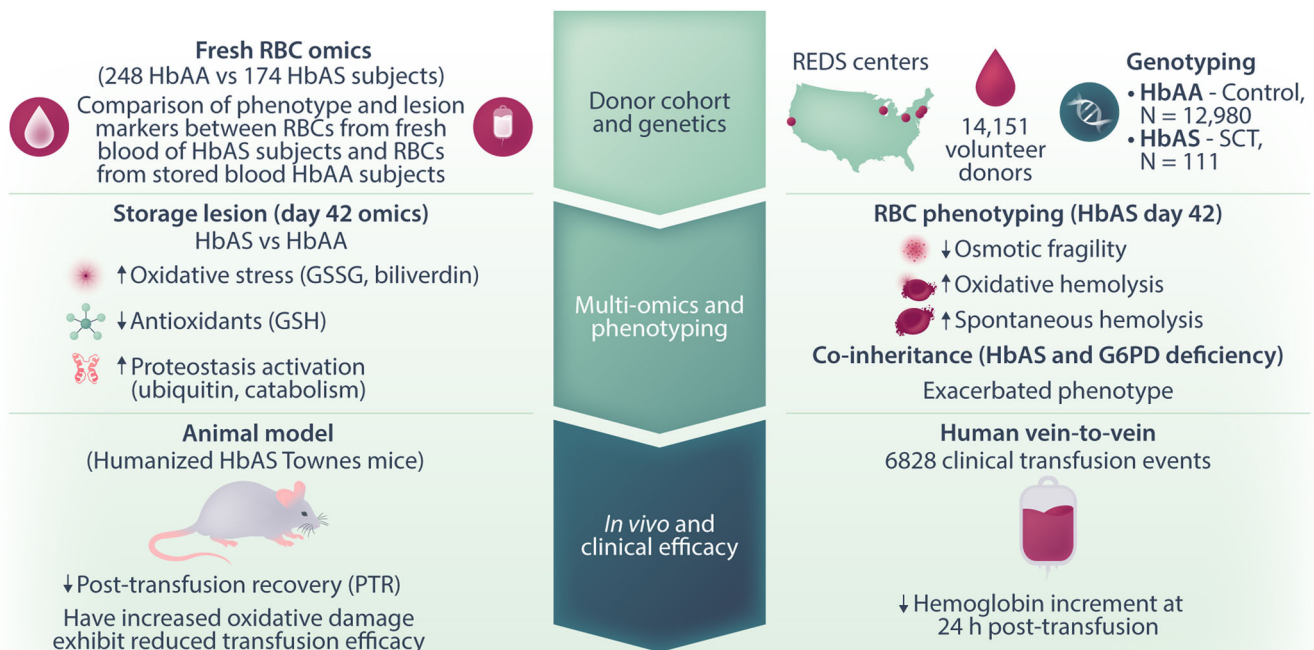


ARTICLE

Lower efficacy of transfusion of red blood cells from donors with sickle cell trait

Monika Dzieciatkowska¹ | Daniel Stephenson¹ | Ariel Hay² | Xunde Wang³ | Gregory R. Keele⁴ | Travis Nemkov^{1,5} | Xutao Deng^{6,7} | Mars Stone^{6,7} | Kirk C. Hansen^{1,5} | Steve Kleinman⁸ | Philip J. Norris^{6,7} | Michael P. Busch^{6,7} | Grier P. Page⁴ | Steven L. Spitalnik⁹ | Nareg Roubinian^{6,7,10} | James C. Zimring^{8,11} | Swee-Lay Thein³ | Angelo D'Alessandro^{1,5} 

Graphical Abstract



Sickle cell trait (SCT) drives an accelerated RBC "aging" phenotype during storage, leading to a reduced hemoglobin increment in recipients

ARTICLE

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Abstract

Sickle cell trait (SCT), the heterozygous state for the hemoglobin S (HbS) mutation, affects roughly 1 in 13 African American individuals and is common among blood donors recruited for antigen-matched transfusions in sickle cell disease (SCD). While individuals with SCT are typically asymptomatic, it is unclear whether red blood cells (RBCs) from SCT donors have impaired storage quality and transfusion efficacy. Here, we integrate multi-omics to characterize RBCs from donors SCT and evaluate their performance post-transfusion. We first profiled RBCs from 174 HbAS and 248 HbAA volunteers, identifying elevated levels of metabolic markers of the storage lesion in SCT RBCs at baseline. We then interrogated the REDS RBC Omics dataset (>13,000 donors), identifying blood donors carrying the HbS E6V variants. SCT RBCs exhibited accelerated metabolic aging, oxidative stress, and proteostatic activation—phenotypes further exacerbated by storage duration and co-inheritance of G6PD deficiency. Functional assays confirmed decreased osmotic fragility and increased oxidative hemolysis in SCT RBCs by storage Day 42. Pre-clinically, stored RBCs from Townes mice carrying one allele of human sickle hemoglobin were characterized by a drop in post-transfusion recovery compared to mice expressing canonical human hemoglobin. Clinically, analysis of 6828 transfusion events revealed that SCT RBCs were associated with lower hemoglobin increments 24 h post-transfusion. These findings provide mechanistic and clinical evidence that SCT influences RBC quality and transfusion outcomes. Given the overrepresentation of SCT in donor pools serving patients with SCD, our study supports a more personalized approach to inventory management and transfusion strategies in high-risk populations.

INTRODUCTION

Sickle cell trait (SCT) is the heterozygous carrier state for the human hemoglobin S (HbS) mutation (HBB: c.20A>T; p.Glu6Val or E6V), which affects ~300 million individuals worldwide and ~10% of Americans of African descent, reflecting the demographic overlap

between regions endemic for malaria and the African diaspora.¹ In 2010, the incidence of SCT in the United States was 15.5 per 1000 newborns, with markedly higher rates observed among Black and Hispanic infants (73.1 and 6.9 per 1000, respectively); state-specific incidence ranged from 0.8 per 1000 screened newborns in Montana to 34.1 per 1000 in Mississippi.² Although SCT is often considered benign, recent studies have challenged this assumption,^{3,4}

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revealing potential associations with exertional rhabdomyolysis, venous thromboembolism, and chronic kidney disease. The implications of SCT may also extend to areas previously considered immunologically or physiologically silent, including blood donation and transfusion medicine.⁵

Modern transfusion medicine increasingly recognizes that donor factors (e.g., age, sex, ethnicity, genetics, and environmental exposures⁶) can modulate red blood cell (RBC) storage quality and post-transfusion performance.^{7–14} The REDS (Recipient Epidemiology and Donor Evaluation Study) RBC Omics program involves large-scale investigation of the genomic, metabolomic, proteomic, and lipidomic determinants of inter-donor variability.¹⁵ By profiling >13,000 RBC units stored for 42 days under standard blood banking conditions, this program revealed reproducible donor-dependent signatures of storage lesion severity, hemolysis propensity, and post-transfusion hemoglobin increments, the latter gleaned by interrogating vein-to-vein databases.¹⁶

Against this backdrop, SCT represents a particularly compelling case. For example, SCT donors are overrepresented in the blood supply, due to population demographics and targeted recruitment to provide antigen-matched transfusions to patients with sickle cell disease (SCD). In addition, increasing evidence suggests that SCT RBCs behave differently during storage and post-transfusion.^{5,17,18} In early biomechanical studies using optical tweezers, HbAS RBCs retained normal elasticity early in storage, but 30% became rigid and undetectable in deformability assays by storage Day 35.¹⁷ In recent murine transfusion studies, stored human SCT RBCs, despite lower osmotic fragility, exhibited increased post-transfusion clearance in mice through splenic or hepatic sequestration.⁵ Nonetheless, caveats were raised regarding xenotransfusion studies in mice, as human RBCs are nearly twice the diameter of murine erythrocytes, promoting splenic sequestration and potentially biasing post-transfusion recovery (PTR) results.¹⁹ Although these limitations can be mitigated using syngeneic murine transfusion models, or by employing humanized mice expressing either normal human hemoglobin (HbA) or HbS, no study has directly assessed the PTR of stored RBCs in this humanized context, which is part of the present study.

