Blood-storage duration affects hematological and metabolic profiles in patients with sickle cell disease receiving transfusions

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Conflicts of Interest: AD is a founder of Omix Technologies Inc and Scientific Advisory Board member for Macopharma Inc and Synth-Med. AD and SLS are Scientific Advisory Board members for Hemanext Inc. SLS is a Scientific Advisory Board member of Alcor, Inc. MSK is a paid consultant for Westat, Inc. RMF serves on a medical advisory board for Pfizer and Cerus, and has received research funding from Cerus; he also serves as a consultant for REDSIV-P which is funded by the NIH/NHLBI. JJF has received honoraria from Bayer, and receives research funding from FORMA Therapeutics, Shire/Takeda, and Rigel. JAL receives research support from Pfizer, Novo-Nordisk, and the American Society of Hematology. The remaining authors declare no competing financial interests.

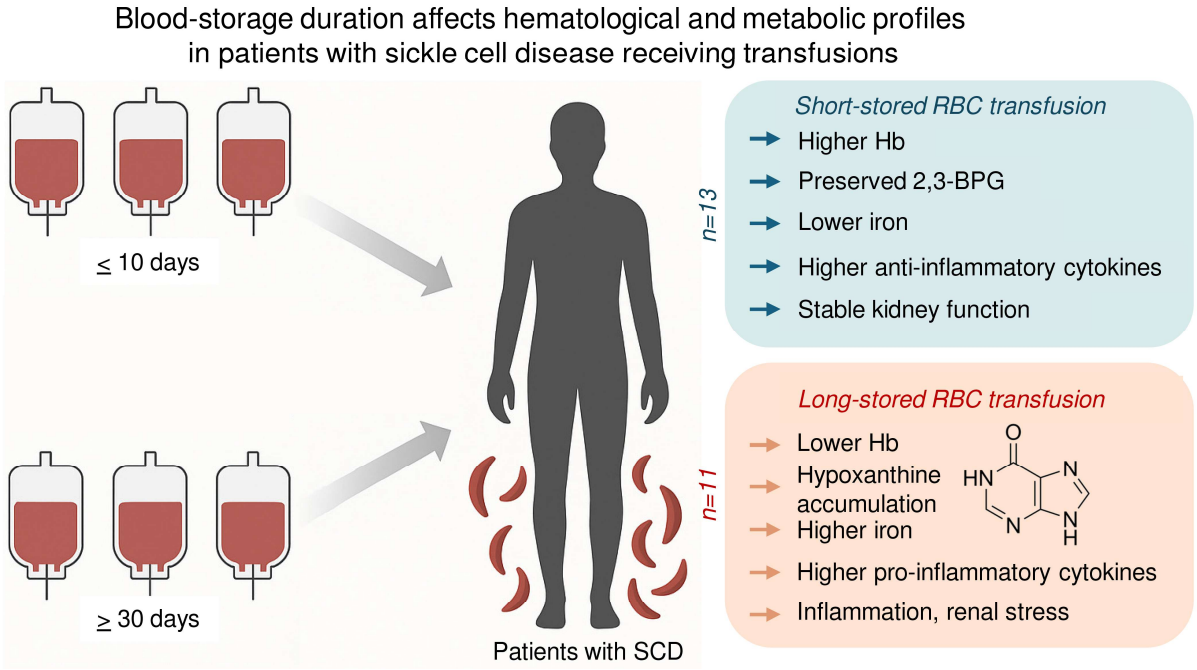
Running title: *Metabolic effects of blood storage duration in patients with sickle cell disease*
Word count: 4210
Figures: 7

Abstract

Patients with sickle cell disease (SCD) frequently receive red blood cell (RBC) units stored near the end of their permissible storage life. To evaluate whether storage duration influences recipient metabolism, clinical chemistry and hematological parameters, we conducted a prospective, randomized, blinded trial comparing transfusions of RBC units stored for ≤10 days versus ≥30 days.

Chronically transfused adults with SCD (N=24) received three consecutive outpatient transfusions with randomized-age RBCs, and blood samples from units and recipients were analyzed by metabolomics and clinical chemistry. Transfusion of short-stored units resulted in significantly higher circulating levels of 2,3-bisphosphoglycerate, an essential regulator of oxygen unloading, up to two weeks post-transfusion. Conversely, transfusions of long-stored RBCs were associated with lower hemoglobin and RBC increments, higher iron and transferrin saturation, pro-inflammatory cytokines and metabolites, oxidative stress and markers of renal dysfunction. Plasma and RBC metabolomic profiles revealed time- and storage-age-dependent alterations, particularly affecting glycolysis, purine, and sphingolipid metabolism. Transfusion of long-stored RBCs consistently worsened laboratory surrogates of oxygen delivery and RBC efficacy, and increased the circulating levels of immunomodulatory metabolites and pro-inflammatory cytokines. These findings highlight metabolic and hematologic advantages associated with transfusing fresher RBCs in adults with SCD, independent of immediate clinical outcomes.

Keywords: Metabolism, Blood, Hypoxanthine, Lactate, Hemoglobin, Iron, Sickle Cell Disease



Introduction

Over a century since its routine introduction into medical practice (1), blood transfusion remains one of the most common medical procedures, with over 11 million red blood cell (RBC) units collected annually in the United States alone (2). RBC units can be refrigerator-stored for up to 42 days, providing critical logistical advantages. However, extended storage induces progressive biochemical, metabolic, and morphological changes collectively termed the “storage lesion” (3). These storage-associated metabolic perturbations include slowed enzyme kinetics (4), disrupted electrolyte gradients (sodium and calcium influx, potassium efflux (5)), and diminished ATP synthesis despite supra-physiological glucose concentrations in modern storage solutions (6).

Specifically, glycolytic flux is markedly impaired (7), leading to lactate accumulation and intracellular acidification (8), further limiting glycolytic efficiency and ATP regeneration (9). Notably, critical metabolic intermediates, such as 2,3-bisphosphoglycerate (2,3-BPG (10)), rapidly decline within two to three weeks (11), impairing the RBCs' capacity to deliver oxygen effectively. Simultaneously, adenylate pools shift toward adenosine monophosphate (AMP), which is subsequently metabolized to hypoxanthine (12, 13), a biomarker closely associated with increased susceptibility to extravascular hemolysis following transfusion (3, 4, 12, 14, 15).

The reduction of 2,3-BPG not only impairs oxygen delivery but also promotes oxidative stress by increasing oxygen retention within hemoglobin molecules, facilitating Fenton and Haber-Weiss reactions in iron-rich mature RBCs. Although RBCs possess multiple antioxidant systems, persistent storage conditions overwhelm these defenses, leading to oxidative modifications of critical enzymes (16), such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (17). These oxidative modifications redirect glucose metabolism from glycolysis to the pentose phosphate pathway (PPP (18)), impacting the availability of NADPH and reducing antioxidant defenses (19), especially glutathione (GSH) (5). Reactive oxygen species accumulate alongside reactive metabolites like methylglyoxal (20) and lipid peroxidation products (5), causing irreversible protein modifications (21, 22), lipid membrane alterations (23), and increased RBC rigidity (24) and fragility, ultimately leading to enhanced hemolysis. Lipid peroxidation particularly compromises membrane fluidity, facilitating splenic sequestration and extravascular clearance of RBCs (25, 26).

Despite the consistent observation of storage lesion phenomena across RBC units, considerable variability exists regarding lesion onset, progression, and severity (27), influenced by donor-specific factors including age, sex, body mass index, genetic background, and environmental exposures (4, 12, 14, 15, 28, 29). These characteristics collectively determine the unit's "metabolic

age," distinct from its chronological age (30, 31). Randomized clinical trials have generally supported standard transfusion practices, showing non-inferiority compared to selective transfusion of fresher units, with caveats noted (32). Nevertheless, specific metabolic markers like kynurenine (33) and hypoxanthine (34) correlate strongly with increased RBC fragility and poorer transfusion outcomes, suggesting potential clinical relevance for tailored transfusion practices in certain patient populations.

The precise impact of RBC storage age on recipient metabolism, particularly in clinical populations, remains poorly defined. Prior omics-based investigations in healthy volunteers (35, 36) have identified elevated levels of metabolites such as hypoxanthine and lipid peroxidation products following transfusion with older RBC units, underscoring metabolic shifts of potential clinical significance. However, important knowledge gaps persist regarding the reversibility of storage-induced metabolic changes post-transfusion (25, 37), especially concerning the PPP (36) and delayed restoration of critical metabolites like 2,3-BPG (up to10 hr to restore 5 mM pools at a rate of 0.5 mM/hr), which can measurably impact organ perfusion and oxygen kinetics (38).

