HHS Public Access

Author manuscript

Transfusion. Author manuscript; available in PMC 2020 January 01.

Published in final edited form as:

Transfusion. 2019 January ; 59(1): 89–100. doi:10.1111/trf.14979.

Heterogeneity of blood processing and storage additives in different centers impacts stored Red Blood Cell metabolism as much as storage time: lessons from REDS III – Omics

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Abstract

Background—Biological and technical variability has been increasingly appreciated as a key factor impacting red blood cell (RBC) storability and, potentially, transfusion outcomes. Here we performed metabolomics analyses to investigate the impact of factors other than storage duration on the metabolic phenotypes of stored RBC in a multi-center study.

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Authors' Contributions JAR, TN, RCH, SG, CZ, ADA performed metabolomics experiments and metabolomics data analysis. XF, MA and JCZ designed the experiments and performed metabolite extractions. TK, MTG, JG, GP, MG, SK, ML, MS, MB, JCZ and the REDS III RBC-Omics team designed the study, enrolled patients and supervised the REDS III Omics – Metabolomics study. ADA prepared all figures and ADA wrote the first draft of the manuscript. All co-authors contributed to final manuscript preparation.

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Disclosure of Conflict of interest ADA is a consultant for Hemanext Inc. TN and, ADA are founders of Omix Technologies Inc. JCZ serves on the scientific advisory board for Rubius Therapeutics. All the other authors disclose no conflicts of interest.

Study design and Methods—Within the framework of the REDS-III RBC-Omics study, 13,403 donors were enrolled from four blood centers across the United States and tested for the propensity of their RBCs to hemolyze after 42 days of storage. Extreme hemolyzers were recalled and donated a second unit of blood. Units were stored for 10, 23 and 42 days prior to sample acquisition for metabolomics analyses.

Results—Unsupervised analyses of metabolomics data from 599 selected samples revealed a strong impact (14.2% of variance) of storage duration on metabolic phenotypes of RBCs. The blood center collecting and processing the units explained an additional 12.2% of the total variance, a difference primarily attributable to the storage additive (AS-1 vs AS-3) used in the different hubs. Samples stored in mannitol-free/citrate-loaded AS-3 were characterized by elevated levels of high-energy compounds, improved glycolysis and glutathione homeostasis. Increased methionine metabolism and activation of the trans-sulfuration pathway was noted in samples processed in the center using AS-1.

Conclusion—Blood processing impacts the metabolic heterogeneity of stored RBCs from the largest multi-center metabolomics study in transfusion medicine to date. Studies are needed to understand if these metabolic differences influenced by processing/storage strategies impact the effectiveness of transfusions clinically.

Keywords

Mass spectrometry; metabolomics; blood storage; transfusion medicine

Introduction

Transfusion of packed red blood cells (RBCs) is the most common invasive medical procedure worldwide, one that helps save millions of lives every year by restoring adequate tissue oxygenation in patients needing acute or chronic intervention. Massively or chronically transfused recipient categories include patients suffering from traumatic injuries (with or without hemorrhagic shock), cardiovascular disease (e.g. perioperative bleeding during cardiac surgery) or cancer patients undergoing chemotherapy/radiotherapy. Storage in the blood bank makes it possible to logistically handle both constant and sudden demands of RBCs and other blood products for rapid transfusion.

Refrigerated liquid storage of RBCs in blood banks has improved over the past ~100 years to achieve the current high standard of practice.[1] However, cold liquid preservation of packed RBCs is a non-physiological process that exposes erythrocytes to low temperatures and storage solutions loaded with supraphysiological concentrations of additives (e.g. glucose, phosphates, and either mannitol or citrate in AS-1 and 3, respectively).[2] As a result, RBC storage induces a series of biochemical and morphological alterations collectively referred to as the storage lesion(s),[3] a phenomenon that ultimately compromises the cells' capacity to generate high energy phosphate compounds (such as adenosine triphosphate – ATP) and cope with oxidative stress.

Ten years ago, a provocative series of retrospective studies suggested that the storage lesion(s) may negatively impact transfusion outcomes in certain categories of recipients.[4]

Since then, omics technologies have helped to elucidate the complexity of the storage lesion, especially through the use of metabolomics, which provides a comprehensive overview of RBC metabolism.[5] While limited in the number of biological replicates and conditions tested in a single study, metabolomics investigations have flooded the literature over the past few years in the attempt to document the effect of different storage additives (SAGM, AS-1, AS-3, AS-5, AS-7, PAGGSM, PAG3M, ESOL-5),[6–13] rejuvenation procedures,[14] anaerobic storage conditions,[15–18] temperature effects (>4°C[19] or cryopreservation[20]) or novel storage additives (e.g. supplementation with adenine, alternative sugars or antioxidants).[21–23] Tracing experiments have provided insights into fluxes through key antioxidant pathways[15] or previously unappreciated metabolic reactions in stored RBCs, such as those involving citrate and other carboxylates,[13,24] expanding our understanding of RBC biology in general. In addition, systems biology elaboration of metabolomics data has contributed a mathematical tool to identify three metabolic phases during routine storage,[25] which can be used to test and predict RBC storability in new storage additives *in silico*.[26] While the clinical relevance of the metabolomics studies of RBC storage is still a matter of debate, metabolomics studies in animal models and preliminary evidence in humans suggest that the severity of the metabolic lesion does indeed correlate with hemolysis *in vitro*[27,28] and post-transfusion recovery,[15,29–31] the two gold standards for the determination of RBC storage quality according to Food and Drug Administration standards.[32]

Recent prospective randomized clinical trials (RCTs) addressing the issue of the "age of blood"[33–37] have provided reassuring evidence to conclude that current standards of practice are non-inferior to the exclusive transfusion of the freshest units available. Notably, none of the RCTs tested the impact on transfusion outcomes of units at the very beginning of storage vs units at the end of storage (last week of shelf-life) owing to ethical concerns.[38] Therefore, the discussion regarding the age of blood has been fueled again by the publication of observational clinical evidence about an effect of transfusion of blood stored >35 days on circulating levels of non-transferrin bound iron and clinical outcomes in high-risk recipients.[39,40] A secondary analysis of the INFORM clinical trial data which had enough data to evaluate the effect of RBCs stored >35 days did not detect differences in in-hospital mortality between patients transfused with at least one unit older than 35 days, and patients transfused with units less than 8 days; however, other clinical outcomes were not evaluated in this trial.[41] The apparent disconnect between studies could possibly in part be explained by the role that biological variability (both of donors and recipients) and processing strategies play on the development of the storage lesion and clinical outcomes.[42]