Despite these reports of altered mechanical and clearance behavior, recent metabolomics studies suggest that RBCs from human SCT donors are not metabolically distinct from HbAA RBCs, at least when supernatants of stored units are analyzed.¹⁸ Specifically, targeted and untargeted profiling of amino acids and metabolites, including key intermediates of glycolysis, oxidative stress, and redox homeostasis, failed to reveal statistically significant differences between HbAS and HbAA units across multiple storage timepoints.¹⁸ This contrasts with larger metabolomics studies of volunteers with SCT, where changes in redox metabolism and acyl-carnitine pools implicated lipid oxidant stress at baseline²⁰ in the absence of storage-induced oxidative stress.²¹ This dissociation between structure and metabolism suggests a more nuanced biology; thus, although HbS in SCT may compromise cytoskeletal integrity and membrane biomechanics over time, it may not necessarily induce major shifts in steady-state metabolism during storage, particularly under normoxic, normothermic conditions, in part explaining the increased osmotic resistance of stored RBCs from SCT donors.⁵

Importantly, other common hemoglobinopathies and red cell disorders may follow opposite trends. For instance, β -thalassemia minor—another hemoglobinopathy frequently encountered in Mediterranean, Middle Eastern, and Southeast Asian populations—was unexpectedly associated with improved RBC storage quality and resilience to oxidative stress.²² Similarly, donor polymorphisms in glucose-6-phosphate dehydrogenase (G6PD),^{12,23,24} the rate-limiting enzyme of the pentose phosphate pathway (PPP), which generates NADPH to fuel virtually all RBC antioxidant pathways,²⁵ influence the severity of storage lesion and post-transfusion efficacy, reinforcing

the need to consider donor genotype in precision transfusion medicine²⁶ protocols. Thus, genotype–phenotype studies identified G6PD deficiency, the most common human enzymopathy, which affects ~13% of routine blood donors of African descent,²⁴ as a key genetic trait affecting storage quality and transfusion performance. However, the role of SCT on storage quality and transfusion efficacy remains untested and is the main focus of the present study.

To this, we first analyzed fresh RBC samples from a cohort of 174 individuals with SCT (HbAS) and 248 ethnically-matched HbAA controls. Even at baseline with freshly drawn blood, SCT RBCs exhibited metabolic and proteomics signatures typically associated with the storage lesion,²⁷ suggesting a predisposition towards storage-related oxidative and proteostatic stress.²⁸ Based on these findings, we hypothesized that SCT may negatively impact RBC storage quality and transfusion performance. To test this, we leveraged the REDS RBC Omics dataset, filtering >13,000 donor records by genome-wide single-nucleotide polymorphism (SNP) data to identify carriers of HbS and other HBB variants. Multi-omics and hemolytic profiling of stored units, combined with clinical outcome data from the vein-to-vein database, demonstrated that SCT RBCs exhibit a more severe storage lesion along with reduced post-transfusion hemoglobin increments. Thus, this integrated approach provides mechanistic insight and clinical context to the impact of SCT on the blood supply.

METHODS

In the interest of space, extensive details for this section are provided in Supporting Information S1.

RBCs from volunteers with HbAA and HbAS genotypes

RBC pellets were obtained from 248 volunteers with HbAA and 174 volunteers with SCT, enrolled under an institutional review board (IRB)-approved protocol NCT03685721. Whole blood (1 mL) was collected and centrifuged at 2000 $\times$ g for 10 min at 4°C before separation of RBC pellets for high-throughput metabolomics,^{29,30} proteomics,³¹ and lipidomics³² analyses.

REDS RBC Omics: Index and recalled donors

Metabolomics analyses were conducted on packed RBCs stored for 42 days from a cohort of 13,091 “index” donors recruited across four participating blood centers in the REDS RBC Omics study. Omics datasets were evaluated in relation to SNPs located within the HBB gene locus, based on genotyping data covering approximately 879,000 SNPs. A subset of donors ($n = 643$), identified as frequent donors, or within the 5th and 95th percentiles for end-of-storage hemolysis, were invited to provide a second (“recalled”) donation. These recalled RBC units were sampled at storage Days 10, 23, and 42, yielding 1929 samples for hemolysis testing and high-throughput metabolomics,^{29,30} proteomics,³¹ and lipidomics³² profiling. Subsequent analyses examined associations between molecular phenotypes (e.g., RBC susceptibility to oxidative, osmotic, and spontaneous storage hemolysis³³) and HBB genotypes, with a particular focus on the HbS variant (E6V rs334 SNP).

Storage and PTR studies in humanized HbAS SCT Townes mice

PTR studies in mice were conducted following established protocols.³⁴ C57BL/6J (good storing mice),⁷ 129S1/SvImJ mice

(poor storing mice),^{7,35–37} and humanized Townes mice expressing either homozygous human HbA or heterozygous HbAS ($n = 3$ per group) were used as RBC donors. The Townes mice used in this study (stock #013 071) are maintained on a mixed C57BL/6J;129S1/SvImJ genetic background. Because prior work^{7,35–37} has demonstrated marked strain-dependent differences in RBC storage quality and PTR between these two parental strains, inclusion of C57BL/6J and 129S1/SvImJ mice provided reference points to contextualize PTR performance of the humanized model within the known spectrum of murine storage resilience. After 12 days of refrigerated storage, donor RBCs were transfused into ubiquitin-GFP transgenic recipient mice (one per donor), enabling discrimination of transfused cells within the GFP-negative population. To account for potential variability in transfusion volume or blood sampling, freshly isolated mCherry-labeled RBCs were included as a tracer population and mixed with the stored test RBCs immediately before transfusion. PTR was determined as the ratio of test to tracer RBCs after transfusion, normalized to the pre-transfusion ratio. Final PTR values were expressed relative to a maximum of 1.0 (i.e., 100% recovery). PTR experiments were repeated twice.

Determination of hemoglobin and bilirubin increment via the vein-to-vein database

Association of the SCT E6V allele with post-transfusion hemoglobin and bilirubin increments was performed by interrogating the vein-to-vein database, as described.¹²

Statistical analysis

Data analysis and statistical analyses, including hierarchical clustering analysis (HCA), linear discriminant analysis (LDA), uniform Manifold Approximation and Projection (uMAP), correlation analyses, and Lasso regression, were performed using both MetaboAnalyst 5.0 and in-house developed code in RStudio (2024.12.1 Build 563).

RESULTS

Fresh RBCs from SCT carriers exhibit baseline elevations in markers of the storage lesion

To investigate whether SCT is associated with pre-existing alterations in RBC biology, freshly collected RBCs from 174 HbAS and 248 ethnic-matched HbAA control donors were analyzed (Figure 1A). Results are provided as a heat map of the top 100 variables by t -test P -value (Figure 1A; zoomed in version with variable names in Supporting Information S1: Figure 1) and a volcano plot (Figure 1B). Combined results from high-throughput metabolomics, lipidomics, and proteomics revealed that SCT RBCs exhibited significant changes, including (i) elevation in ubiquitin-dependent protein catabolism via the proteasome (e.g., USP5, USP7), a hallmark of the storage lesion^{28,38}; (ii) decreases in antioxidant systems, including decreased reduced glutathione (GSH), increased oxidized glutathione (GSSG), and decreased glutathione synthesis (GCLM) and GSH-dependent systems; (iii) decreases in proteins involved in blood coagulation and platelet aggregation; (iv) increases in tri- and diacylglycerols (TAG and DAG), consistent with membrane lipid remodeling and vesiculation; (v) changes in metabolites associated with the storage lesion, even prior to storage (Figure 1C), including decreased 2,3-bisphosphoglycerate (2,3-BPG)³⁹ and increased hypoxanthine,⁴⁰ and kynurenine, the latter being markers of hemolytic propensity.¹⁰ Taken

together, these findings reveal that even in freshly drawn blood, RBCs from individuals with SCT exhibit a phenotype consistent with mild oxidative stress, metabolic exhaustion, and early features of the storage lesion even at baseline.