Patients with sickle cell disease (SCD) present unique clinical vulnerabilities due to chronic hemolysis, systemic inflammation, endothelial dysfunction, and frequent transfusion requirements, potentially exacerbating susceptibility to adverse metabolic consequences of transfusion. Existing studies in SCD have primarily evaluated clinical endpoints such as hemoglobin increments and transfusion-related complications (39, 40), with minimal emphasis on metabolic profiling. Retrospective analyses and limited prospective studies have revealed metabolic disturbances post-transfusion (41) (42); however, these studies have often been limited by variability in patient demographics, transfusion practices (43), and incomplete characterization of transfused RBC units.

Given the distinctive pathophysiological landscape of SCD, characterized by chronic oxidative stress and inflammation, patients may exhibit unique sensitivities to transfusion-associated metabolic disturbances. Thus, we hypothesized that transfusing RBC units stored for longer durations (≥30 days) would lead to discernible hematologic and metabolic alterations in recipients compared to transfusions with shorter-stored units (≤10 days). These metabolic changes may occur independently of immediate clinical endpoints and reflect fundamental donor- and storage-dependent factors with potential implications for transfusion efficacy and recipient metabolic integrity.

Results

Short-stored and long-stored RBC units are metabolically distinct

First, we performed a metabolomic analysis on RBCs and supernatants from short-stored (≤10 day old) and long-stored RBC units (≥30 day old), which were transfused during the three independent transfusion events after randomization of the recipients with SCD (**Figure 1.A; Supplementary table 1**). Significant differences were observed, as determined by uniform Manifold Approximation and Projection (uMAP) for both the unit's RBCs and their corresponding supernatants (**Figure 1.B**). These profiles showed clear separation based on storage duration, with no apparent clustering or overlap related to the type of additive solution used or the transfusion event sequence, supporting the robustness of storage age as the primary driver of observed differences. Two-way ANOVA by RBC storage age group and transfusion event identified a significant impact of the former, but not the latter on the unit's RBC and supernatant metabolomes (**Figure 1.C**). This signature included several markers of the RBC storage lesion in the RBCs and supernatants of long-stored units, such as decreased ATP, sphingosine 1-phosphate, and GSH, and increased lactate, hypoxanthine, 5-oxoproline, cystine, and nicotinamide. Biomarker analysis (received operating characteristics (ROC) curves - **Figure 1.D**) based on these parameters confirmed high specificity and sensitivity in discriminating long-stored RBC units from short-stored ones, consistent with the literature (14). The heat map in **Figure 1.E** summarizes the most significant metabolic changes between the short-stored and long-stored RBCs. timelines transfusion A summary overview of the metabolic storage lesion is provided in **Figure 1.F**.

Transfusing short-stored RBCs affects RBC metabolism in transfusion recipients

We longitudinally assessed the impact on recipients' RBC metabolism of three independent transfusion events using either short-stored (≤10 day old) or long-stored (≥30 day old) RBCs (**Figure 2.A**). All patients were SS genotypes, self-identified as Black, with no significant differences in age (median 28.5 – 23-34) or body mass index. However, females were over-represented (n=10 out of 13) in the ≥ 30 day study arm patient group compared to the ≤ 10 day arm (n=6 out of 13 - **Supplementary table 1**). No significant differences were reported with respect to comorbidities, emergency department or hospital admission in the previous year. Most patients were transfused with two RBC units per event (60/68, 88.2%) and 80.9% of units transfused (55/68) were of the appropriate storage age. As such, despite metabolomics data having been collected (reported in **Supplementary Table 1**), two subjects receiving at least one of the RBC units not meeting age criteria were excluded from further analysis, leaving 13 subjects in the short-stored and 11 subject in the long-stored RBC study arm, respectively. At baseline, 84.6% of subjects were receiving iron chelation therapy, and 15.4%

were receiving hydroxyurea, with comparable distributions across the two study arms. Important to interpret the results described henceforth, the RBCs analyzed herein are a mixture of the patients' own RBCs and the transfused ones at an approximate ratio of 4:1. First, uMAP analysis identified stable trajectories based on the recipient, rather than the randomization arm (**Figure 2.B**). In parallel, we performed mixed-effects modeling incorporating time, study arm, their interaction, and adjustment for unit age compliance, with results provided in **Supplementary Table 1**. Consistent with mixed-effect modeling, linear discriminant analysis (LDA) based on RBC storage age group – either unadjusted or adjusted by time and subject – identified differences in glycolysis, and in purine, NAD, carnitine, and kynurenine metabolism, as a function of RBC storage age (**Figure 2.C**). An overview of the significant metabolites from this analysis is in **Figure 2.D**. 2,3-BPG levels were higher in recipients of ≤10 day old units through the whole study time-course, with a spike noted at 2-24h after the second transfusion event (**Figure 2.E**). Elevated levels of the glycolysis metabolites fructose 1,6-bisphosphate, phosphoenolpyruvate, and pyruvate, but not of glucose or hexose phosphate (combined isomers), were observed in recipients of short-stored RBCs (**Figure 3**). Trends in metabolites (statistics including Two-Way ANOVA, LDA and mixed-effect models, in **Supplementary Table 1**) such as RBC ATP, hypoxanthine (ATP breakdown and oxidation product), urate (oxidation product of hypoxanthine-derived xanthine), GSH, PPP intermediates (e.g., 6-phosphogluconate, pentose phosphate isomers), and lactoyl-GSH varied across the three transfusion timepoints and between study arms (**Figure 3**). Although some of these metabolites showed directional differences that aligned with expectations based on RBC storage lesion severity by storage age, their levels did not consistently differ between the short- and long-stored groups across all transfusion events. Therefore, we interpret these data as indicative of complex and time-dependent metabolic remodeling, rather than as definitive evidence of a persistent storage-age effect on these individual pathways. NAD(P) pools were better preserved in RBCs from recipients of short-stored RBCs, with lower breakdown to nicotinamide (**Figure 3**), consistent with lower CD38/BST1 activation as a function of storage duration (34). Similarly, acyl-carnitine pools were better preserved in recipients of short-stored RBCs, consistent with a storage-induced depletion of carnitine pools (44). Elevated mannitol was observed immediately after each transfusion event in both randomization arms, presumably due to infusion of the mannitol present in the various Additive Solutions (45).

Markers of hypoxia are elevated in the plasma of recipients of long-stored RBC units

Metabolomics analysis of plasma from recipients of short-stored vs long-stored RBC units showed more marked differences as compared to recipients' RBCs. Except for two patients clustering with the long-stored RBC arm, recipients of short-stored RBCs clustered separately across uMAP 1,

although their trajectories did not deviate significantly based on intervention arm (**Figure 4.A; Supplementary table 1**). LDA identified significant effects of the age of transfused RBCs (**Figure 4.B-D**) on short-chain acyl-carnitines, bilirubin (unexpectedly higher in the short-stored RBC arm in the mass spec analysis), ascorbate, and markers of hypoxia (e.g., hypoxanthine (46), urate (47), lactate (48), fumarate (49), sphingosine 1-phosphate (50-52)), all higher in recipients of long-stored RBCs. Recipients of long-stored RBCs also had higher baseline levels of a metabolite whose chemical and physical properties are consistent with its identification as either serotonin (platelet-derived) or cotinine (derived from smoking or other nicotine exposures) (53). An overview of all the above results is shown as a volcano plot of merged plasma and RBC data comparing the 1h and 24h post-transfusion time points vs pre-transfusion values (**Supplementary Figure 1**).

Circulating levels of pro-inflammatory cytokines are higher in recipients of long-stored RBCs, while anti-inflammatory cytokines are higher in recipients of short-stored RBCs

Cytokine measurements identified significant increases in the levels of pro-inflammatory cytokines interleukin (IL)-6, IL-8, IL-1β and Chemokine (C-X-C motif) ligand 9 (CXCL9) in recipients of long-stored RBCs at any given time point after the first transfusion (**Figure 4.E-G**). On the other hand, recipient of short-stored RBCs showed higher levels of anti-inflammatory cytokines IL-12, IL-10, IL-1receptor antagonist (IL-1ra) after transfusion (**Figure 4.E-G**). Pro-inflammatory interferon gamma (IFNy) was higher at baseline, before transfusion in patients enrolled in the short-stored RBC study arm, but its levels became comparable to the rest of the cohort after the first transfusion (**Figure 4.G**).

