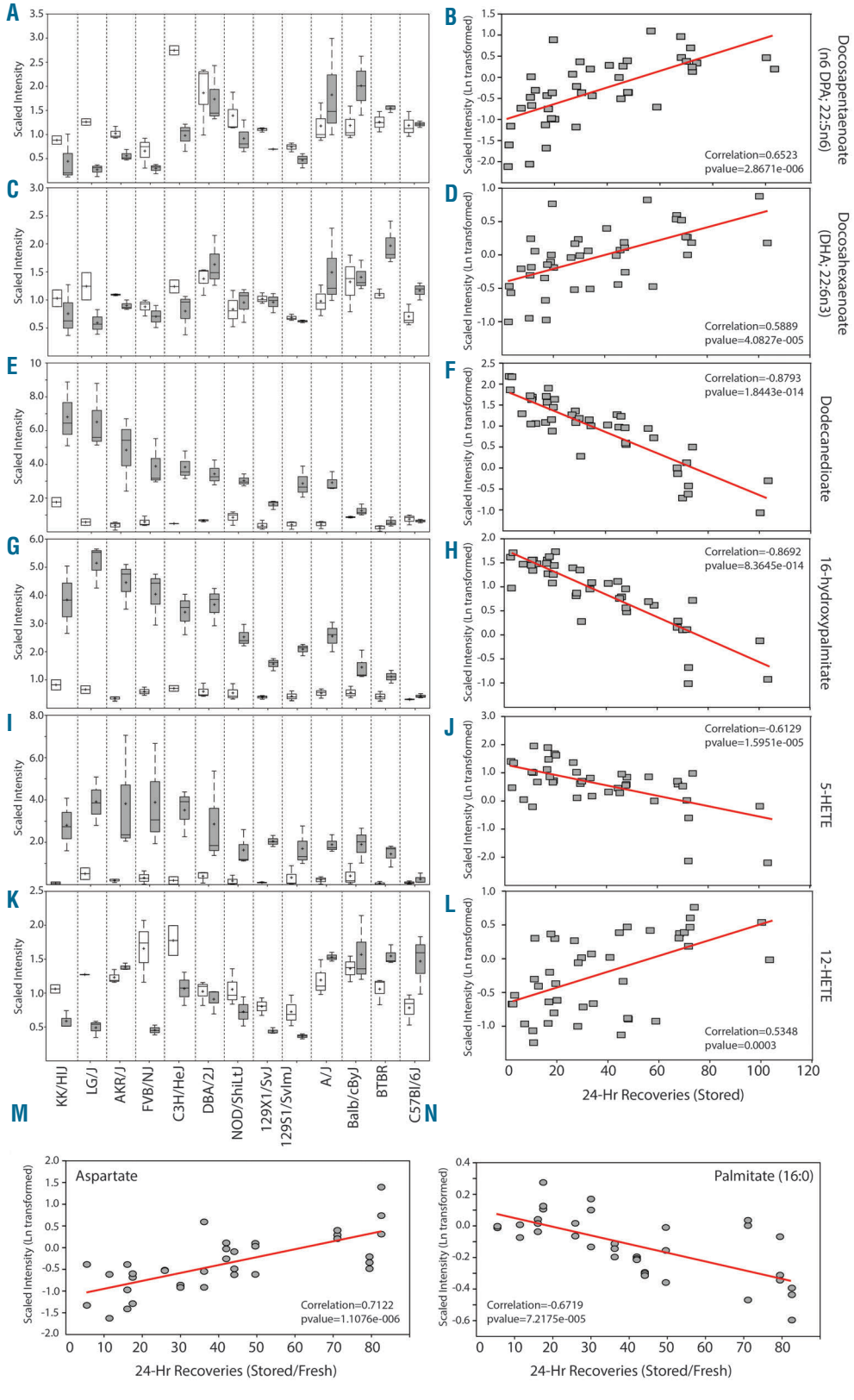

Figure 4. Levels of the indicated metabolite are shown in fresh (white) and stored (gray) samples, as are the correlations of the metabolite with 24-h red blood cell (RBC) recovery. (A and B) DPA, (C and D) DHA, (E and F) dodecanedioate, (G and H) 16-hydroxypalmitate, (I+J) 5-HETE, (K and L) 12-HETE. Correlations of levels of aspartate (M) and palmitate (N) in freshly isolated RBCs are shown *versus* the 24-h RBC recovery after seven days of storage. Open boxes represent levels at time of collection whereas gray boxes indicate levels after storage. Correlation calculations represent Pearson's coefficient. All levels of metabolites are relative concentrations based upon areas under the peaks and are averages for all three experiments shown in Figure 1. Correlation plots show combined data from all three of the indicated experiments in Figure 1.