Genomic profiling of the REDS cohort identifies associations of SCT and other *HBB* variants with storage-linked hemolysis

The REDS RBC Omics dataset includes 13,091 end-of-storage (i.e., Day 42) RBC samples from donors genotyped for 879,000 SNPs. These data identified 111 SCT (HbS heterozygotes), along with donors heterozygous for other *HBB* variants, including 38 with HbC (E6K, rs33930165 c.19G>A), 35 with a β -thalassemia major-associated mutation (Q128Stop, rs33971634 c.382C>T), 11 with a β^+ thalassemia-associated mutation (E27K, rs33950507 c.79G>A); 2 with a β^0 thalassemia-associated mutation (Q40E/K Stop, rs11549407 c.118C>T); 4 with hemoglobin Tyne (P6S, rs33912272 c.16C>T); and 2 with hemoglobin D (E121Q, rs33946267, c.364G>C) (Figure 2A).

Donors carrying SCT were mostly females of African descent, under 50 years of age, and overweight (body mass index [BMI] ≥ 30 kg/m²—Figure 2B), factors that are also linked to higher oxidative hemolysis and lower osmotic fragility.^{13,41} The RBCs of SCT donors exhibited the lowest osmotic fragility (Figure 2C), confirming prior results⁵; they also contained the lowest levels of GSH and the highest levels of GSSG and PPP products, consistent with the elevated oxidant stress seen in the 174 volunteers from the other cohort (Figure 1) and prior metabolomics studies of fresh SCT RBCs.²⁰ In addition, SCT RBCs showed significantly altered metabolic networks (Figure 2D), with stronger intra-class metabolite correlations (e.g., among PPP intermediates and redox cofactors) than controls, suggesting reorganization of core metabolic modules in response to oxidative load. Linear regression models adjusted for age, sex, ethnicity, and BMI confirmed that SCT status was associated with increased levels of lactate, methionine, hydroxyphenyl-pyruvate, cysteate, multiple long-chain fatty acids (e.g., FA 22:6, 20:4), and decreased mean corpuscular volume (MCV) (Figure 2E,F), but also with lower osmotic fragility (independent of storage additives or donor blood groups—Supporting Information S2: Table 1), higher oxidative hemolysis, and lower total hemoglobin levels (Figure 2F). Of note, a significant association was also noted between osmotic fragility and hemoglobin C trait or β^+ trait (Supporting Information S1: Figure 2).

SCT RBCs display exacerbated hemolytic phenotypes during storage

While the REDS RBC Omics index cohort offers a uniquely large population to explore the impact of gene-metabolite association, a major limitation is that these RBCs were only collected at the end-of-storage, Day 42. However, the REDS RBC Omics study also involved a recalled donor cohort, in which a second, independent donation from these donors was sampled on storage Days 10, 23, and 42. Seven donors with SCT were to enroll in this phase of the study (Figure 3A). Clustering SCT donors in the index and recalled cohort via uMAP showed some overlap with a subgroup of the donor population (Figure 3B,C). Principal component analysis (Figure 3D) confirmed this observation and identified that PPP and glutathione homeostasis metabolites informed this clustering; thus, these pathways were completely rewired in SCT donors, as determined by proteomics, metabolomics, and lipidomics results, throughout the

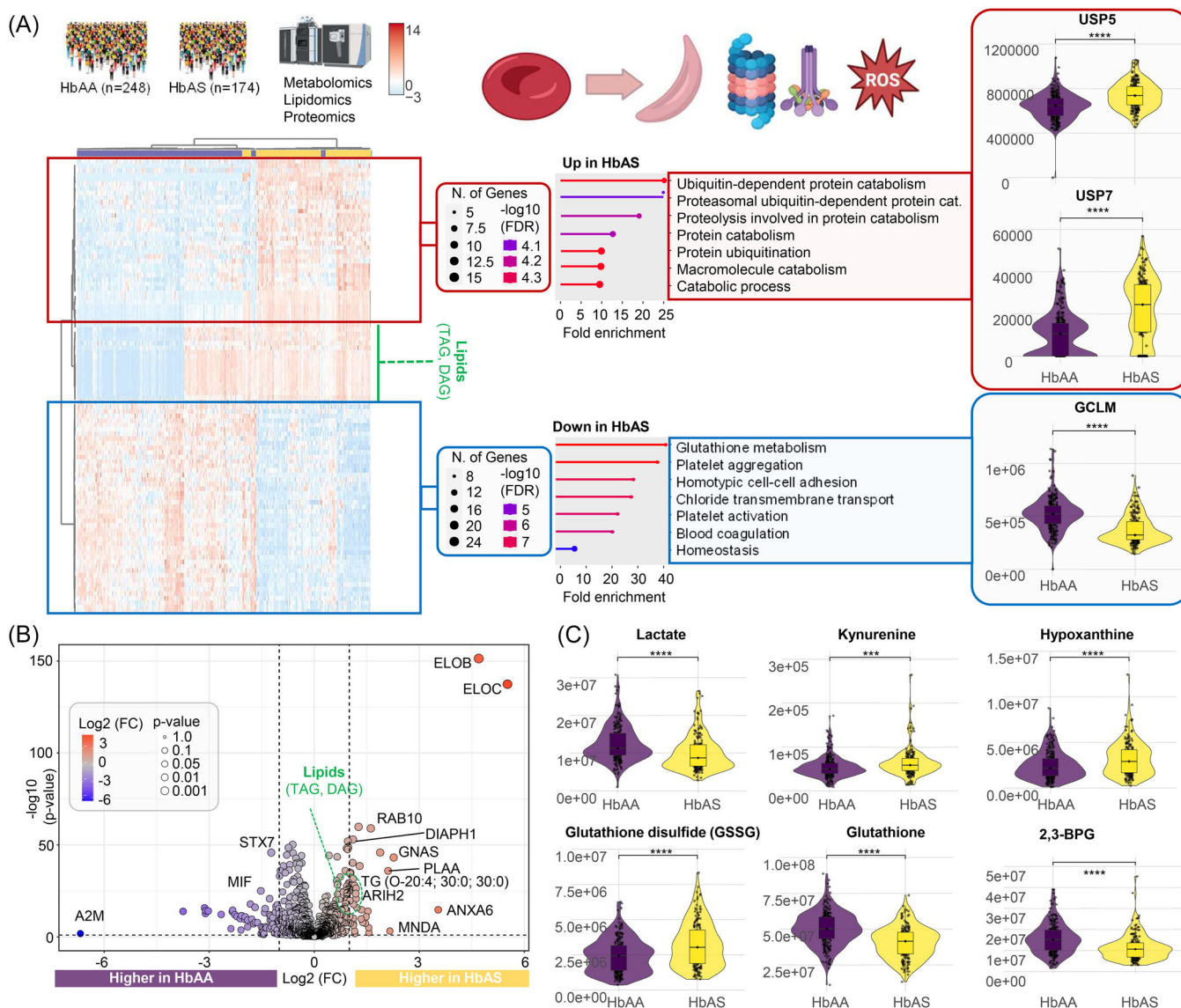


FIGURE 1 Baseline multi-omics signatures of red blood cells (RBCs) from subjects with sickle cell trait (SCT). Freshly collected RBCs from 174 HbAS and 248 HbAA subjects were analyzed by metabolomics, lipidomics, and proteomics. **(A)** Heatmap of the top 100 variables ranked by P-value, showing widespread metabolic remodeling in SCT RBCs, including activation of ubiquitin-dependent proteolysis and depletion of antioxidant pathways. **(B)** Volcano plots highlighting statistically different proteins and lipids between HbAA and HbAS RBCs pathway enrichment analyses indicate upregulation of proteasomal protein catabolism and lipid remodeling (tri- and diacylglycerols [TAG/DAG]) and downregulation of glutathione metabolism and platelet-activation pathways. **(C)** Violin plots highlight differential levels of lactate, kynurenine, hypoxanthine, glutathione (GSH), and oxidized glutathione (GSSG) and 2,3-bisphosphoglycerate (2,3-BPG). These data demonstrate that SCT RBCs already display early markers of the storage lesion before storage.

storage period (Figure 3E). A version of this network highlights PPP metabolites as the nodes with the highest betweenness centrality in the network of omics variables in these donors (Figure 3F).