Transfusion of long-stored RBCs yields significantly lower RBC and hemoglobin increments, and greater dysregulation of iron and renal metabolism, hemoglobin glycation

Measurements of clinical chemistry and hematological parameters showed a significant impact of long-stored RBC transfusion (**Figure 5.A-D; Supplementary table 1**). These parameters were also available 2 weeks after each transfusion event, and trends for each post-transfusion time point (2h, 24h, 2 week), normalized to pre-transfusion values, are shown in **Supplementary Figure 2**. Taken together, we observed cumulative effects across all 3 transfusion visits (**Figure 5.B-F**) on: (i) RBC and hemoglobin parameters: RBC count and hemoglobin A levels were higher in recipients of short-stored RBCs, despite higher starting levels of HbS% in patient from this arm. Both transfusion of short- or long-stored RBC units resulted in comparable drops in HbS% after transfusion (**Figure 5.B-E**); unfortunately, reticulocyte % were only captured in a subset of patients from the long-stored RBC study arm at a limited number of time points (**Figure 5.D-E**) (ii) iron metabolism (unsaturated iron-binding capacity: higher in recipients of short-stored RBCs; total iron and

transferrin iron saturation: higher in recipients of long-stored RBCs); measurements of heme metabolism, in particular total and direct bilirubin, were only captured for a subset of patients in the long-stored RBC study arm, though they increased after transfusion (**Figure 5.E**); (iii) renal function (higher BUN in recipients of long-stored RBCs); (iv) white blood cell (WBC) counts (higher WBCs and neutrophils in recipients of long-stored RBCs, consistent with the cytokine measurements); (v) hemoglobin glycation (higher HbA1c in recipients of long-stored RBCs); (vi) acidosis (lower bicarbonate in recipients of long-stored RBCs); (vii) hemolysis markers (comparable LDH in the two arms). These effects were more marked when directly comparing trajectories at 24h post-transfusion to pre-transfusion levels (**Figure 5.G**).

Correlations between metabolic measurements and clinical chemistry or hematological parameters

We then combined metabolomics measurements of recipients' plasma and RBCs and clinical chemistry or hematological covariates, to compare the main changes between the two study arms, irrespective of the time point (**Supplementary Figure 3**). We then leveraged the merged data to: (i) determine the plasma vs RBC metabolites that correlated the most across matrices (**Figure 6**); (ii) identify cross matrix correlates (i.e., metabolites from the transfused RBCs and supernatants that correlate with metabolites in plasma and RBCs of recipients after transfusion); and (iii) identify the top omics correlates to clinical chemistry or hematological parameters (**Figure 7**).

The first analysis identified a cluster of strongly correlated metabolites intra-matrix (**Figure 6.A**), especially with respect to fatty acid metabolism (e.g., FA 18:2 vs FA 18:3 in RBCs), glycolysis (e.g., 2,3-BPG vs phosphoenolpyruvate), and carboxylic acid metabolism (e.g., fumarate vs malate); most of these overlapped between recipients of short-stored or long-stored RBCs (**Figure 6.B**). A network overview of the top 25% correlations (by Spearman rho and p-value) is shown in **Figure 6.C**, with the respective matrix view in the heat map in **Figure 6.D**. After noting a robust core of intra-matrix metabolite-metabolite correlates, we then sought to understand which of these correlations were most significantly altered by the study arm, an analysis suggestive of an impact of the selective transfusion of short- or long-stored RBCs on that specific metabolic reaction sub-network (**Figure 6.E**). Deltas of short- or long-stored RBC correlations identified altered taurine, citrulline/creatinine, cystine/ascorbate, bilirubin, pyruvate, thymidine, fatty acid, and carnitine metabolism as the most affected pathways as a function of the storage age of the transfused RBC units (**Figure 6.E**).

We then sought to determine the metabolites whose levels correlated the most or the least between matched plasma and RBCs from the same transfusion recipient, (**Figure 6.F**), identifying a

strong cross matrix reproducibility in the levels of thymidine and 5,6-dihydrothymine, sulfocatechol, creatinine, and urate (**Figure 6.G**). In contrast, poor correlations were observed between plasma and RBC levels of hypoxanthine, L-arginine, 2-oxoglutarate, glutamyl-glutamine, and butanoyl-carnitine, suggesting matrix specific metabolism (e.g., catabolism of hypoxanthine to urate (**Supplementary Figure 4**).

Correlation of metabolomics data to clinical chemistry or hematological parameters (**Figure 7.A-B**) identified several key clusters. The top two clusters are highlighted in the Debiased Sparse Partial Correlation network in **Figure 7.C-D**, and identify a strong association between: (i) *Network 1* (**Figure 7.C**): acidosis and circulating carboxylates (e.g., methylcitrate), transaminases (AST, ALT) and sphingosine 1-phosphate/taurine, direct bilirubin (clinical chemistry) and ethanolamine/sphingolipid metabolism, total protein/albumin and kynurenine metabolism (kynurenine, quinolinic acid); (ii) *Network 2* (**Figure 7.D**): hemoglobin A and citrulline; MCH, MCV, MCHC, RDW and ornithine; neutrophil and GSH metabolism (ɣ-glutamyl-cysteine/glutamine; cysteinyl-glycine).

Focusing on selected clinical chemistry covariates, such as creatinine (**Figure 7.E**), confirmed the quality of the mass spectrometry-based metabolomics data, with strong association between CLIA-regulated clinical chemistry assays for creatinine and the MS measurements in these patients (**Supplementary Figure 5**). Of note, several metabolites involved in arginine metabolism (e.g., citrulline, aspartate) also correlated with creatinine levels.

Hemoglobin levels strongly and positively correlated with total GSH, iron levels, sphingosine 1-phosphate (in RBCs), and octanoic or nonanoic acid (FA 8:0 and 9:0; **Figure 7.F**). Clinical chemistry measurements of bilirubin, for the subset of recipients of long-stored RBC transfusions for which this measurement was available, were negatively associated with total GSH pools (both reduced and oxidized GSH; **Figure 7.G**).

Finally, correlation analysis of metabolites from RBC units (either the RBCs or supernatants) to circulating plasma or RBC metabolites in the recipients did not identify specific metabolites whose levels in the RBC unit impacted post-transfusion levels of the same metabolite in the recipient (e.g., no association between hypoxanthine in stored RBCs or supernatants and its levels in post-transfusion plasma or RBC; **Supplementary Figure 6**). Even when significant associations were noted (e.g., sphingosine 1-phosphate in stored RBCs and circulating RBCs in transfusion recipients; **Supplementary Figure 7**), the actual correlation was not compelling. However, specific metabolite (RBC unit)-metabolite (recipient) associations were noted, including a negative association between hypoxanthine in the RBC units and urate in transfusion recipients.

On the other hand, metabolite levels, both in transfused RBCs and in the plasma or RBCs of transfusion recipients strongly correlated (module of Spearman rho $\geq$ 0.85) with several pro-inflammatory cytokines in recipients of long-stored RBCs, and anti-inflammatory cytokines in recipients of short-stored RBCS (**Figure 7.H**). A network of strongly and positively intercorrelated pro-inflammatory cytokines (above all IL-6, TNFα, IL-8 – **Figure 7.I**) was linked to circulating levels of kynurenine.

Discussion

The present study provides the first direct evidence of a metabolic effect of the storage age of transfused RBCs on clinical chemistry or hematological parameters and related metabolic measurements in transfusion recipients in a prospective randomized trial in adults with SCD. The impact of the storage lesion is well established and confirmed here by direct measurements of the metabolic changes in supernatant and RBCs of short- and long-stored RBC units. These results confirm a depletion of adenylate (34), NAD(P) and GSH pools(54), the accumulation of byproducts of their oxidation and catabolism (e.g., hypoxanthine(34), nicotinamide (34) and 5-oxoproline(14)), and depletion of acyl-carnitine pools (44), methionine (55), and sphingosine 1-phosphate (50).

Nonetheless, questions remained whether these metabolites affected the bloodstream characteristics of patients requiring allogeneic transfusions, such as those with SCD enrolled in the present study. The current results demonstrate that both recipients' plasma and RBCs are significantly impacted, one notable effect related to increased circulating levels of mannitol (a storage additive component), irrespective of the storage age of the RBC unit. More physiologically relevant, transfusion of short-stored RBC units was associated with significantly higher recipient circulating levels of 2,3-BPG; this result is consistent with improved oxygen kinetics in recipients of short-stored RBCs and supports recent studies of renal perfusion (38). While transfusion of short-stored RBC units was associated with higher recipient levels of glycolysis intermediates such as 2,3-BPG and phosphoenolpyruvate, interpretation of downstream metabolites such as hypoxanthine, 6-phosphogluconate, and lactoyl-GSH requires caution. These metabolites exhibited variability across the transfusion timeline and did not consistently segregate by study arm. As such, although trends in hypoxanthine and its catabolite urate aligned with known oxidative pathways (12, 34, 47), these findings likely reflect a combination of storage lesion severity, recipient-specific factors, and the temporal dynamics of metabolic remodeling post-transfusion, rather than a uniform storage-age effect. These metabolomics results were corroborated by clinical chemistry measurements of acidosis, with significantly lower bicarbonate in the plasma of recipients of long-stored RBCs. Lower sphingosine 1-phosphate in recipients with SCD is clinically relevant, because this metabolite stabilizes deoxyhemoglobin, thereby potentially promoting crystallization of sickle hemoglobin in these patients (39, 51, 56). Similarly, higher hypoxanthine levels in long-stored RBCs and their supernatants positively correlated with plasma and RBC urate in transfusion recipients, suggesting rapid metabolism of hypoxanthine to urate by xanthine dehydrogenase/oxidase , a reaction that also generates hydrogen peroxide. In this context, the negative association between hypoxanthine levels and the post-transfusion circulatory capacity of stored RBCs may not be just correlative, but may be mechanistically explained by the utilization of this metabolite as a substrate for pro-oxidant reactions

following transfusion, consistent with mechanistic models of reperfusion injury upon ischemic hypoxia (49).