To investigate the impact of donor demographic and genetic variability on metabolic phenotypes of stored RBCs in a large prospective cohort, four blood collection centers involved in the REDS-III RBC-Omics Study enrolled ~14,000 donors of different race-ethnicities, gender and age to determine how donor biology impacts spontaneous and stress-induced hemolysis of RBC *in vitro* after 42 days of storage.[43] Donors whose RBCs were at the extreme end of storage hemolytic phenotypes were recalled for a second donation, from which RBCs were manufactured according to standard operational procedures at each blood center and then stored under routine blood bank conditions and sampled at storage days 10, 23 and 42 to test for spontaneous, oxidative, osmotic and mechanical hemolysis *in vitro*. Metabolomics analysis was performed on a total of 599 samples from 250 recalled donors.

As a result, we are reporting the outcome of the largest to date multi-center metabolomics study of RBC stored under blood bank conditions. Results confirm and expand on the previous observations about the progressive accumulation of RBC metabolic changes during storage. While evaluation of the impact of donor biology (age, sex, ethnicity) and correlations of metabolomic findings with hemolytic phenotypes is beyond the scope of this preliminary analysis, here we report a significant impact of RBC collection/processing strategies associated with the hubs where the units were processed and stored, a phenomenon primarily explained by differences in storage additives. In light of these results, we comment on the need for physiological studies and *in vivo* RBC transfusion outcomes analyses that evaluate the effectiveness and safety of blood units while taking into account differences in processing methods and additive solutions.

Materials and Methods

REDS-III Omic

Donor selection criteria and recruitment procedures for RBC-Omics were previously described by Kanias et al[43] Donors were enrolled at the American Red Cross (ARC - Farmington, CT), the Institute for Transfusion Medicine (ITxM - Pittsburgh, PA), Blood Center of Wisconsin (BCW - Milwaukee, WI), and Blood Centers of the Pacific (BCP/BSRI - San Francisco, CA). Of 14,520 consenting donors, 13,770 (95%) were classified as fully enrolled after successfully donating a whole blood unit. Of those, 97% (13,403) were fully evaluable for hemolysis parameters. Detailed information about donor enrollment protocols, donor demographics, blood collection and processing strategies is provided in Endres-Dighe et al. in this issue of Transfusion.

Recall Samples:

RBC-Omics donors with either low or high hemolysis (top or bottom 5%) results on 4ºC stored leukocyte-reduced (LR)-RBC samples from enrollment donations stored for 39–42 days were recalled 2–12 months later to donate LR-RBCs, as detailed in Lanteri et al. in this issue of Transfusion. Each RBC component was filtered to generate a LR-RBC unit in additive solution-1 or 3 (CPD/AS-1 in BCW or CP2D/AS-3 in the other three centers). Three blood centers (BCP, BCW, and ARC) performed pre-storage LR immediately after the RBC-component was manufactured. One blood center (ITxM) delayed LR until after negative donor screening results were received and until after the RBC unit was transferred to the central transfusion service, which was generally 48–72 hours after collection. Samples of stored RBCs from the unit and from transfer bags were evaluated for spontaneous and stress-induced hemolysis at selected time points. 664 donors were successfully recalled. A subset of the recalled donors were selected for metabolomic analysis at Day 10 (250 donor samples), Day 23 (173 samples) and Day 42 (176 samples - figure 1B).

Sample processing and metabolite extraction

Commercial reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. An isotopically labeled internal standard mixture including a mix of $^{13}C^{15}N$-labeled amino acid standards (2.5μM) was prepared in methanol. [30] RBCs were separated by centrifugation (10 min at 4ºC and 2500 *g*) then 100 μL was mixed with water and the

mixture of isotopically labeled internal standards (1:1:1, vol/vol/vol).[30] The samples were extracted with methanol (final concentration of 80% methanol). After incubation at −20°C for 1hr, the supernatants were separated by centrifugation and stored at −80°C until analysis.[44,45]

UHPLC-MS metabolomics

Analyses were performed using a Vanquish UHPLC coupled online to a Q Exactive mass spectrometer (Thermo Fisher, Bremen, Germany). Samples were analyzed using a 3 minute isocratic condition[46] or a 9 min gradient as described.[45] Solvents were supplemented with 0.1% formic acid for positive mode runs and 1 mM ammonium acetate for negative mode runs. MS acquisition, data analysis and elaboration was performed as described.[45,46] Additional analyses, including untargeted analyses and Fish score calculation via MS/MS were calculated against the ChemSpider database with Compound Discoverer 2.0 (Thermo Fisher, Bremen, Germany). Graphs and statistical analyses (either t-test or repeated measures ANOVA), principal component analysis (PCA) or partial least-square discriminant analyses (PLS-DA) were prepared with GraphPad Prism 5.0 (GraphPad Software, Inc, La Jolla, CA) and MetaboAnalyst 3.0.

Results

From blinded to unblinded through unsupervised analyses

Metabolomics analyses were performed on 599 frozen RBC samples processed at day 10, 23 or 42 of storage from LR-RBC units collected from 250 recalled RBC-Omics donors at four different blood centers in the United States (Figure 1.A-B). Metabolomics workflow and procedures were extensively described and reviewed (Supplementary Figure 1).[5] Sample identifiers for snap-frozen aliquots selected for metabolomics analysis were blinded and randomized by the REDS-III central laboratory (BSRI) prior to shipping to Bloodworks Northwest in Seattle, where the samples were extracted and the extracts sent to the University of Colorado Denver by overnight shipping on dry ice. Once received in Denver, samples were randomized a second time prior to analysis via UHPLC-MS.