LDA of multi-omics results in this cohort identified a strong association between SCT and histones (H2A1B, H4, perhaps due to reticulocytosis, although this was not measured), as well as multiple complement and coagulation components (CO3, CO4A, C4BPA, CO6, FIBA, FIBG, APOB, ITIH1, KNG1, HEMO, SAA4, HRG, and PLMN—Figure 4A), proteomics phenotypes previously linked to osmotic fragility,¹⁰ and untoward clinical consequences in people with SCD.⁴²

Consistent with the index data, SCT donors had significantly lower levels of osmotic and spontaneous storage hemolysis, lower

hemoglobin levels, and higher levels of acetyl-spermine, arginine, spermine, calcium, sodium, and dehydroascorbate (Figure 4B).

By proteomic analysis, stored SCT RBCs were enriched in stress response pathways, including increased abundance of proteasomal, endocytotic, ubiquitination, and complement proteins (e.g., CO3, CO4A, CO5, CO6, and ELOC), and alterations of cholesterol biosynthesis, glutathione homeostasis, and the PPP (Figure 4C,D; Supporting Information S1: Figure 2D). In parallel, multiple RBC indices, including MCV, hematocrit (HCT), and hemoglobin concentration (HGB by CBC—Figure 4E); and HBB peptide containing the E6V mutation by proteomics—Figure 4F were significantly decreased in SCT units, suggesting a shift toward microcytic and hypochromic

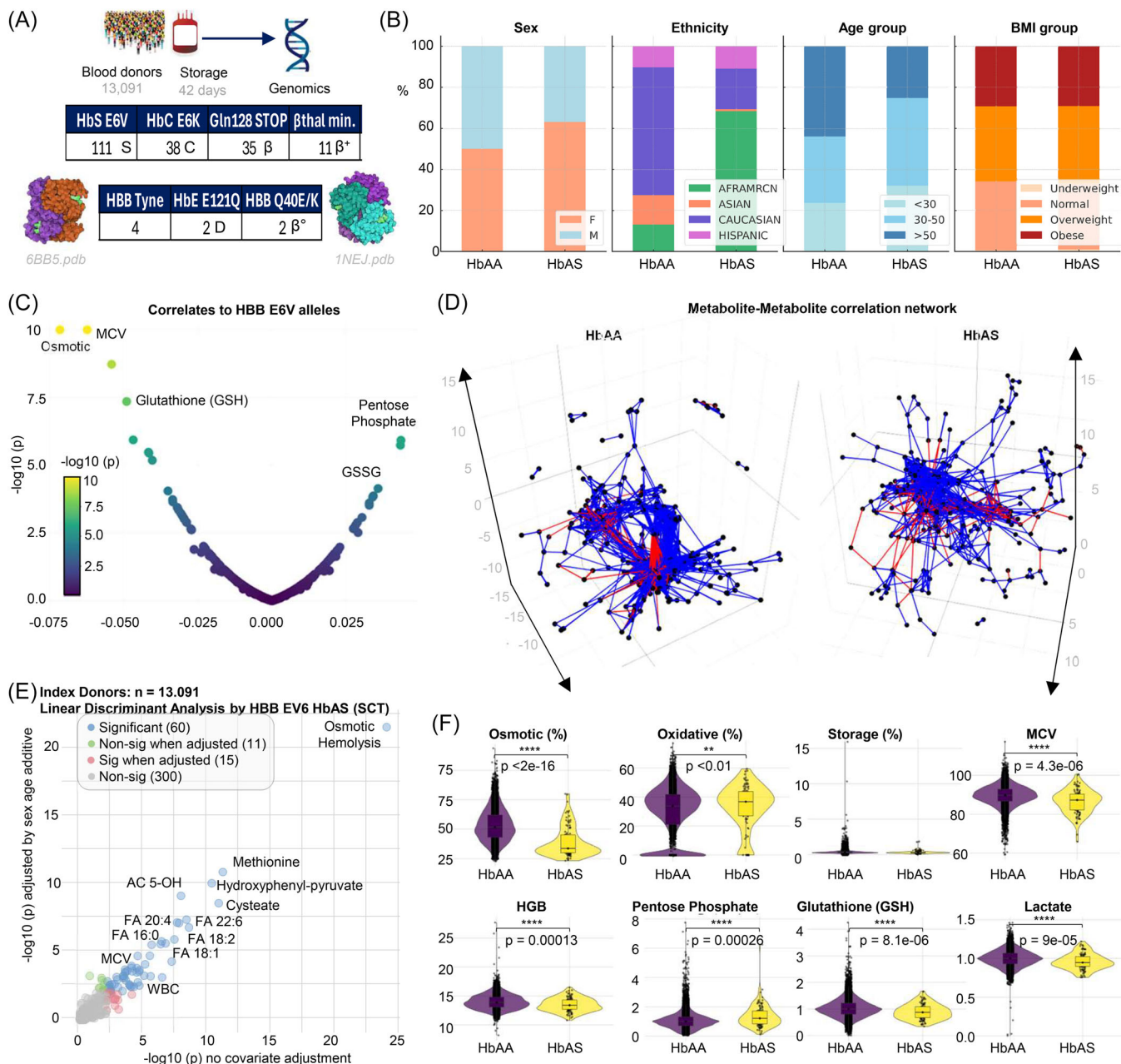


FIGURE 2 Genomic and metabolomic correlates of the HbS E6V variant in over 13 thousand blood donors from the Recipient Epidemiology and Donor Evaluation Study (REDS) red blood cell (RBC) Omics index cohort. **(A)** Distribution of HBB variants (E6V, E6K, E27K, Q128Stop, etc.) identified among 13,091 donors in the REDS III RBC Omics. **(B)** Demographic characteristics of HbAS donors by sex, ancestry, age, and body mass index (BMI). **(C)** Osmotic fragility and glutathione redox balance showing increased oxidative stress and reduced osmotic fragility in sickle cell trait (SCT) RBCs. **(D)** Metabolite-metabolite correlation networks illustrating rewiring of the RBC metabolic modules in HbAS donors. **(E, F)** Linear regression and linear discriminant analysis (LDA) results (adjusted for age, sex, ethnicity, and BMI) highlight increased lactate, methionine, cysteate, and long-chain fatty acids, with lower mean corpuscular volume (MCV) and HGB, and enhanced oxidative and osmotic hemolysis.

profiles in SCT RBCs as a function of storage duration (Supporting Information S1: Figure 3).