Transfusion of long-stored RBCs was associated with poorer overall efficacy (e.g., lower hemoglobin increments, RBC count, and hemoglobin A levels) and increased circulating iron, transferrin saturation, all consistent with elevated hemolysis and with prior results in healthy recipients of autologous RBCs stored for >35 days (57). This observation is explained, in part, by better preservation of antioxidant systems (e.g., GSH pools, pentose phosphate pathway metabolites) in RBCs of recipients of short-stored RBC units in the current study. Transfusion of long-stored RBCs was associated with renal dysfunction (increased BUN and creatinine, the latter observed by both clinical chemistry and mass spectrometry-based assays); this is likely due to increased hemolysis and membrane lipid remodeling in long-stored RBCs (58), due to depletion of carnitine pools (44). In addition, the association between these parameters and kynurenine and its metabolites (e.g. quinolinic acid, a neurotoxicant) is consistent with the recently reported linkage of dysregulated kynurenine metabolism in RBCs at the time of donation with increased osmotic fragility and lower post-transfusion hemoglobin increments (33) or elevated pro-inflammatory cytokines like IL-6 and IFNɣ in the context of COVID-19 (59) or after strenuous exercise (60). Circulating kynurenine levels were here linked to IL-6 and to a series of pro-inflammatory cytokines. Elevated WBC and neutrophil counts in recipients of long-stored RBCs is consistent with activation of pro-inflammatory processes in transfusion recipients, and with the established roles of metabolites (e.g., lactate (61), citrulline (62), sphingosine 1-phosphate (63-65), and carboxylic acids - e.g., fumarate and succinate (66)) in immunometabolic reprogramming towards pro-inflammatory phenotypes. These observations were corroborated by direct measurements of circulating cytokines, which identified the elevation of pro-inflammatory cytokines and key drivers of poor outcomes in patients with sickle cell anemia (67), IL-6, IL-1β, IL-8 and CXCL9 in recipients of long-stored RBCs, while recipients of short-stored RBCs had higher levels of anti-inflammatory cytokines IL-10, IL-12, CXCL11. Of note, CXCL11 can drive the polarization of CD4+ T cells into IL-10-producing Tr1 cells,(68) which are a type of regulatory T cell known for their immunosuppressive properties. As such, CXCL11 has been linked to the modulation of tolerogenic immune responses, which is important in light of the clinical role of alloimmunization in chronically transfused patients with SCD (69). On the other hand, pro-inflammatory CXCL9 and 10, which were instead elevated after transfusion of long-stored, but not short-stored RBCs. Transfusion of short-stored RBCs also lowered the levels of pro-inflammatory IFNɣ, which were higher before transfusion in the patients enrolled in that study arm. Notably, the metabolism of arginine to ornithine was recently linked to the age of the donor, to the storage age of the donated RBCs, and, ultimately, to post-transfusion hemoglobin increments (29). Finally, altered

post-transfusion levels of glucose and HbA1c in recipients of long-stored RBC units is consistent with previous hypotheses, and is reported here for the first time in prospective, randomized clinical trial (70).

In addition to the hematologic and metabolic outcomes, we systematically captured severe and non-severe adverse events, including vaso-occlusive episodes (VOEs) and hospitalizations. While the present study was primarily powered to detect metabolic differences, exploratory analyses revealed that subjects randomized to receive longer-stored RBC units (≥30 days) experienced a numerically higher incidence of pain crises and non-severe adverse events requiring medical attention. Although these observations suggest that storage duration may influence clinical outcomes in patients with sickle cell disease, the study was not powered to definitively evaluate these endpoints. A comprehensive analysis of adverse event rates, hospitalization frequency, and clinical predictors of post-transfusion morbidity in this cohort will be reported separately in a forthcoming companion manuscript. These preliminary findings, however, further highlight the potential importance of donor and storage factors in optimizing transfusion support for individuals with sickle cell disease.

This study has several notable limitations. First, the small sample size (13 subjects per arm originally planned, with 11–13 subjects ultimately analyzed) limits the statistical power to robustly detect subtle differences or conclusively establish causation between RBC storage age and clinical outcomes. Recruitment targets were affected primarily by changes in clinical practice favoring exchange transfusions and interruptions due to the COVID-19 pandemic. Second, despite using rigorous statistical modeling (e.g., repeated measures and mixed-effects modeling) to control for donor and unit variability (e.g., hemoglobin dosage, processing sites, anticoagulants), residual confounding likely persists. Third, the study design did not include a crossover arm, which would have helped control for individual patient variability, thus potentially affecting interpretation of some metabolic and hematological parameters. Fourth, alloimmunization status and comprehensive antigen-matching details beyond CEK were not systematically captured, limiting the ability to explore their impact on transfusion efficacy and RBC survival; these represent important areas for future studies. Fifth, while cytokine measurements and white blood cell flow cytometry data were obtained, these complex analyses were beyond the scope of the current manuscript and will be addressed comprehensively in a companion manuscript currently under preparation. Sixth, although hydroxyurea and iron chelation therapies were recorded and distributed similarly between groups, potential effects of these disease-modifying treatments on transfusion outcomes cannot be excluded. Lastly, most subjects (84.6%) received iron chelation, and a smaller subset (15.4%) received hydroxyurea; variations in these therapies might influence transfusion outcomes and are explicitly

detailed in the supplementary demographics data. Further large-scale studies are required to validate these findings and their broader clinical implications, particularly in the context of current clinical practices.

In conclusion, this prospective and randomized clinical trial identified clinical chemistry, hematological and metabolic effects resulting from the storage age of RBC units, by longitudinally monitoring three independent transfusion events in chronically transfused patients with SCD. Our results indicate that selective transfusion of RBCs units stored for $\leq$10 days has some beneficial effects on metabolic regulators of oxygen kinetics, whereas transfusion of long-stored RBCs is associated with potentially harmful increased circulating markers of hypoxia, hemolysis, iron metabolism, inflammation, and renal dysfunction.

Methods

Study design

This study is part of a prospective, randomized, blinded pilot clinical trial conducted at three sites (Medical College of Wisconsin (MCW), University of North Carolina at Chapel Hill (UNC), and Emory University), under protocols approved by their respective institutional review boards. Between 2017 and 2024, we recruited 26 adults (16-60 years) with SCD who were treated with chronic outpatient RBC transfusions (i.e. one or two RBC units transfused every 3-8 weeks per a medically-defined protocol) (71). Consented subjects were randomized to receive ≥30 day-old (n=13) or ≤10 day-old (n=13) units for 3 consecutive outpatient simple transfusion events. Three subjects receiving units not meeting (RBC unit) age criteria were excluded from the old group, despite samples being collected and metabolomics analyses being performed (**Supplementary Table 1**). A baseline venous blood sample for flow cytometry was obtained just prior to the first randomized transfusion.

Sex as a biological variable The study enrolled 10 female patients (out of 13) in the ≥ 30 day study arm group and 6 (out of 13) female patients in the ≤ 10 day arm.

Subjects were asked to hold their iron chelation for 72 hours before each study transfusion so that the post-transfusion change in circulating serum iron could be clearly defined. Pre-transfusion unit link samples were obtained from each RBC units for flow cytometry and metabolomics. RBC and supernatant samples (100 µl via 10 min centrifugation at 4C at 1,500 x *g*) were collected from residual transfusates from each unit immediately after transfusion. Patient venous blood samples were also obtained pre-transfusion and at 2- and 24-hours post-transfusion for flow cytometric and metabolomic analyses (100 µl via 10 min centrifugation at 4C at 1,500 x *g*). Participants completed standardized diaries each day until the end of the study to document subjective symptoms of infection, pain, and emergency department or hospital utilization. All data were captured with standardized case report forms and entered into an electronic database. This database also included a record of basic medical and surgical histories (e.g. age, gender, reason for receiving chronic transfusion therapy, past medical histories, medication list, previous hospitalizations) (71).

For cytokine measurements, all Luminex assays were performed by the UNC Respiratory TRACTS Core Laboratory according to the manufacturer's instructions with the exception of the standard curve, which was extended to a 7-point curve for all assays to increase the quantifiable range. All Luminex assays were read on an Intelliflex Single Reporter system using the high sensitivity settings.

Consecutive outpatient transfusions involved age-appropriate, crossmatch compatible, CEK antigen-matched, ABO/RhD compatible RBC units negative for sickle trait, and sickle-negative RBC units stored in either AS-1, AS-3, or AS-5. Detailed patient demographics (all SS genotypes),

including age, sex and baseline laboratory data) are provided in **Supplementary Table 1.** Information on chronic therapies, including hydroxyurea use and iron chelation therapy, was collected at enrollment and also summarized in **Supplementary Table 1.** Neither the patient nor the clinical staff overseeing the subject's participation in the trial, including the study principal investigator and study coordinators, had access to the treatment arm assignment. However, no alterations were made to the labels on the RBC units; therefore, unintended unblinding of study participants was possible. Medical infusion clinic personnel physically providing the RBC transfusions verified product and patient identity according to hospital-specific procedures.