Unsupervised hierarchical clustering analyses (HCA) and principal component analyses (PCA) were performed on targeted and untargeted metabolomics results generated in Denver (Figure 1.C and 2.A, Supplementary Figures 2 and 3). HCA identified three main clusters: compounds informing this discrimination are highlighted in Supplementary Figure 2, and include metabolites involved in energy homeostasis (ATP, ADP, ADP-ribose, Glucose, Fructose bisphosphate, etc), carboxylates (citrate, oxaloacetate), and metabolic markers of the age of blood (lactate, hypoxanthine, 5-oxoproline).[25] Based on the latter group of metabolites (for which absolute quantities were determined against stable isotope labeled internal standards – Supplementary Figure 3), we predicted that the three main clusters grouped samples on the basis of storage day (Figure 2.B). A total of 242, 180 and 177 samples were predicted as samples at storage day 10, 23 and 42 (Figure 1.C). Once unblinded, we noted that only 8 samples were misclassified in the day 23 group and one at day 42, for a total of 590 correct predictions out of 599 samples (98.5% accuracy – Figure 2.B).

In addition to the identification of three main clusters, sub-clusters including 102 and 54 samples were noted at days 10 and 23 (Figure 1.C). This phenomenon was even more evident in HCA graphs of the results from unsupervised analyses (based on ~200,000 features), clearly showing an impact of factors other than storage time on >40% of the total features monitored in this study (Supplementary Figure 3). Although PCA clearly revealed a significant effect of storage time on metabolic phenotypes of stored RBCs, explaining 14.5% of the total variance across PC1 (Figure 2.A), an additional 12.2% of variance was explained by PC2, which was apparently affected by factors other than storage duration (Figure 2.B). Once unblinded to information about donors and day of RBC storage, PLS-DA analyses revealed that PC2 explained 10.5% of the total variance, suggesting that factors other than storage duration alone (9.1% of the variance) impacted the metabolic phenotypes of the samples tested here (Figure 1.D shows a 3D representation, while Figure 2.B illustrates the interactions of the top 5 PCs on 2D projections).

Metabolic markers of the RBC storage age – revisited

While limited in the number of time points tested, the present study offers a unique opportunity to expand our understanding of the metabolic effects of RBC storage. Trends observed here are consistent with previous studies[6–13,24] and identified increases with prolonged storage time in hypoxanthine (lowest FDR-corrected p-value: 1.21 e-217 of increasing metabolites), 5-oxoproline, lactate, homocysteine and hydroxisourate and decreases in methionine (lowest FDR-corrected p-value: 1.52 e-75 of decreasing metabolites), glutamine, ATP, adenine, and 2,3-DPG as the most significant metabolic changes by ANOVA (Figure 3.A), with >100 metabolites showing FDR-corrected p-values < 7 e-05 (Supplementary Table 1). A series of fatty acids and oxylipins, (including eicosapentaenoic acid, icosapetntaenoic acid, docosapentaenoic acid, docosahexaenoic acid, eicostatraenoic acid, icosatrieonoic acid), all increased significantly over storage duration and made it to the top 50 significant metabolites by ANOVA (Figure 3.B). Of note, relative and absolute quantitative measurements for metabolites including 5-oxoproline, methionine, lactate, glutathione glutamine, fructose bisphosphate, glucose, 2,3-DPG clustered together in the HCA in Figure 3.B, further strengthening the significance of the observation and providing reassurance of the reliability of relative quantitative measurements.

Processing hub identified as a main factor impacting metabolic heterogeneity of REDS III RBC-Omics samples

In an attempt to identify the main factors informing the discrimination of REDS III RBC-Omics samples across PC2 in PCA (Supplementary Figure 5.A provides a 3D representation), samples were classified on the basis of the storage day and the hub that processed the units, and the statistical analyses repeated as a two-factor (time series + hub) analysis via MetaboAnalyst. Of note, PCA revealed a clear effect of processing hub on RBC metabolic heterogeneity (Figure 4.A). ANOVA revealed that the top factor contributing to this clustering was the storage additive used in each hub (AS-3 at ARC, BSRI and ITxM and AS-1 at BCW). Metabolites like citrate and diphosphates (higher in AS-3) or mannitol (absent in AS-3) were identified as key discriminant metabolites across groups (to present results in a readable format, Figure 4.B and subsequent figures show line plots for 25 representative samples in each group). HCA of metabolic phenotypes based on the

combined effect of storage day, hub and storage additives clearly shows that metabolic heterogeneity was attributable to AS-1 (Figure 4.C). AS composition promoted stark differences across hubs in the levels of several metabolites involved in purine metabolism/oxidation (Figure 4.D), glycolysis and carboxylate metabolism (Figure 5), glutathione and sulfur homeostasis (Figure 6), citrate metabolism (Figure 7) and fatty acid/signaling lipid homeostasis (Supplementary Figure 5.B)

Metabolic pathways impacted as function of processing Hubs

Metabolomics analyses revealed a center-dependent impact on purine oxidation – partly explained by the formulation of storage additives – with decreased levels of ATP, ADP, IMP and increased levels of adenosine and purine oxidation byproduct urate (but not hydroxyisourate) in samples stored in AS-1 (Figure 4.D). No significant differences in the levels of hypoxanthine were noted across hubs (Supplementary Figure 5). This observation is in part explained by decreased glucose and hexose phosphate and fructose bisphosphate levels in the same subset of samples stored in AS-1, which were however characterized by higher levels of late trioses and triose phosphates (glyceraldehyde 3-phosphate and lactate – Figure 5), suggestive of heterogeneity in glycolytic phenotypes across samples. The same AS-1 group of samples was characterized by significantly lower levels of glutamine, gamma-glutamyl-cysteine (a precursor to glutathione), dehydroascorbate and lactoyl-glutathione, and higher levels of 5-oxoproline, S-adenosyl-methionine and S-adenosyl-homocysteine – suggestive of a differentially regulated glutathione homeostasis and trans-sulfuration pathway in this group in comparison to blood processed at other hubs (Figure 6). AS-1 stored RBCs showed significantly lower levels of free fatty acids, pyruvoyl-tetrahydropterin (a guanosine metabolism product) and the highest levels of sphingosine 1-phosphate (Supplementary Figure 5). Samples processed at ITxM - which had leukoreduction performed several days after collection whereas the other three hubs performed pre-storage leukoreduction - had the highest levels of a sulfur-containing compound taurine and the short odd-chain fatty acids heptanoic acid (used to ease the formation of methyl-ester in the industrial processes that generate phthalates[47]) and 9-oxonanoic acid (a common water soluble ketoacid[48] - Supplementary Figure 5). Finally, since mannitol free AS-3 is loaded with citrate, we focused on carboxylate metabolism[13,16,24] in RBCs from different hubs. Samples stored in AS-1 were characterized by lower levels of citrate (absolute and relative quant graphs are shown in Figures 4.B and 7, respectively), oxaloacetate, homoaconitate and acyl-carnitine C6-DC (Figure 7). The same samples were instead characterized by higher levels of malate and fumarate – byproducts of reactions preserving NADH homeostasis and purine salvage in the presence of aspartate (an almost completely inactive pathway in RBCs[31]).