Proteomic/metabolomic signatures reveal accelerated dysfunction of oxidative and energy metabolism in SCT RBCs

Longitudinal profiling of S human RBCs across storage Days 10, 23, and 42 revealed progressive and faster metabolic deterioration in SCT RBCs,

as compared to HbAA RBCs (Figure 5). Conjugated bile acids (e.g., glycocholate, taurocholate), divalent cations (e.g., calcium, iron), glycolytic intermediates (e.g., PEP, pyruvate), PPP components (e.g., 6PGL, R5P), and nucleotide pools (e.g., ATP, AMP, and IMP) were depleted more rapidly in SCT RBCs (Figure 5A–D). Concurrently, oxidative stress markers, including GSSG, hypoxanthine, urate, and xanthine, accumulated more rapidly in SCT units (Figure 5D). By lipidomics analyses (Figure 5E,F; Supporting Information S1: Figure 3C,D), SCT RBCs showed increased accumulation of specific oxidized sphingolipids and lysophosphatidylcholines (e.g., SPH d18:1,

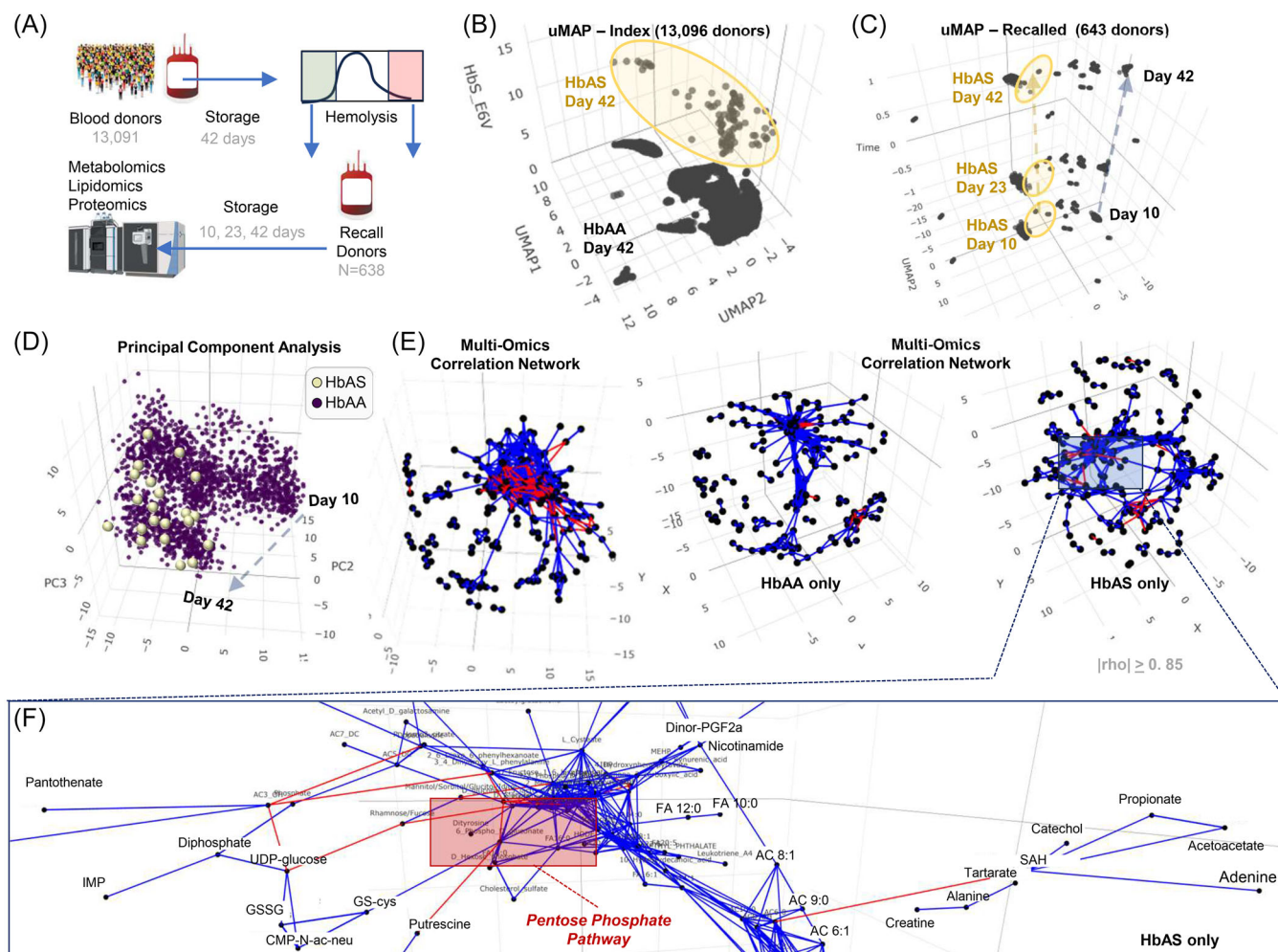


FIGURE 3 Storage-linked hemolytic phenotypes in recalled donors with sickle cell trait. (A) Study design for the Recipient Epidemiology and Donor Evaluation Study (REDS) red blood cell (RBC) Omics recalled cohort ($n = 643$ donors ranking <5 th or >95 th percentile for hemolysis in REDS Index, including 7 sickle cell trait [SCT] donors). (B, C) Uniform Manifold Approximation and Projection (uMAP) projections comparing index and recalled cohorts; SCT donors cluster with a distinct subgroup. (D) Principal component analysis showing strong segregation of SCT samples driven by pentose-phosphate and glutathione pathways. (E, F) Multi-omics correlation networks reveal metabolic rewiring in SCT RBCs with pentose phosphate pathway (PPP) metabolites showing high betweenness centrality.

LPC 20:4) and sphingosine-1-phosphate, consistent with accelerated membrane oxidation and vesiculation.⁴³

A subset of SCT donors are also G6PD deficient, which further exacerbates the phenotype

Because SCT donors were predominantly of African descent, we evaluated whether a subset of these donors also carried the rs1050828 SNP encoding the V68M “Common African” variant of X-linked G6PD, which is also prevalent in this ethnic background.¹⁴ Of the 111 donors with SCT, 11 heterozygous females and 7 hemizygous males exhibited this G6PD variant (Figure 6A), 15 of African descent, 1 of Hispanic descent, and 1 Caucasian. The combined influence of these two traits was most evident in the levels of S-adenosyl-homocysteine or sphingosine-1-phosphate/sphingosine—markers of oxidant isopartyl-damage to proteins⁴⁴ and of poor post-transfusion survival,⁴³ respectively. After confirming lower G6PD protein expression in donors with SCT who were also G6PD deficient (Figure 6C), we identified the top 50 significant variables (metabolites and hemolysis traits) affected by these two simultaneous

genetic variants (Figure 6D). These results identified a combined effect of both traits, especially regarding oxidative and osmotic fragility, the former worsened and the latter mitigated by G6PD status (Figure 6E). Similar compounding effects were noted for carboxylic acids (i.e., malate, fumarate), GSH and GSSG, NAD(P)H-homeostasis-linked glycerol 3-phosphate and biliverdin, 6-phosphogluconate, and cysteine (Figure 6E). A schematic overview of the pathways affected by SCT as a function of G6PD status is in Figure 6.

Post-transfusion recovery is reduced in humanized HbAS mice

To functionally assess whether the metabolic and proteomic alterations observed in HbAS RBCs translated into impaired *in vivo* performance, we performed PTR studies using humanized transgenic Townes mice expressing either canonical human HBB (HbAA) or the HbS E6V variant (HbAS) (Figure 7A). Because Townes mice (stock #013071) are maintained on a mixed C57BL/6J;129S1/SvImJ background—two strains known to exhibit markedly different storage resilience^{7,35–37}—we included both parental strains as reference comparators to contextualize