Sample preparation for metabolomics

Metabolites from plasma and supernatants from RBC units were extracted at a ratio of 1:25 and from RBCs at a ratio of 1:10 with cold MeOH:MeCN:H_2O (5:3:2, v:v:v) and 100% MeOH for metabolomics/oxylipins/bile acid analysis and lipidomics analysis, respectively. Suspensions were vortexed vigorously for 30 min at 4 °C. Insoluble material was pelleted by centrifugation (18,213 *g*, 10 min, 4 °C) and supernatants were isolated for analysis by UHPLC-MS (72).

UHPLC-MS analysis

A Vanquish ultra-high pressure liquid chromatography (UHPLC) system (Thermo) was coupled to an Orbitrap Exploris 120 mass spectrometer (Thermo). Sample injections (10 µL) were resolved across a 2.1 x 150 mm, 1.7 µm Kinetex SB-C18 column (Phenomenex) using a 5 minute, reversed-phase gradient, as described previously (72) (details in **Supplementary Methods**). Run order of samples was randomized and technical replicates were included to assess quality control. Raw files were converted to .mzXML using RawConverter. The resultant files were processed with El-Maven (Elucidata) alongside the KEGG database for metabolite assignment and peak integration as previously described (34, 73).

Statistical analyses

Raw metabolomics data were autoscaled via MetaboAnalyst 5.0.[58] Two-way ANOVA, repeated measures ANOVA, or two-tailed Student's t-test were used to determine significance by matrix, treatment arm over time, or at a specific time point. A P value less than 0.05 (FDR adjusted < 0.05 for ANOVA) was considered significant. Uniform Manifold Approximation and Projection (uMAP), a non-linear dimensionality reduction technique that transforms high-dimensional data into a lower-dimensional representation while preserving its structure was used to analyze metabolomics data as a function of study arm and time (74). To account for repeated measures within subjects and

minimize the impact of confounding variables such as donor hemoglobin dosage, donor biology, processing site differences, and additive solution type, we performed additional analyses employing mixed-effects models with random intercepts for subjects and fixed effects for storage duration, transfusion event, and additive solution (details in **Supplementary Methods**). This approach allowed for robust adjustment for both intra-subject and inter-unit variability. In-house code for R-4.4.1 and MetaboAnalyst were used to perform non-parametric two-tailed Spearman's rank correlation tests with the un-normalized, raw data to generate correlation plots for variables of interest, including correlations across matrices (RBCs and supernatants from transfused units, plasma and RBCs in transfusion recipients) for metabolomics data and/or clinical biochemistry or hematological variables. This software was also used to calculate and plot linear regressions and to generate a central network plot. Additional figures were generated using GraphPad Prism 10 and BioRender.com.

Data availability: All raw data and elaborations are available in **Supplementary Data Table 1.xls**.

Authorship Contribution: Clinical trial design and review of the manuscript: MSK, JJF, JAL, implementation and review of the manuscript: MSK, ASG, RMF, AI, DW, AC, SMJ, HEB, OK, MCC, DS, JAR, NSK, manuscript writing: MSK, AD; Writing and analysis: AD.

Acknowledgements This study was supported by funds by the National Heart, Lung, and Blood Institute (NHLBI) (K23HL136787 and R01HL148151 to MSK; R01HL146442and R01HL149714 to AD). The authors would like to thank the staff of the UNC Respiratory TRACTS Core Laboratory. Summary graphics in Figure 1F and Figure 3 were generated with the software Biorender (2025) under licenses accessible at https://BioRender.com/xygxjhd and https://BioRender.com/17rezum.

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FIGURE LEGENDS

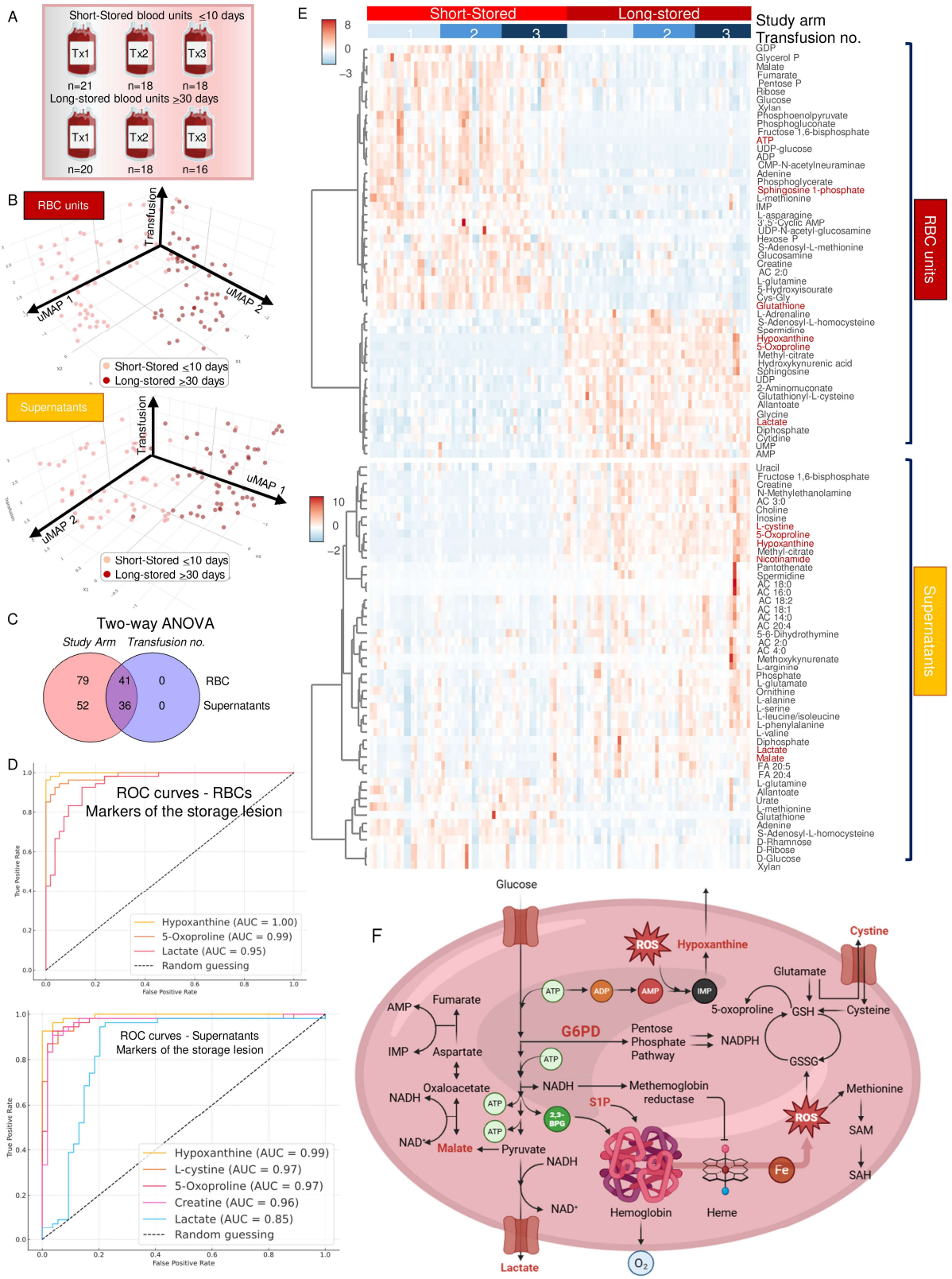

FIGURE 1 – Blood units stored longer than 30 days are metabolically distinct from units stored less than 10 days. In **A**, overview of the experimental design. Numbers indicate total units transfused per each one of the transfusion events

(Tx1-3). In **B**, Uniform Manifold Approximation and Projection (uMAP) of metabolomic data for all blood units transfused at any of the 3 transfusion events for units stored less than 10 days (short-stored) or longer than 30 days (long-stored). In **C**, as per study design, age of blood, but not transfusion sequence was associated with significant metabolic changes (Two-way ANOVA). Transfusion event sequence is shown merely to confirm reproducibility of storage-age related effects across events. In **D**, Receiver Operating Characteristics (ROC) curves for RBC and supernatant levels of the metabolic markers of the storage lesion (Paglia et al. Blood 2016) discriminant between short-stored and old blood units. In **E**, heat map of the most significant metabolic changes in RBCs and supernatants as a function of the storage age of the unit (Two-way ANOVA). A summary overview of the RBC storage metabolic lesion is provided in **F**.