Discussion

In this study, we report observations from an extensive metabolomics analysis of stored packed RBCs (599 samples tested from 250 recalled RBC-Omics donors) from four different centers across the United States. First, previously reported markers of the metabolic age of stored RBCs[25] were confirmed. Second, additional markers were identified with methionine and sulfur metabolism (specifically S-adenosyl-methionine and S-adenosyl-

homocysteine) emerging as significant variables informing storage time and additive solution-specific clustering. Although this and other studies have pointed out the potential relevance of storage-induced methionine consumption and activation of the trans-sulfuration pathway in relation to purine homeostasis and polyamine synthesis,[8,21,24] further studies will be necessary to mechanistically assess the relevance of this pathway.

Most importantly, we noted a substantial metabolic heterogeneity across blood centers using different storage additives and/or processing workflows. In particular, PCA clearly revealed that these factors are as impactful as storage duration with respect to metabolic heterogeneity. This observation is relevant in the light of the increased awareness about the role of biological/processing variability across transfused units and their potential impact on transfusion outcomes.[42]

Pathways affected by storage additives were consistent with previous reports on the metabolic differences between AS-1 and AS-3 in paired[13] or independent studies.[8,9,24] AS-3 is mannitol free and is loaded with citrate to compensate for osmolarity. Citrate is a carboxylic acid that can be metabolized by RBCs despite their lack of mitochondria.[13,16,24,49] Metabolism of citrate into malate, lactate and oxaloacetate has been extensively documented through metabolic tracing with deuterated or ^{13}C-tracers;[13,16,24,49] our study suggests a role for citrate metabolism in fueling homoaconitate via ketoglutarate and homocitrate intermediates.

Sphingosine 1-phosphate plays a key role in RBC responses to hypoxia.[50] Previous studies have reported storage-induced decreases in sphingosine 1-phosphate,[24,51] a finding here recapitulated in all tested samples except from those coming from units stored in AS-1.

Altered energy metabolism was noted in units processed in the hub using AS-1, as inferred from lower levels of high energy phosphate compounds (including ATP and ADP) and differential levels of hexose and triose (phosphate) compounds. Since energy homeostasis is intertwined with redox homeostasis (e.g. glutathione synthesis is an ATP dependent process), it is interesting to note an impairment of glutathione homeostasis and sulfur metabolism (especially methionine catabolism) in the very same group of samples that show dysregulated energy homeostasis.

Previous smaller scale (in comparison to RBC-Omics) controlled studies on twins have shown a donor-dependency of energy and redox homeostasis.[27,28] Our larger study shows that these same metabolic pathways are significantly impacted by the formulation of storage additives in a manner that goes beyond the direct impact of the unique components in the AS (e.g. mannitol vs citrate in AS-1 vs AS-3). Appreciation of the influence of processing and storage additives on the metabolic heterogeneity of stored RBCs in a multi-center study of this scale raises the possibility that this and other manufacturing variations may confound results from randomized clinical trials addressing, for example, the clinical impact of days of in-vitro RBC storage. Metabolites deriving from processing workflows (e.g. odd-chain fatty acids and dicarboxylates derived from industrial processes to generate methyl-esters for the production of phthalates) were here associated to specific hubs, suggesting that even industrially controlled processes such as the production of storage bag plasticizers may

inadvertently impact the final heterogeneity of the manufactured RBC (which, again, does not necessarily imply an impact on its safety and efficacy).

A limitation of this study is that it did not address the impact of other variables (e.g., donor sex, age, ethnicity, and prior donation frequency) that are recognized to correlate with hemolysis. [43,52] Future studies will address the correlations of the present findings with those variables, as well as the propensity to hemolyze in several *in-vitro* hemolytic assays and with donor genetics. Analyses are also planned to determine the *in vivo* consequences of these findings following transfusions of RBC components from RBC-Omics donors into recipients represented in the REDS-III linked donor-recipient database.