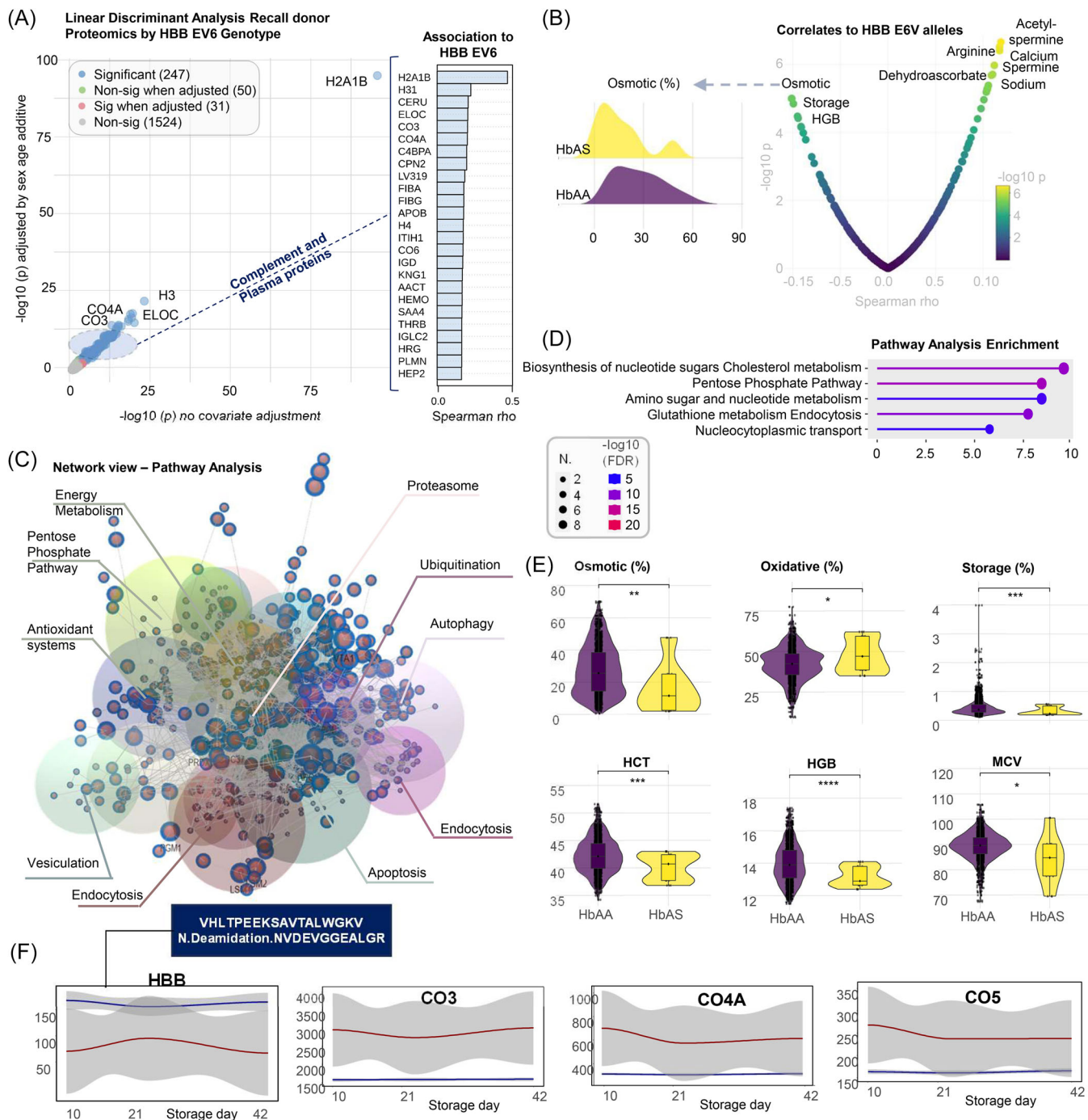


FIGURE 4 Proteo-metabolomic features of stored sickle cell trait (SCT) red blood cells (RBCs). (A) Linear discriminant analysis (LDA) of proteomics data identifies enrichment of histones, complement, and coagulation proteins in HbAS donors. (B) Metabolite-level differences showing higher acetyl-spermine, arginine, calcium, sodium, and dehydroascorbate in SCT RBCs, and reduced osmotic fragility and HGB. (C, D) Pathway enrichment for proteasome, endocytosis, autophagy, ubiquitination, apoptosis, and antioxidant systems. (E, F) Decreased mean corpuscular volume (MCV), HGB, hematocrit (HCT), and reduced HBB E6V peptide intensity during storage (Days 10–42) indicate microcytosis and progressive proteo-oxidative stress.

PTR values within the known spectrum of murine storage biology (Figure 7B). After 6 days of refrigerated storage, HbAS or HbAA RBCs were transfused into ubiquitin-GFP recipient mice alongside tracer fresh mCherry RBCs. PTR was calculated as the normalized ratio of test to tracer RBCs. As expected based on prior studies of strain-dependent storage performance, C57BL/6J RBCs exhibited the highest PTR, whereas 129S1/SvImJ RBCs showed lower PTR (Figure 7B). HbAS RBCs

showed a trend toward reduced PTR compared to HbAA Townes controls ($P = 0.0518$; Figure 7C). These findings are consistent with the proteo-metabolic signatures observed in HbAS RBCs, suggesting that alterations in redox homeostasis, membrane stability, and vesiculation propensity may contribute to reduced post-transfusion persistence in vivo, while acknowledging that this experiment demonstrates association rather than direct mechanistic causality.

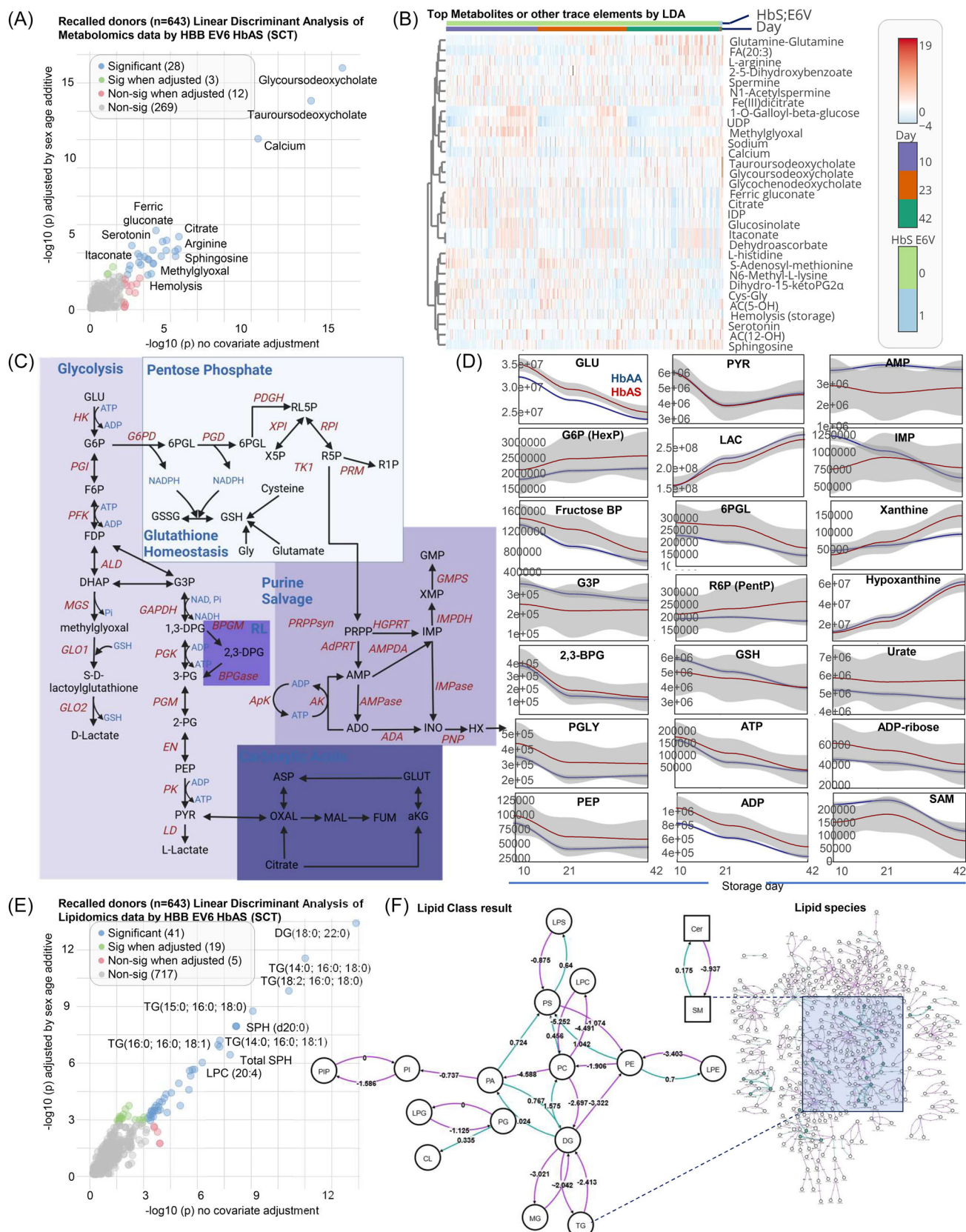


FIGURE 5 Accelerated oxidative and energetic decline during storage of sickle cell trait (SCT) red blood cells (RBCs). **(A–D)** Longitudinal metabolomics profiles of recalled donors showing faster depletion of glycolytic and pentose-phosphate intermediates (e.g., PEP, G6P, 6PGL, and R5P) and nucleotide pools (ATP, AMP, and IMP) and accumulation of oxidative by-products (hypoxanthine, urate, xanthine, and GSSG) in HbAS RBCs. **(E, F)** Lipidomics analyses identify increased oxidized sphingolipids, lysophosphatidylcholines, and sphingosine species, consistent with enhanced membrane oxidation and vesiculation. Results indicate accelerated storage-lesion kinetics in SCT units.