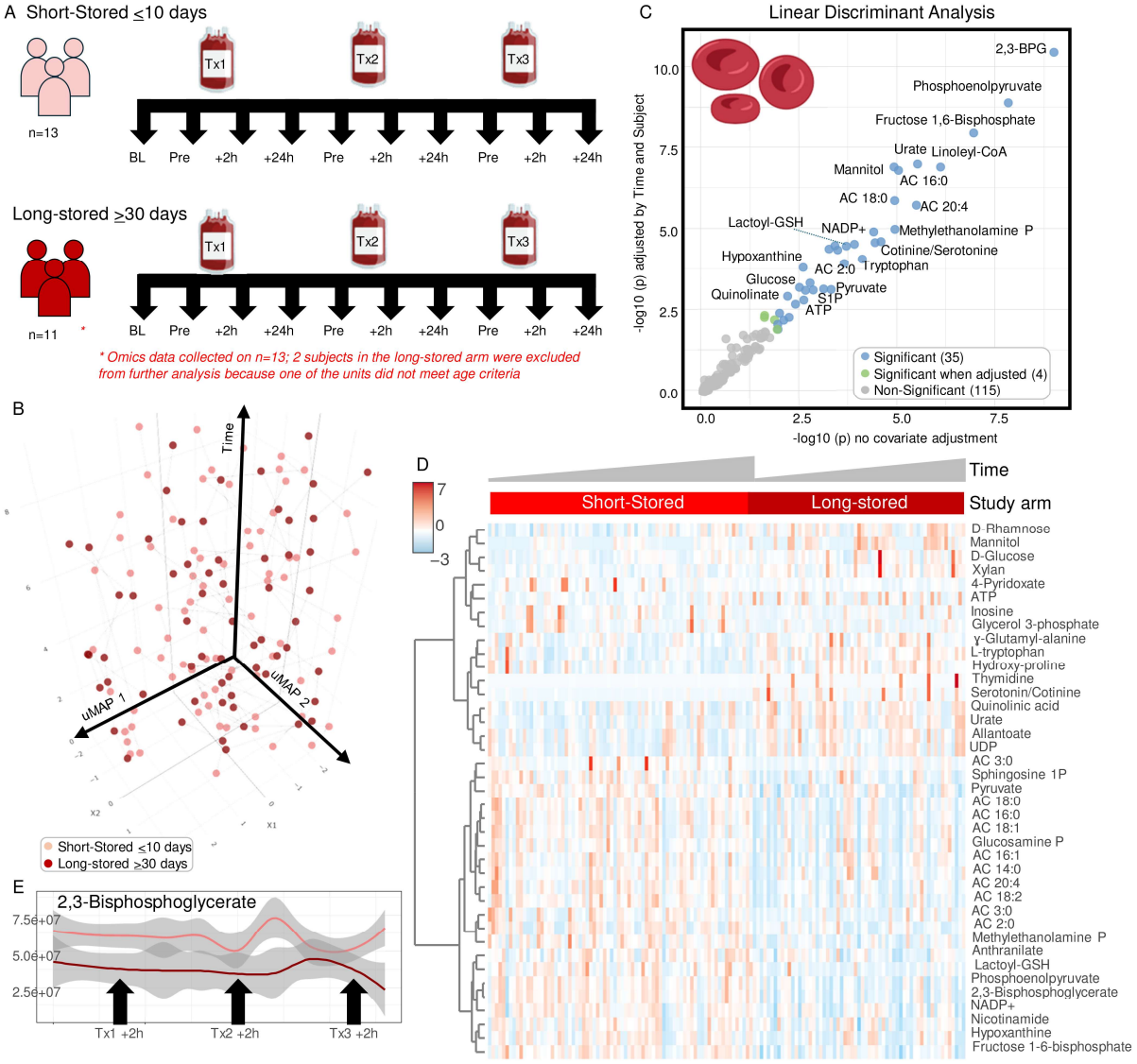

FIGURE 2 – Metabolic impact of transfusion on the recipients' RBC metabolome. Twenty-six patients with sickle cell disease received three consecutive transfusion with short-stored (<10 days) or long-stored (>30) pRBCs (**A**). RBC samples were drawn for metabolomics analysis of plasma and RBCs from the recipient at baseline, before transfusion, and 2 or 24h after each one of the transfusion events. Two patients were excluded from long-stored RBC study arm because some of the units they received did not match the age criteria of the study protocol. In **B-C-D**, uMAP, linear discriminant analysis (LDA) and heat map of significant RBC metabolites by time and storage age of blood by LDA. In **E**, line plot of temporal changes in 2,3-bisphosphoglycerate (BPG) over multiple transfusion as a function of the storage age of blood (light and dark red for short-stored or long-stored units, respectively).

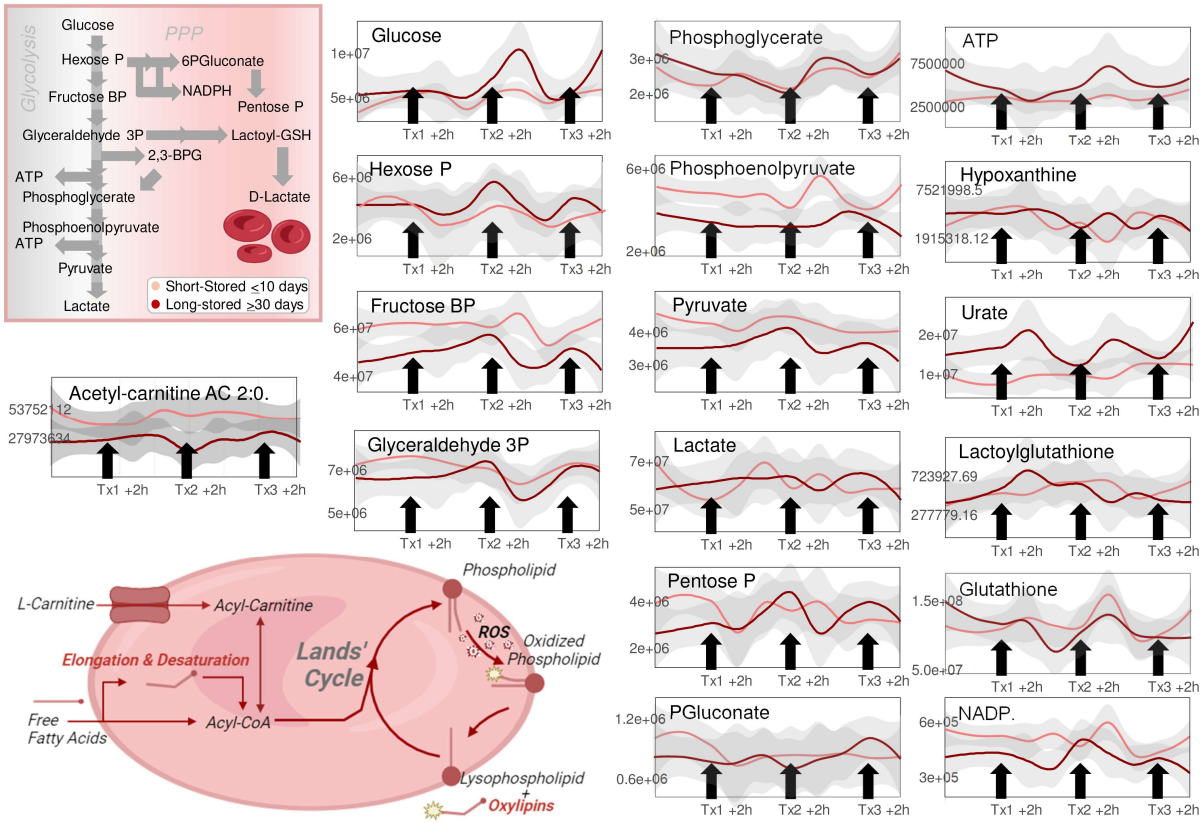

FIGURE 3 – Impact of short-stored vs long-stored blood on glycolysis, pentose phosphate pathway and redox homeostasis in the RBCs of transfusion recipients. Line plots show temporal changes after transfusion (Tx) 1, 2 and 3 (light and dark red represent median ± interquartile ranges for short-stored and long-stored units, respectively).