Conclusion

In this manuscript, we report a data-driven analysis of metabolomics results from stored RBC samples from the REDS III RBC-Omics study. A total of 599 samples generated from 250 donors in 4 different blood centers were processed and analyzed, making this the largest multi-center metabolomics study of stored RBCs to date. Unsupervised analyses of metabolomics data revealed that hub/processing/additives significantly impact the metabolic heterogeneity of stored RBCs, to an extent comparable to storage duration. Pathways impacted by hub/processing/additives included glycolysis, purine (including ATP) and glutathione homeostasis, carboxylate and sulfur metabolism; these pathways have been previously shown to depend on the storage age and donor variability. Appreciation of the metabolic heterogeneity of stored RBCs and the relevance of processing strategies/additives does not necessarily imply any impact on transfusion outcomes. Future studies will be necessary to investigate whether variables such as processing/storage additives may have masked the impact of age of blood on transfusion outcomes in recent randomized clinical trials. This study further stresses the opportunity to further improve standardization of blood product processing[42] and the formulation of storage additives. Finally, this study demonstrates the relevance of metabolomics technologies in transfusion medicine and the potential transformative impact that data generated through this technology can have when correlated with transfusion outcomes, as recommended by recent meetings on the state of science in transfusion medicine sponsored by the National Heart, Lung, and Blood Institute and the Food and Drug Administration.[32,53]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research reported in this publication was supported in part by funds from the Boettcher Webb-Waring Investigator Award (ADA), the Shared Instrument grant by the National Institute of Health (S10OD021641 to KCH). Trainee support was provided by NIH T32 HL007171 (TN). The NHLBI Recipient Epidemiology and Donor Evaluation Study-III (REDS-III) was supported by NHLBI contracts NHLBI HHSN2682011–00001I, −00002I, −00003I, −00004I, −00005I, −00006I, −00007I, −00008I, and −00009I. The authors would like to express their deep gratitude to the RBC-Omics research staff at all participating blood centers and testing labs for their contribution to this project, and to all blood donors who agreed to participate in this study. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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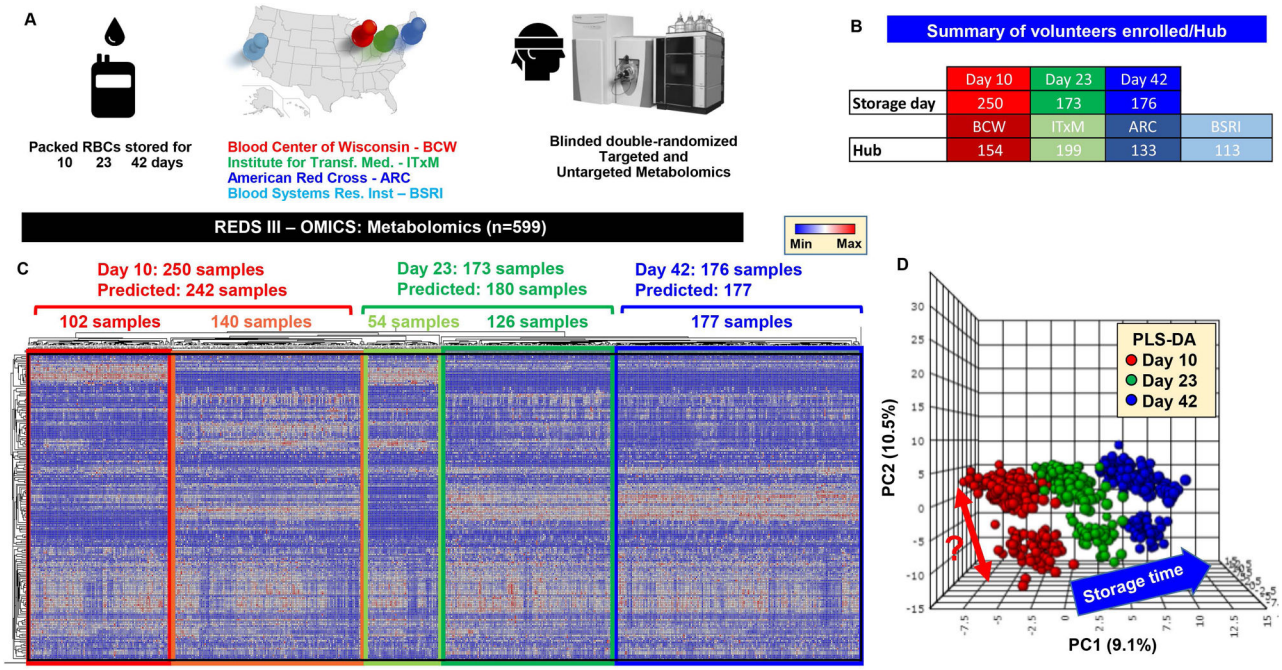

Figure 1 - Metabolomics analyses of REDS III samples.

In A, an overview of the study design. In B, a breakdown of the number of patients enrolled in this arm of the study at four different blood centers (BCW: Blood Center of Wisconsin; ITxM: institute for Transfusion Medicine, Pittsburgh; ARC: American Red Cross at Yale; BSRI: University of California at San Francisco and Blood Systems Research Institute). In C, the heat map shows the results from unsupervised hierarchical clustering of 599 samples on the basis of metabolic phenotypes. A total of 242, 180, and 177 samples were predicted to belong to storage day 10, 23 and 42 categories, with only 8 samples misclassified as storage day 23 actually belonging to the day 10 and day 42 groups. Upon unblinding, partial least square discriminant analysis was performed in D, showing sample clustering on the basis of storage time across principle component 1 (explaining 9.1% of the total variance). Of note, a clear subcluster was observed across PC2 (10.5% of the total variance. Color code: Red: day 10 samples; Green: day 23 samples; Blue: day 42 samples.

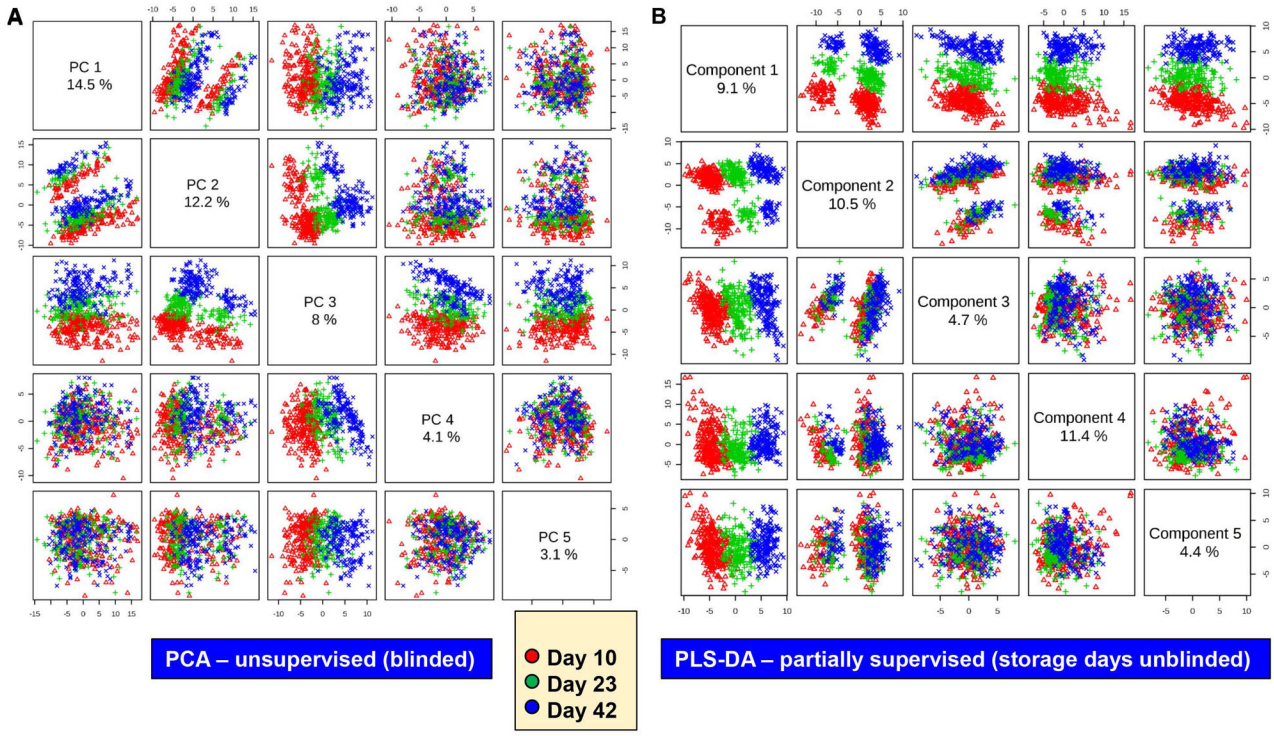

Figure 2 - Multivariate analysis of metabolomics data discriminates two major variables in RBC-Omics samples,