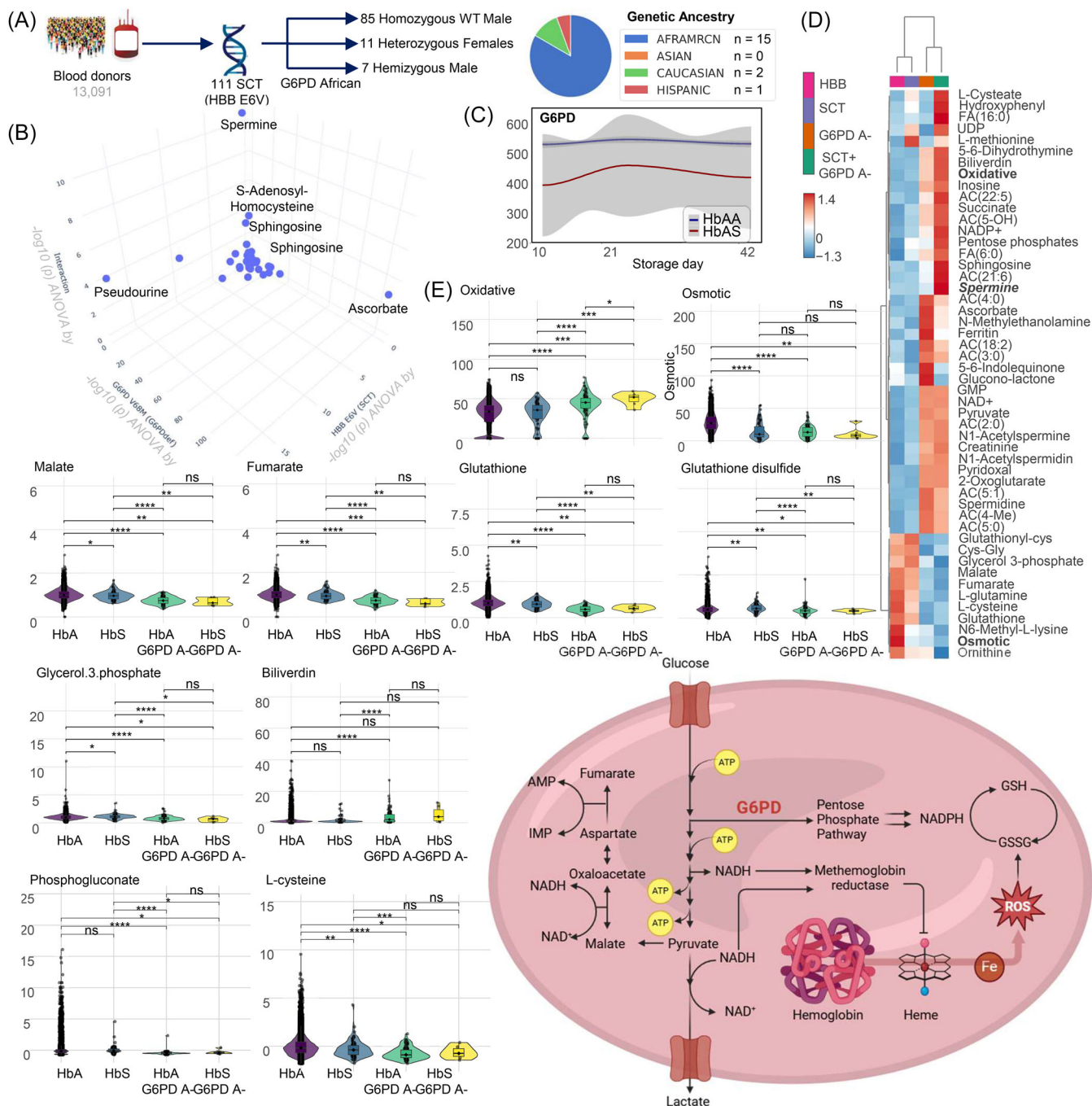


FIGURE 6 Combined effects of sickle cell trait and G6PD deficiency. (A) Frequency of G6PD African variant (V68M) among HbAS donors (11 heterozygous females, 7 hemizygous males). (B, C) Representative metabolites altered by dual inheritance of HbS E6V and G6PD deficiency, including elevated S-adenosyl-homocysteine and sphingosine-1-phosphate; reduced G6PD protein confirmed by proteomics. (D, E) Top 50 variables distinguishing HbAA, HbAS, G6PD-deficient, and double-trait donors, showing compounding effects on oxidative (GSSG, biliverdin, malate, and fumarate) and osmotic fragility. Pathway map summarizes overlapping oxidative-stress and metabolic vulnerabilities.

Transfusions of patients with human SCT donor units are associated with lower post-transfusion hemoglobin increments

To assess the clinical significance of SCT-related storage defects, we interrogated the vein-to-vein database comprising 6828 single-unit RBC transfusion events. After adjusting for confounding variables

(e.g., storage age; donor and recipient demographics; and recipient weight, sex, and comorbidities), SCT unit transfusions were associated with significantly lower hemoglobin increments at 24 h post-transfusion, as compared to HbAA unit transfusions (Figure 7D,E). This difference persisted across subgroup analyses and was independent of storage duration. These decreases in hemoglobin increment were further exacerbated by concomitant G6PD deficiency

DISCUSSION

The goal of this study was to characterize the biology of SCT RBCs at baseline, and after refrigerated storage and transfusion. Thus, multi-omics analyses of RBCs obtained from 174 HbAS and 248 ethnically-matched HbAA donors, suggested that RBCs from subjects with SCT exhibit elevated “metabolic markers of the storage lesion,”⁴⁵ even when freshly obtained. By genotyping >13,000 donors in the REDS RBC Omics study, volunteers carrying the HbS variant, as well as other HBB locus variants (e.g., HbC, HbE, E121Q, and β -thalassemia-associated mutations) were identified. Among these, the HbS allele was the most prevalent hemoglobinopathy-associated polymorphism, consistent with population-wide epidemiology and REDS donor ancestry distribution. Multi-omics profiling of these donors revealed that intracellular metabolite concentrations in SCT RBCs exhibited subtle, yet consistent, changes, with signatures associated with an accelerated storage lesion, faster depletion of high-energy (e.g., ATP) and antioxidant (e.g., glutathione, carnitine) pools, increased accumulation of oxidant damage and hemolysis markers, and exacerbated activation of the proteasomal machinery, the latter a hallmark of storage-induced small microcytic RBCs that are primed for splenic sequestration post-transfusion.³⁸

The progressive loss of ATP and redox buffering capacity during storage, paired with upregulation of ubiquitin-proteasome system components, highlights an early and persistent struggle to maintain homeostasis during storage. These cellular stress signatures align with previous evidence of increased rigidity and decreased deformability in SCT RBCs after prolonged storage,¹⁷ and are consistent with our findings of increased proteasomal degradation and lower MCV, hallmarks of small microcytic erythrocytes,³⁸ a sub-population of <43 μm^2 projected surface area that is prone to splenic sequestration after transfusion.⁴⁶ It must be noted that reticulocyte counts were not captured in this study, leading to potential confounders to MCV measurements. However, reticulocytosis would be expected to increase MCV, whereas units from donors with SCT show lower MCV both at baseline and during storage, suggesting that the microcytic trend is unlikely to be explained by reticulocytes alone and is more consistent with progressive membrane loss and vesiculation.