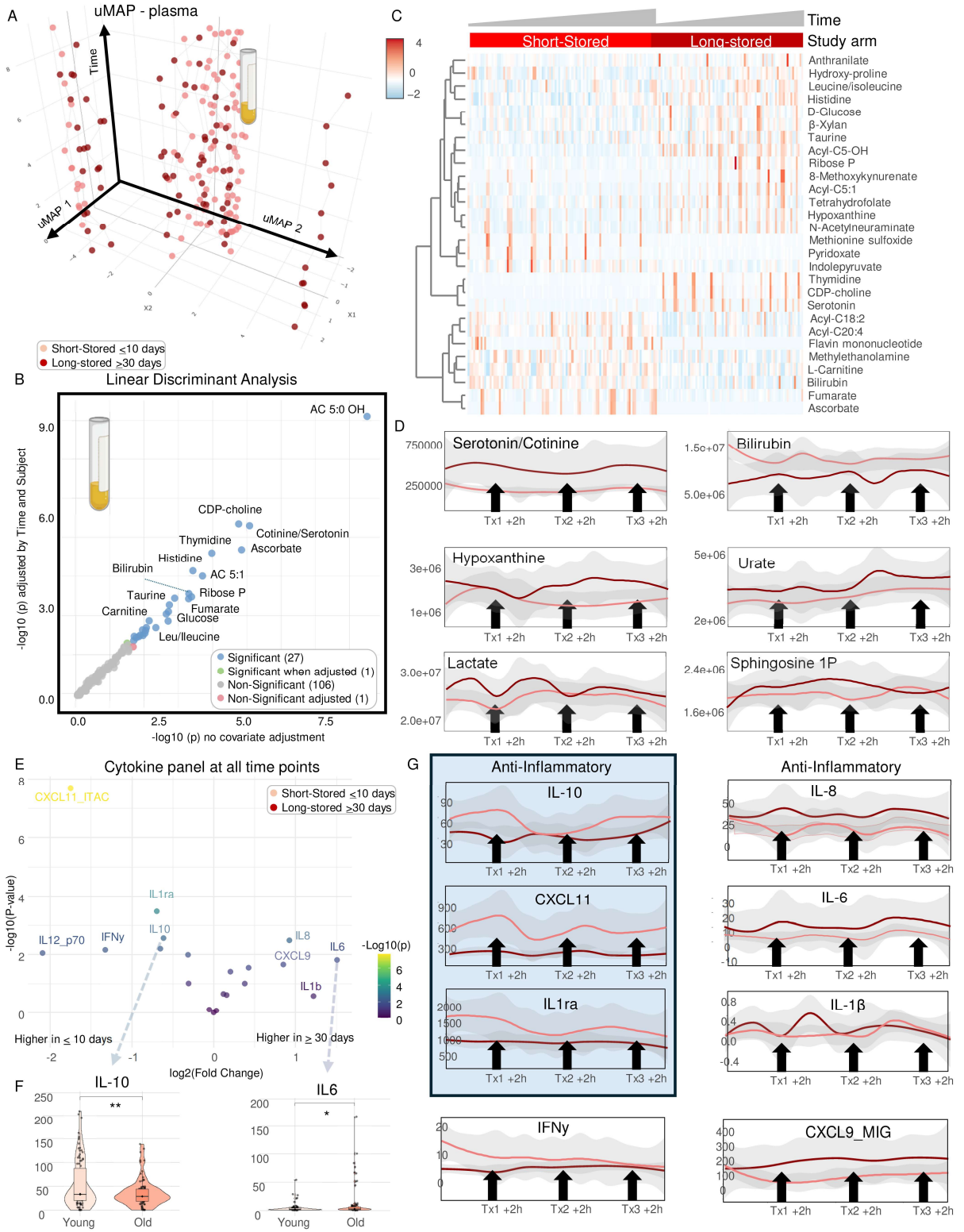

FIGURE 4 - Metabolic impact of transfusion on the recipients' plasma metabolome and cytokines. In **A-B-C**, uMAP, linear discriminant analysis (LDA) and heat map of significant plasma metabolites by time and storage age of blood by LDA. In **D**, line plot of temporal changes in the most significantly impacted plasma metabolites (by LDA) over multiple transfusion as a function of the storage age of blood (light and dark red for short-stored and long-stored units, respectively). In **E**, volcano plot comparing changes in circulating levels of cytokines in recipients of long- or short-stored pRBCs shows significant effects (two-tailed T-test, adjusted) on anti- or pro-inflammatory cytokines (e.g., IL-10 or IL-6,

respectively) at all tested time points (**F**). Line plots are shown for the time course effects for the most significantly affected pro- or anti-inflammatory cytokines (Two-way ANOVA – **G**).

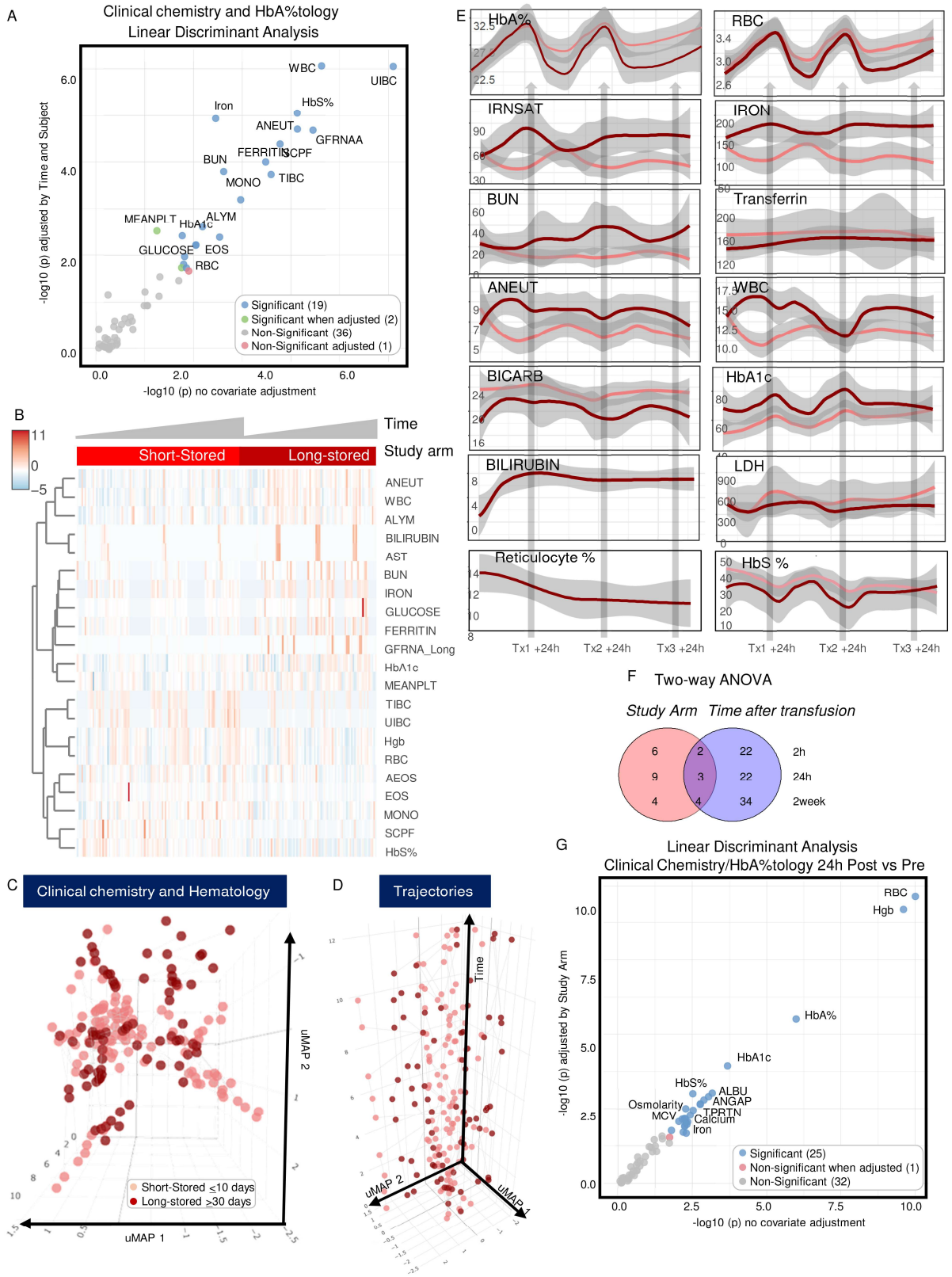

FIGURE 5 – Impact of transfusion of short-stored vs long-stored blood on the recipients' clinical chemistry panels and complete blood counts. In **A-B**, linear discriminant analysis (LDA), heat map of significant clinical chemistry and hematological parameters affected by time and storage age of blood. In **C-D**, uMAP (2D and 3D with temporal trajectories). In **E**, line plot of temporal changes in the most significantly affected (two-way ANOVA in **F**) clinical

chemistry and hematological parameters over multiple transfusion as a function of the storage age of blood (light and dark red for short-stored and long-stored units, respectively). In **G**, LDA of long-stored vs short-stored blood at the 24h vs pre-transfusion time point for each transfusion.