as determined by unsupervised principal component analysis (PCA) and partial least square discriminant analysis. One of the major variables was immediately identified as the storage age (Color code: Red: day 10 samples; Green: day 23 samples; Blue: day 42 samples).

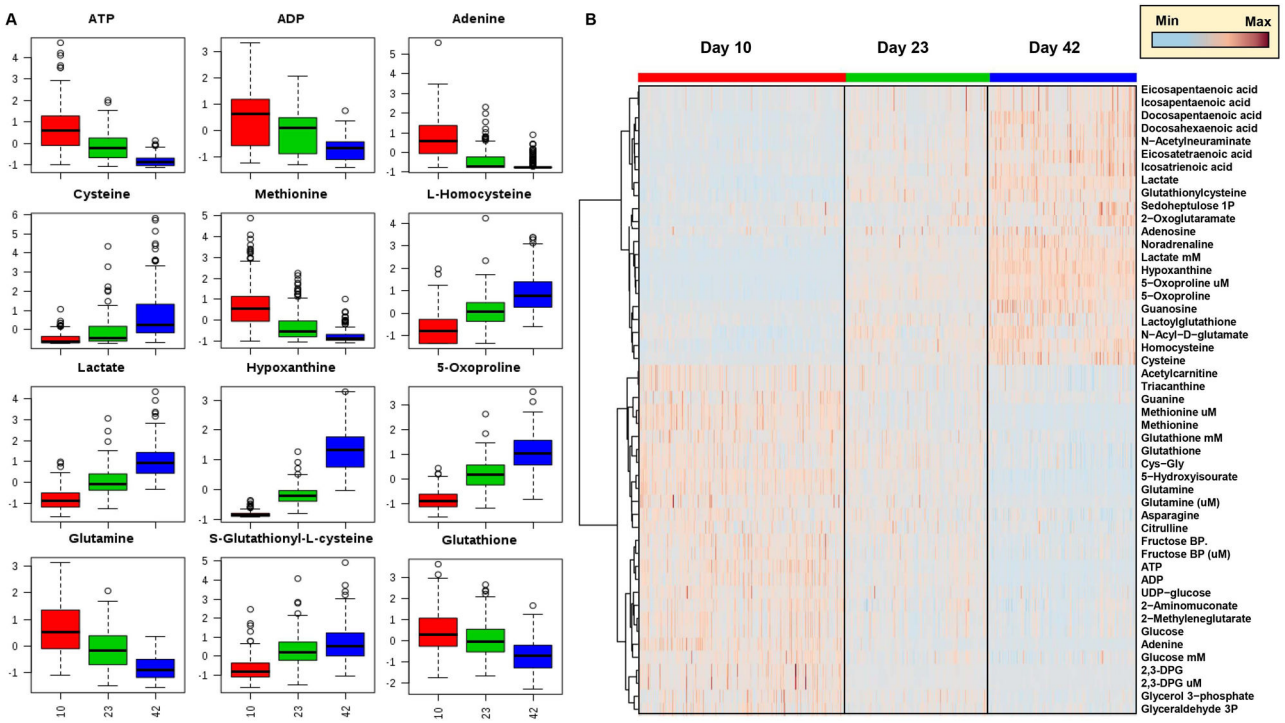

Figure 3 - Top 50 metabolites affected by RBC storage age in RBC-Omics samples, as shown by box and whisker plots and heat map (blue to red: low to high). Color code for sample grouping: Red: day 10 samples; Green: day 23 samples; Blue: day 42 samples. In B, relative and absolute quantitative measurements for most metabolites clustered together, providing additional confidence about the reproducibility of the observation.