the less alpha-tocopherol present at the time of collection and the more alpha-tocopherol that remained after storage, the better the RBC recoveries. These data are inconsistent with a simple model of increased alpha-tocopherol providing increased resistance to oxidation. However, juxtaposition of starting and ending levels of alpha-tocopherol showed that strains that stored poorly started with higher alpha-tocopherol levels than strains that stored well; however, by the end of storage, strains that stored poorly had lower levels of alpha-tocopherol than strains that stored well (Figure 5A). Strains that stored well had little change in alpha-tocopherol at all, whereas poorly storing strains rapidly depleted their alpha-tocopherol. This trend becomes clear when the correlation(s) are analyzed with regards to the ratio of alpha-tocopherol from fresh RBCs to stored RBCs (Figure 5F, bottom panel). These data are equally consistent with poorly storing strains generating more oxidative stress, having decreased anti-oxidant regeneration, or both. Alternatively, alpha-tocopherol could represent non-causal association and be a surrogate marker.

Although less dramatic than the pattern seen with alpha-tocopherol, GSH showed a similar trend (Figures 5B and G). Since the most common anti-oxidant pathway of GSH is for 2 GSH molecules to form GSSG; GSSG levels were examined (Figure 5C); however, no obvious pattern emerged to correlate with changes in GSH levels. In contrast, a clear trend was seen with the analysis of 4-hydroxy-nonenal-glutatione, a common product of GSH anti-oxidant activity upon a product of lipid peroxidation (Figure 5D). Of note, increases in 4-hydroxy-nonenal-glutathione from fresh RBCs to stored RBCs correlated with poor RBC recoveries (*Online Supplementary Table S2* and *data not shown*). Because vitamin C serves as an intermediate between GSH and alpha-tocopherol, vitamin C levels were examined, but no meaningful trend was observed (Figure 5E) and ascorbate levels did not correlate with RBC storage (*data not shown*). Finally, although its correlation was 0.48 (and thus technically below the 0.5 cut off), N-acetylcysteine (which is both an antioxidant and also a GSH precursor) had levels in stored RBCs that correlated well with 24-h RBC recovery (Figure 5H).

Discussion

The current report makes the observation that, similar to donor-to-donor variation in storage of human RBCs, genetically distinct stains of mice have a wide range of RBC storage biology regarding both metabolome and post-storage circulation. The ability of an RBC to circulate does not guarantee its full function (e.g. traversing microcapillary beds, delivering oxygen to tissues, removing CO_2, etc.); however, it seems a fair statement that an RBC that does not circulate will certainly not function. Thus, 24-h recovery is a meaningful, if not all encompassing, metric and is currently used as licensing criteria for RBC storage systems.

The current studies are carried out in a tractable animal model; however, like all models, it suffers the potential that it may not translate into human biology. Nevertheless, it serves to generate new knowledge of mammalian RBC storage biology which may translate into humans, and which provides (from amongst the numerous changes in RBCs during storage) a focused list of compounds and pathways to test in human RBC storage. It is worth noting that lipid peroxidation is a well-known component of storage of human RBCs.[19-23] In addition, a number of studies have been reported regarding metabolomics of human RBCs and these show heritable differences amongst donors and different storage conditions;[6,7,24-28] to the best of our knowledge no human studies have been reported that have combined metabolomics and *in vivo* recovery.