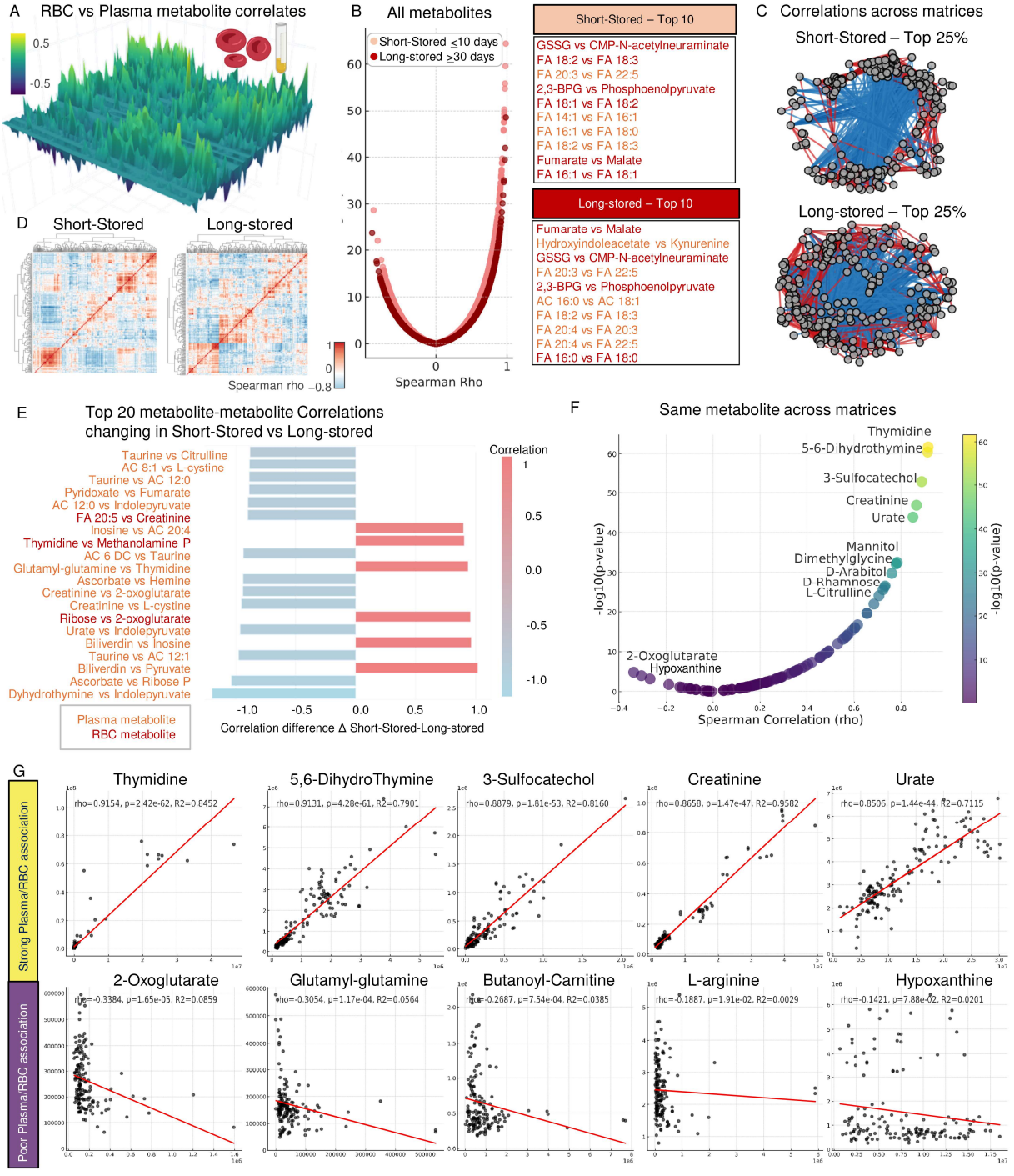

FIGURE 6 – Correlation analysis of plasma vs RBC metabolic phenotypes after all transfusion events as a function of the storage age of transfused pRBCs. In **A**, 3D map of Spearman rho correlations (z axis) of transfusion recipient plasma vs RBC metabolites (x and y axes). In **B**, volcano plots of these correlations, with the top 10 most significant correlations highlighted for short-stored and long-stored blood units, for plasma (light orange) and RBCs (dark red). In **C-D**, network and heat map view of the correlation matrix (top 25% significant same-matrix correlations are shown). In **E**, top 20 metabolite-metabolite correlations affected by transfusion of short-stored vs long-stored blood units. In **F-G**,

volcano plot and scatter plot of the most significantly positive and negative correlations for the same metabolite in plasma vs RBCs.

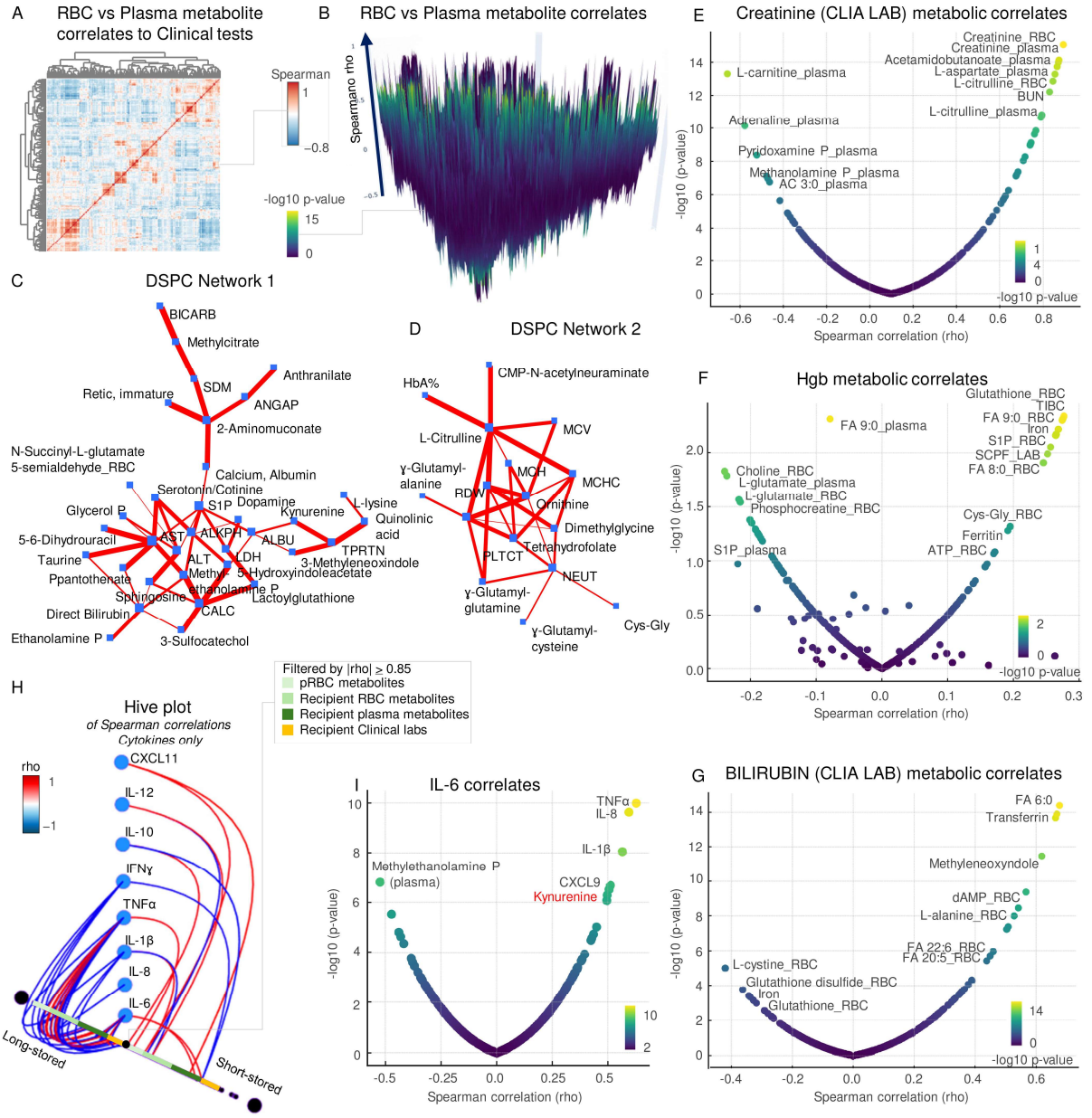

FIGURE 7 – Metabolic correlates to clinical chemistry and hematological parameters. In **A**, correlation matrix (Spearman rho) between metabolites and clinical chemistry and complete blood count parameters in transfusion recipients. In **B**, same as A, with the z axis representing Spearman rho positive vs negative values, and colors proportional to the -log10 p-value of the correlation significance. In **C-D**, DSPC Network 1 and 2 of the top metabolite-metabolite and metabolite-clinical covariates in this study. In **E-G**, volcano plots (Spearman rho vs -log10 p for x and y axis, respectively), for clinical chemistry measurements of creatinine, hemoglobin (g/dL) and bilirubin in transfusion recipients. In **H**, hive plot summarizing correlations (module of Spearman rho $\geq$ 0.85) for cytokines vs metabolites in RBC units, recipient RBCs or plasma or clinical labs identifies a stronger association between pro-inflammatory cytokines and metabolites levels in SCD recipients of units stored longer than 30 days. In **I**, volcano plot of Spearman correlations to IL-6 levels in the recipient shows strong positive correlations among pro-inflammatory cytokines and circulating levels of kynurenine.