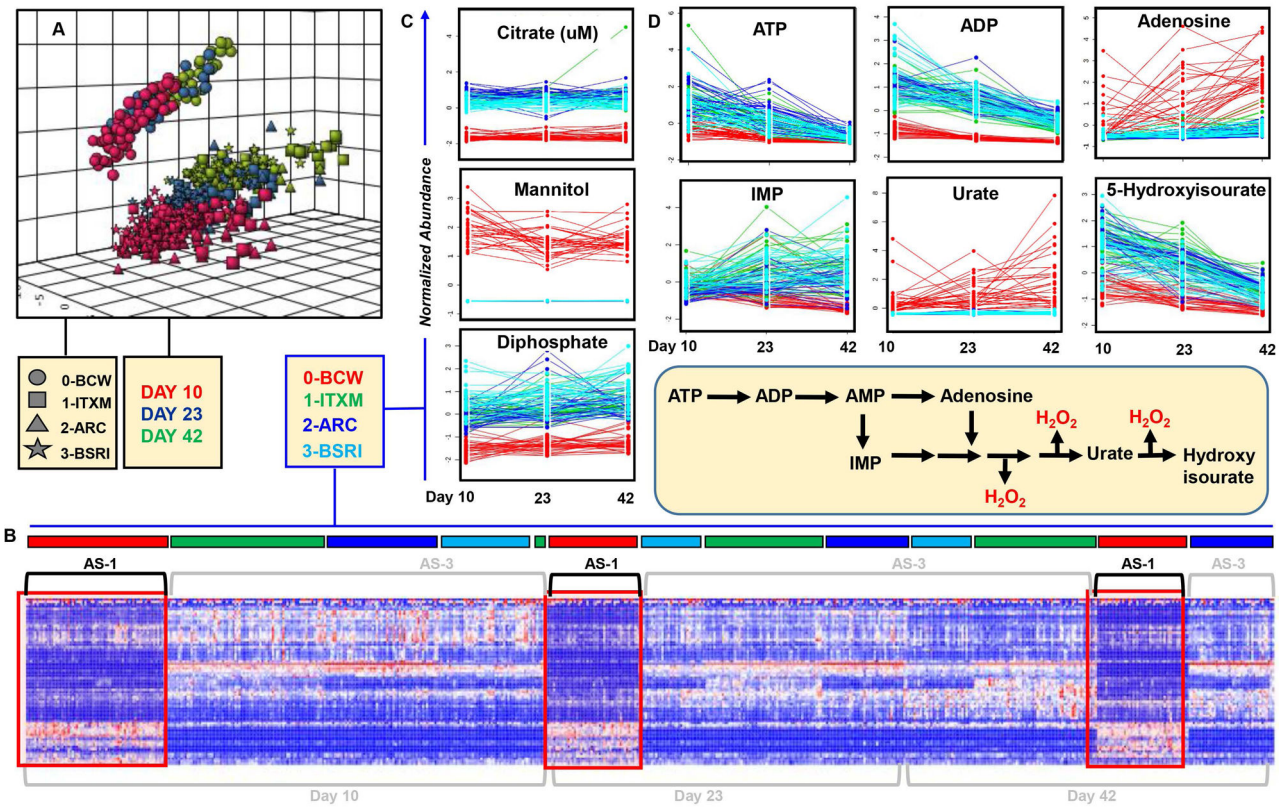

Figure 4 - The effect of Hub collection/processing and storage additives on RBC-Omics samples clustering on the basis of metabolomics data.

In A, unsupervised principal component analysis reveals that the main factor contributing to sample clustering across PC2 is the Hub where the units were collected/processed (each shape indicates a different center, according to the legend on the bottom left corner of panel A; colors from red, blue and green indicate storage day 10, 23 and 42, respectively). In B, the heat map reveals metabolites differing specifically in the BCW hub (red) in comparison to the other hubs (ITXM: green; ARC: dark blue; BSRI: light blue) independently of the storage age and entirely explained by the different storage additive (AS-1) adopted at BCW for the units tested in this study. In C and D, line plots indicate time dependent changes for metabolites derived from storage additives (C) or purine oxidation/catabolism (D), according to the color scheme in the bottom right corner of panel A.

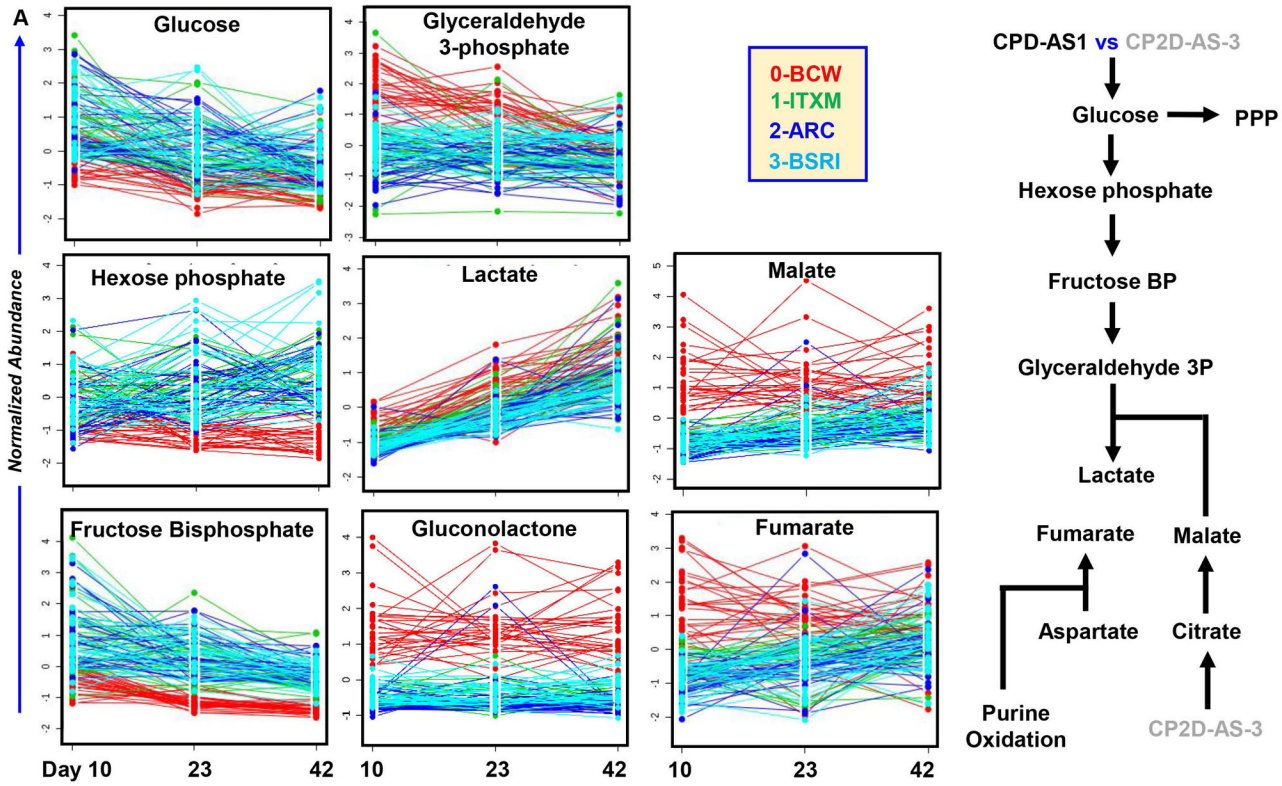

Figure 5 - Heterogeneity in glycolysis and carboxylate metabolism in RBC-Omics samples as a function of processing Hubs.

Results are graphed as line plots that indicate time dependent changes for named metabolites according to the color scheme in the top right corner. An overview of the main pathways relevant to this figure is provided on the right hand side of the figure.