It is worth noting that, in the current study, the stored RBCs were not leukoreduced. The majority of (although certainly not all) stored RBCs in humans are leukoreduced. Given the volume of mouse blood required for leukoreducction, this approach was not considered feasible in the setting of broad screening of multiple strains. However, we have previously reported metabolomics analysis of B6 and FVB strains, using filter leukoreduced products. In this setting, the same lipid oxidation pathways are associated with poor storage.[29] In addition, dicarboxylic acids remain associated with poor storage in leukoreduced RBCs (*data not shown*). Thus, while some of the associated change may be due to contaminating leukocytes and/or platelets, the major findings of lipid peroxidation persist even with leukoreduction. A second consideration is that a 7-day storage time was chosen for this study, since this allowed the widest range of differences between strains to be observed, while still allowing for the best storing strains to have recoveries of greater than 75% (in line with FDA standards for human RBC storage).

To control for potential differences in recipient phagocytic biology, a single common recipient strain of mice was used for all donors in the current study. This approach also allowed an experimental design in which donor RBCs did not have to be manipulated or labeled prior to transfusion. However, there is a theoretical risk that one is crossing alloimmune barriers between strains, which could account for some differences in survival. Naturally occurring RBC alloantibodies (analogous to ABO in humans) have not been described in mice, and 24-h recovery is typically too short a period of time for adaptive humoral responses to occur; thus, we did not predict any problems with alloimmunity in the current studies. In support of this notion, there were no statistically significant differences in 24-h recovery of freshly isolated RBCs, thus indicating that, even if alloantibodies were present, they had no clear functional outcome. Nevertheless, one must give theoretical consideration to possible effects of crossing strain barriers.

The biological underpinnings that regulate lipid oxidation across strains are unclear; however, alpha-tocopherol and GSH are candidates for being involved in the underlying processes that lead to lipid peroxidation. Alpha-tocopherol does have the ability to inhibit chemical oxidation of RBCs;[30] however, the extent to which such is a normal pathway during RBC storage is unclear and has only been tested in an *in vitro* setting.[31] Of interest, it has recently been reported that vitamin C and N-acetylcysteine mitigate oxidative stress in *in vitro* human RBC studies.[32] *In vivo* studies in mice have shown that vitamin C supplementation can improve storage as measured by 24-h recovery.[33]

The generation of products of lipid oxidation not only gives insight into underlying RBC storage biology, but may in themselves represent a biologically significant component of transfused RBCs. Among the lipid oxidation products that were observed to both increase with

storage and negatively correlate with RBC recoveries are 5-HETE and the bioactive lipids 9,10-DiHOME, and (13-HODE+9-HODE). In addition, 4-hydroxy-2-nonenal has biological and signaling properties, in addition to being a common indicator of lipid peroxidation. Interestingly, in contrast to 5-HETE, 12-HETE had a significant positive correlation to RBC storage. Both 5-HETE and 12-HETE are arachidonic acid metabolites generated by separate lipoxygenase enzymes. Bioactive lipids are known to be involved in complex biologies including inflammation, coagulation, vascular tone and immunity. Bioactive lipids have been implicated in the pathogenesis of transfusion-related acute lung injury (TRALI), a potentially lethal sequela of blood transfusion.[34-36] It is also worth considering the source of substrates for lipid oxidation pathways, such as eicosanoid generation. Levels of both medium and long chain fatty acids were strongly correlated with RBC recovery, and lysolipids and monoacylglycerols increased with storage, suggesting release of the observed free fatty acids from glycerophospholipid breakdown. It is unclear if release of free fatty acids from phospholipids precedes lipid oxidation as a separate step or is the result of lipid peroxidation, but it is clear that both correlate.

Increases in long chain fatty acids at the time of RBC collection strongly correlated with the post-storage RBC recovery. Of these, 4 particular long chain fatty acids had a nega-