Notably, the observed alterations in stored SCT RBCs were not only of biochemical interest but also translated into functional phenotypes. Thus, SCT RBCs exhibited significantly increased osmotic fragility and oxidative hemolysis by storage Day 42, along with modest, but reproducible, differences in HCT, HGB, and MCV. Importantly, these *in vitro* findings were paralleled by clinical data from the vein-to-vein database; that is, among 6828 single-unit transfusion events, transfusion of SCT RBCs was associated with lower hemoglobin increments at 24 h post-transfusion, as compared to HbAA units; this effect remained significant even after adjusting for storage age and recipient covariates.

The observed increased resistance to osmotic stress links the observation from the present study to the existing literature regarding SCT RBC storage,⁵ and further corroborates similar observations about a disconnect between osmotic resistance and oxidative fragility in RBCs from donors of certain backgrounds (e.g., donors African descent, donors with higher BMI, and G6PD-deficient donors).¹⁴ In this regard, the current study has direct implications for transfusion practices, particularly within programs designed to provide antigen-matched RBCs to patients with SCD. Because these patients are predominantly of African descent, donor recruitment initiatives have appropriately focused on racially and ethnically matched populations. As a result, RBC units from individuals with SCT are disproportionately represented in inventories reserved for recipients with SCD. Although SCT donors are currently eligible to donate blood

in the United States, and their units are not routinely flagged for differential handling, our findings argue for a more nuanced approach. Specifically, if other alternatives are available, the transfusion of SCT RBCs may not be optimal in clinical contexts where post-transfusion efficacy is critical, such as in chronic transfusion regimens, exchange transfusions, and perioperative anemia management.

Importantly, these transfusion outcomes are underpinned by molecular and metabolic evidence of RBC fragility, and these results provide critical insights into the mechanisms that likely drive these functional deficits. Elevated baseline levels of oxidative stress markers, premature depletion of antioxidant defenses, and increased proteasomal activity suggest that SCT RBCs experience heightened proteo-oxidative pressure even prior to storage. These vulnerabilities are further exacerbated by refrigerated storage and by co-inheritance of traits that impair redox homeostasis. Notably, up to 16% of SCT donors in the REDS Index cohort were also heterozygous for the “common African” G6PD-deficient variant (i.e., V68M),⁴⁷ which provides only 10%–50% of residual enzyme activity. In the context of antigen-matched transfusion strategies, where donor and recipient ancestries frequently overlap,⁴⁸ this co-inheritance may lead to repeated transfusions with RBCs with an increased susceptibility to oxidative damage and premature clearance. This highlights the need for future transfusion algorithms that account not only for alloantigen compatibility but also for donor-intrinsic RBC quality traits that may affect therapeutic efficacy.

Although this study provides a comprehensive molecular and clinical portrait of SCT RBC biology, there are several limitations. First, although our fresh cohort and REDS RBC Omics data are large, the number of donors with rare *HBB* variants remains relatively small, limiting our ability to draw definitive conclusions about alleles such as HbC, HbE, or β -thalassemia mutations. Second, although the murine model leverages humanized Townes mice expressing human hemoglobins to approximate human transfusion outcomes, murine hemoglobin biology differs substantially from human hemoglobin in cysteine content and redox-active residues,^{7,35–37} and parental mouse strains (C57BL/6J and 129S1/SvImJ) are known to exhibit markedly different storage resilience and PTR driven by hemoglobin-dependent ferroptosis and redox pathways. For this reason, parental strains were included as reference comparators to contextualize PTR performance within the known spectrum of murine storage biology. Additionally, functional readouts such as RBC deformability, oxygen dissociation kinetics, and tissue oxygen delivery were not assessed. Thus, the absence of these data precludes conclusions about whether reduced RBC PTR meaningfully impacts tissue perfusion or clinical benefit. Further studies incorporating RBC deformability, vesiculation kinetics, RBC lifespan *in vivo*, and recipient measures of transfusion benefit (e.g., symptom relief, exercise tolerance) are necessary to refine mechanistic understanding. Finally, while we found statistically significant differences in post-transfusion hemoglobin increments in human patients, the clinical magnitude and relevance of these differences remain to be established.

Taken together, these findings provide a comprehensive systems-level view of SCT RBC storage biology, reconciling previous inconsistencies by demonstrating that SCT RBCs undergo progressive structural and redox deterioration during storage, culminating in reduced transfusion efficacy *in vivo*. Notably, we report that freshly drawn SCT RBCs already display metabolic hallmarks of the storage lesion, suggesting that these signatures may serve as donor-intrinsic biomarkers of storage resilience. These insights argue for renewed consideration of SCT status in donor screening and RBC inventory management, particularly in clinical scenarios where maximizing transfusion efficacy is critical. Thus, the clinical magnitude and consequences of these differences remain to be determined in

prospective human studies. Importantly, these results should not be interpreted as a call to exclude donors with SCT from the blood supply. Rather, they support the advancement of precision transfusion medicine, where donor-recipient matching extends beyond blood group antigen compatibility to include molecular and functional traits that influence RBC quality and clinical efficacy. Future clinical trials are essential to assess whether incorporating donor genotype information, including SCT and co-inherited variants, such as G6PD deficiency,⁴⁷ into transfusion decision-making improves outcomes in vulnerable patient populations, particularly those with high transfusion burdens such as individuals with SCD.

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AUTHOR CONTRIBUTIONS

Monika Dzieciatkowska: Investigation; methodology; formal analysis. **Daniel Stephenson:** Investigation; methodology. **Ariel Hay:** Investigation; methodology. **Xunde Wang:** Resources; project administration; investigation. **Gregory R. Keele:** Investigation. **Travis Nemkov:** Methodology. **Xutao Deng:** Data curation. **Mars Stone:** Resources; data curation; investigation. **Kirk C. Hansen:** Investigation; methodology; resources. **Steve Kleinman:** Supervision; resources; investigation; funding acquisition. **Philip J. Norris:** Investigation; funding acquisition; supervision; resources. **Michael P. Busch:** Investigation; funding acquisition; supervision; resources. **Grier P. Page:** Methodology; software. **Steven L. Spitalnik:** Conceptualization; writing—review and editing. **Nareg Roubinian:** Formal analysis; supervision; data curation. **James C. Zimring:** Investigation; methodology; formal analysis; supervision; funding acquisition. **Swee-Lay Thein:** Resources; investigation; validation; formal analysis; supervision. **Angelo D'Alessandro:** Conceptualization; investigation; funding acquisition; writing—original draft; visualization; formal analysis; project administration; supervision; data curation; resources.

CONFLICT OF INTEREST STATEMENT

The authors declare that A.D., K.C.H., and T.N. are founders of Omix Technologies Inc. A.D., T.N., and S.L.S. are Scientific Advisory Board (SAB) members for Hemanext Inc. A.D. is an SAB member for Macopharma Inc. and SynthMed Bio. S.L.S. is a SAB member for Alcor, Inc. All the other authors have no conflicts to disclose in relation to this study.

DATA AVAILABILITY STATEMENT

The NHLBI Recipient Epidemiology Donor Evaluation Study (REDS)-III Red Blood Cell Omics (RBC-Omics) and vein-to-vein databases are accessible at https://biolncc.nhlbi.nih.gov/studies/reds_iii/, and Genomics data are deposited at dbGaP Study Accession: phs001955.v1.p1. All raw data and elaborations are included in Supporting Information S2: Table 1. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Angelo D'Alessandro (angelo.dalessandro@cuanschutz.edu).

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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