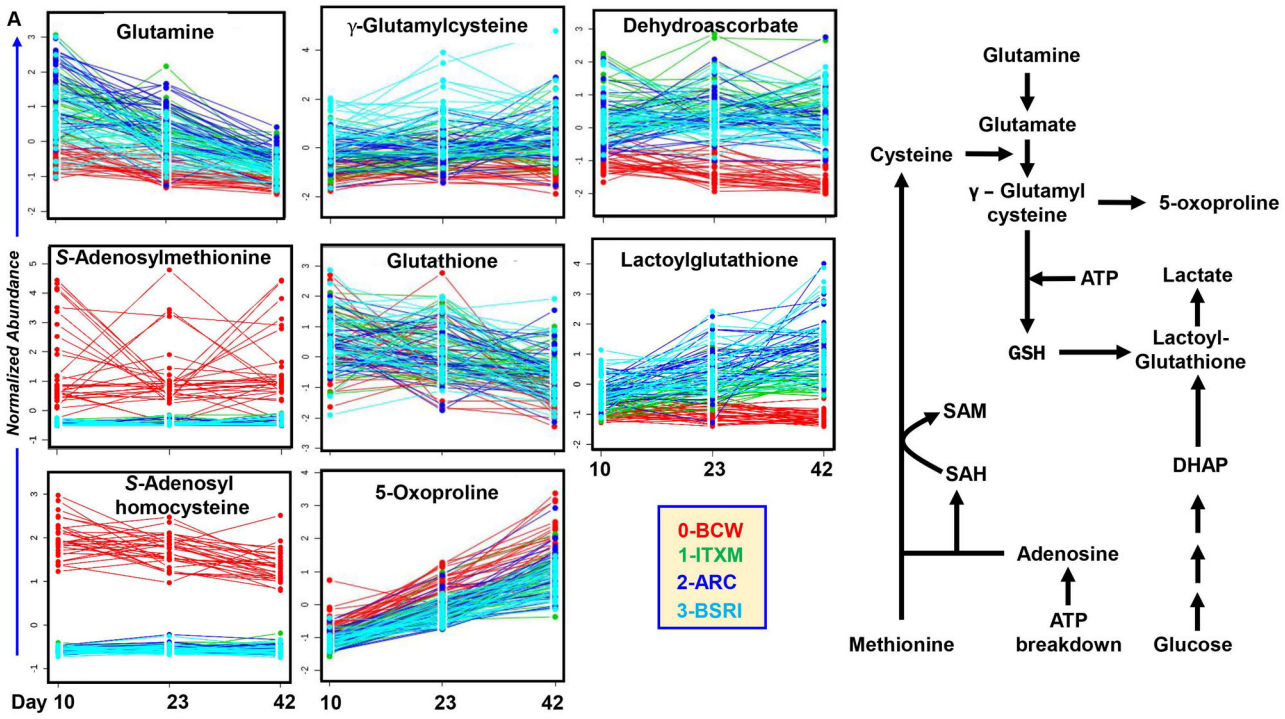

Figure 6 - Heterogeneity in glutathione homeostasis and sulfur metabolism in RBC-Omics samples as a function of processing Hubs.

Results are graphed as line plots that indicate time dependent changes for named metabolites according to the color scheme in the bottom right corner. An overview of the main pathways relevant to this figure is provided on the right hand side of the figure.

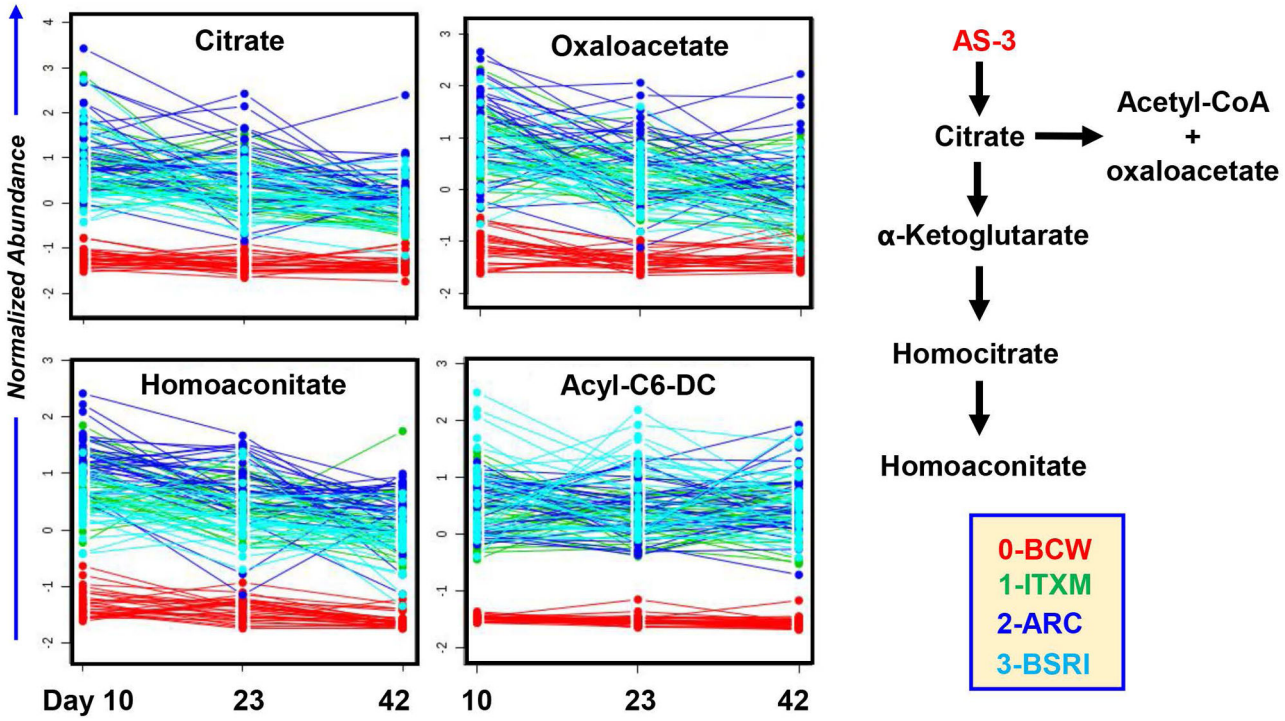

Figure 7 - Heterogeneity in citrate metabolism in RBC-Omics samples as a function of processing Hubs.

Results are graphed as line plots that indicate time dependent changes for named metabolites according to the color scheme in the top right corner. An overview of the main pathways relevant to this figure is provided on the right hand side of the figure.