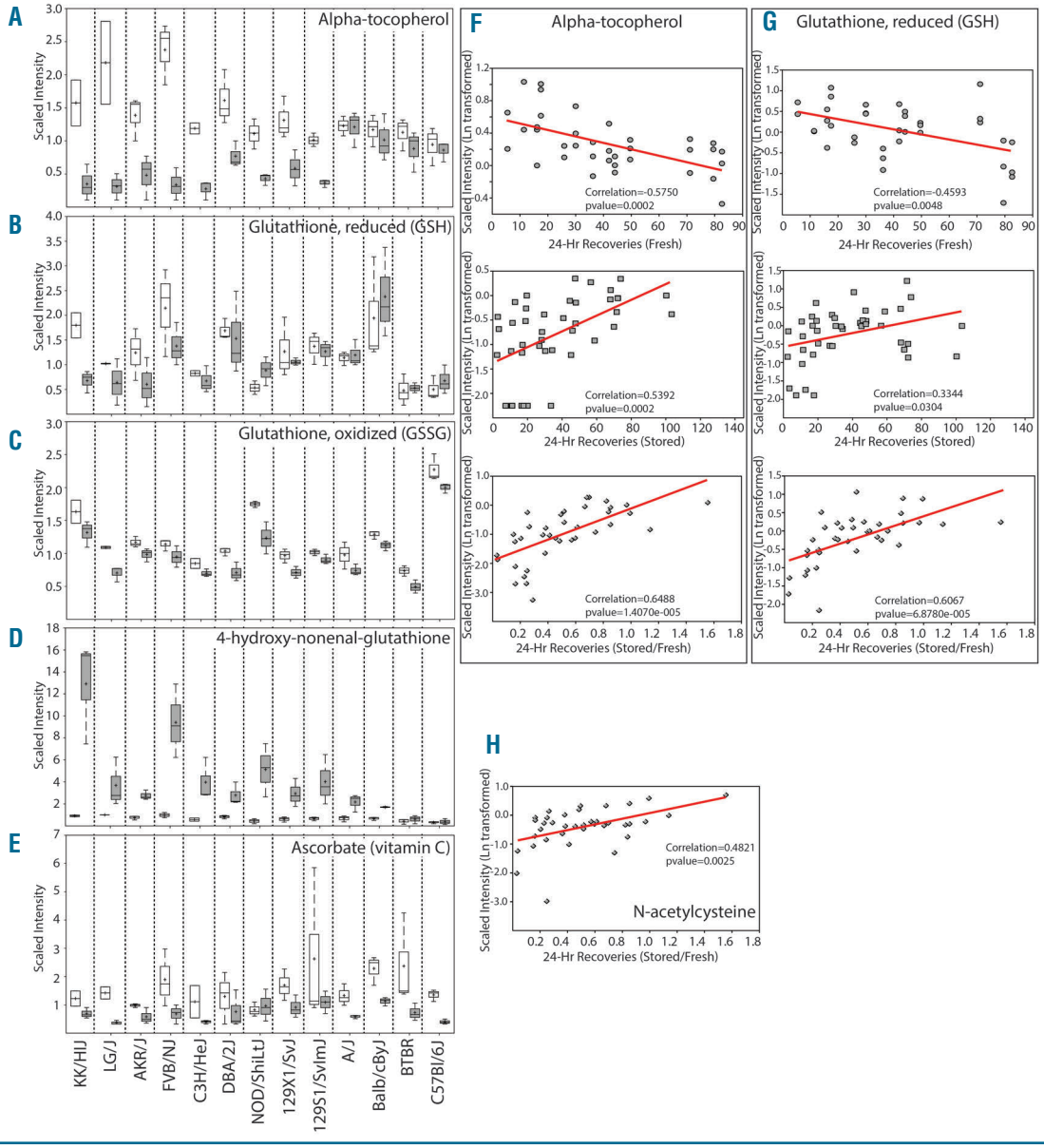

Figure 5. Anti-oxidant pathways during red blood cell (RBC) storage. Levels of alpha-tocopherol in freshly collected RBCs and after storage are shown (A). Correlation plots are shown for alpha-tocopherol for levels in fresh, stored, and the ration of fresh/stored (F). Levels of GSH in freshly collected RBCs and after storage are shown (B). Correlation plots are shown for GSH for levels in fresh, stored, and the ration of fresh/stored (G). Levels of GSSG (C), 4-hydroxy-nonenal-glutathione (D), and ascorbate (E) are shown in freshly collected RBCs and after storage. Correlations between the ratio of N-acetylcysteine (fold change) is shown (H). Open boxes represent levels at time of collection; gray boxes indicate levels after storage. Correlation calculations represent Pearson's coefficient. All levels of metabolites are relative concentrations based upon areas under the peaks and are averages for all three experiments shown in Figure 1. All horizontal axes labeled 24-h recovery represent RBCs circulating 24-h post transfusion, as described in *Methods*. Correlation plots show combined data from all three of the indicated experiments in Figure 1.

tive correlation both in fresh RBCs and stored RBCs (palmitate, palmitoleate,10-heptadecenoate, and cis-vaccenate). There was no significant change in the levels of these 4 long chain fatty acids between time of collection and after storage. Although one cannot rule out a simultaneous increase in production and consumption resulting in an unaltered steady state, a more likely explanation is that increased lipid breakdown (or processes that are associated with it), as a function of the normal RBC biology, predispose RBCs to poor storage. In contrast to the above long chain fatty acids, omega-3 fatty acids DPA and DHA had a positive correlation with 24-h recovery when measured after storage. EPA can be converted into DPA, which then can be converted to DHA. EPA was detected in this panel, but had no correlation to RBC recoveries. These findings are of potential practical value, as they may serve as criteria to evaluate how RBCs will store through screening at time of donation.

In summary, a model emerges from the current studies in which lipid peroxidation is associated with poor 24-h recovery. Future experimental studies in mice will be required to test the functional relevance of the tocopherol-ascorbate-GSH axis and lipid peroxidation. Human studies will also be required to assess the extent to which the observations generated herein predict human RBC storage biology. The further resolution of these issues is a much needed step in advancing the ability to predict and control the quality of stored human RBCs.

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References

1. Dumont LJ, AuBuchon JP. Evaluation of proposed FDA criteria for the evaluation of radiolabeled red cell recovery trials. Transfusion. 2008;48(6):1053-1060.
2. Hess JR. Red cell changes during storage. Transfus Apher Sci. 2010;43(1):51-59.
3. Hess JR. Red cell storage. J Proteomics. 2010;73(3):368-373.
4. Brewer GJ. Genetic and population studies of quantitative levels of adenosine triphosphate in human erythrocytes. Biochem Genet. 1967;1(1):25-34.
5. Dern RJ, Wiorkowski JJ. Studies on the preservation of human blood. IV. The hereditary component of pre- and poststorage erythrocyte adenosine triphosphate levels. J Lab Clin Med. 1969;73(6):1019-1029.
6. van 't Erve TJ, Doskey CM, Wagner BA, et al. Heritability of glutathione and related metabolites in stored red blood cells. Free Radic Biol Med. 2014;76:107-113.
7. van 't Erve TJ, Wagner BA, Martin SM, et al. The heritability of metabolite concentrations in stored human red blood cells. Transfusion. 2014;54(8):2055-2063.
8. van't Erve TJ, Wagner BA, Martin SM, et al. The heritability of hemolysis in stored human red blood cells. Transfusion. 2015;55(6):1178-1185.
9. van 't Erve TJ, Wagner BA, Ryckman KK, Raife TJ, Buettner GR. The concentration of glutathione in human erythrocytes is a heritable trait. Free Radic Biol Med. 2013;65:742-749.
10. Zimring JC. Established and theoretical factors to consider in assessing the red cell storage lesion. Blood. 2015;125(14):2185-2190.
11. Fergusson DA, Hebert P, Hogan DL, et al. Effect of fresh red blood cell transfusions on clinical outcomes in premature, very low-birth-weight infants: the ARIPI randomized trial. JAMA. 2012;308(14):1443-1451.
12. Lacroix J, Hebert PC, Fergusson DA, et al. Age of transfused blood in critically ill adults. N Engl J Med. 2015;372(15):1410-1418.
13. Steiner ME, Ness PM, Assmann SF, et al. Effects of red-cell storage duration on patients undergoing cardiac surgery. N Engl J Med. 2015;372(15):1419-1429.
14. Qu L, Triulzi DJ. Clinical effects of red blood cell storage. Cancer Control. 2015;22(1):26-37.
15. Desmarets M, Cadwell CM, Peterson KR, Neades R, Zimring JC. Minor histocompatibility antigens on transfused leukoreduced units of red blood cells induce bone marrow transplant rejection in a mouse model. Blood. 2009;114(11):2315-2322.
16. Petkov PM, Ding Y, Cassell MA, et al. An efficient SNP system for mouse genome scanning and elucidating strain relationships. Genome Res. 2004 Sep;14(9):1806-1811.
17. Gilson CR, Kraus TS, Hod EA, et al. A novel mouse model of red blood cell storage and posttransfusion in vivo survival. Transfusion. 2009;49(8):1546-1553.
18. Uchida K. 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. Prog Lipid Res. 2003;42(4):318-343.
19. Arun P, Padmakumaran Nair KG, Manojkumar V, Deepadevi KV, Lakshmi LR, Kurup PA. Decreased hemolysis and lipid peroxidation in blood during storage in the presence of nicotinic acid. Vox Sang. 1999;76(4):220-225.
20. Karon BS, van Buskirk CM, Jaben EA, Hoyer JD, Thomas DD. Temporal sequence of major biochemical events during blood bank storage of packed red blood cells. Blood Transfus. 2012;10(4):453-461.
21. Knight JA, Searles DA, Blaylock RC. The effect of metal chelators on lipid peroxidation in irradiated erythrocytes. Ann Clin Lab Sci. 1992;22(6):417-422.
22. Knight JA, Voorhees RP, Martin L. The effect of metal chelators on lipid peroxidation in stored erythrocytes. Ann Clin Lab Sci. 1992;22(4):207-213.
23. Knight JA, Voorhees RP, Martin L, Anstall H. Lipid peroxidation in stored red cells. Transfusion. 1992;32(4):354-357.
24. D'Alessandro A, Blasi B, D'Amici GM, Marrocco C, Zolla L. Red blood cell subpopulations in freshly drawn blood: application of proteomics and metabolomics to a decades-long biological issue. Blood Transfus. 2013;11(1):75-87.
25. D'Alessandro A, Hansen KC, Silliman CC, Moore EE, Kelher M, Banerjee A. Metabolomics of AS-5 RBC supernatants following routine storage. Vox Sang. 2015;108(2):131-140.
26. D'Alessandro A, Nemkov T, Hansen KC, Szczepiorkowski ZM, Dumont LJ. Red blood cell storage in additive solution-7 preserves energy and redox metabolism: a metabolomics approach. Transfusion. 2015;55(12):2955-2966.
27. D'Alessandro A, Nemkov T, Kelher M, et al. Routine storage of red blood cell (RBC) units in additive solution-3: a comprehensive investigation of the RBC metabolome. Transfusion. 2015;55(6):1155-1168.
28. Roback JD, Josephson CD, Waller EK, et al. Metabolomics of ADSOL (AS-1) red blood cell storage. Transfus Med Rev. 2014;28(2):41-55.
29. Zimring JC, Smith N, Stowell SR, et al. Strain-specific red blood cell storage, metabolism, and eicosanoid generation in a mouse model. Transfusion. 2014;54(1):137-148.
30. Claro LM, Leonart MS, Comar SR, do Nascimento AJ. Effect of vitamins C and E on oxidative processes in human erythrocytes. Cell Biochem Funct. 2006;24(6):531-535.
31. Leonart MS, Weffort-Santos AM, Munoz EM, Higuti IH, Fortes VA, Nascimento AJ. Effect of vitamin E on red blood cell preservation. Braz J Med Biol Res. 1989;22(1):85-86.
32. Pallotta V, Gevi F, D'Alessandro A, Zolla L. Storing red blood cells with vitamin C and N-acetylcysteine prevents oxidative stress-related lesions: a metabolomics overview. Blood Transfus. 2014;12(3):376-387.
33. Stowell SR, Smith NH, Zimring JC, et al. Addition of ascorbic acid solution to stored murine red blood cells increases posttransfusion recovery and decreases microparticles and alloimmunization. Transfusion. 2013;53(10):2248-2257.
34. Silliman CC, Moore EE, Kelher MR, Khan SY, Gellar L, Elzi DJ. Identification of lipids that accumulate during the routine storage of prestorage leukoreduced red blood cells and cause acute lung injury. Transfusion. 2011;51(12):2549-2554.
35. Silliman CC, Bjornsen AJ, Wyman TH, et al. Plasma and lipids from stored platelets cause acute lung injury in an animal model. Transfusion. 2003;43(5):633-640.
36. Silliman CC, Voelkel NF, Allard JD, et al. Plasma and lipids from stored packed red blood cells cause acute lung injury in an animal model. J Clin Invest. 1998;101(7):1458-1467.