# SUPPLEMENTARY MATERIAL for

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

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## SUPPLEMENTARY METHODS

### Detailed metabolomics methods

Filtered extracts were transferred to an ultra-high-pressure liquid chromatography (UHPLC-MS — Vanquish) equipped with a plate charger. A blank containing a mix of standards detailed before (1) and a quality control sample (the same across all plates) were injected 2 or 5 times each per plate, respectively, and used to monitor instrument performance throughout the analysis. Metabolites were resolved on a Phenomenex Kinetex C18 column (2.1 x 30 mm, 1.7 um) at 45 °C using a 5-minute gradient method in positive and negative ion modes (separate runs) over the scan range 65-975 m/z(2) exactly as previously described (3). The UHPLC was coupled online to a Q Exactive mass spectrometer (Thermo Fisher). The Q Exactive MS was operated in negative ion mode, scanning in Full MS mode (2 μscans) from 90 to 900 m/z at 70,000 resolution, with 4 kV spray voltage, 45 sheath gas, 15 auxiliary gas. Following data acquisition, .raw files were converted to .mzXML using RawConverter then metabolites assigned and peaks integrated using ElMaven (Elucidata) in conjunction with an in-house standard library (4).

### Statistical analysis of metabolomics data

To evaluate the impact of storage duration on recipient RBC and plasma metabolomes, we applied mixed-effects linear modeling to each metabolite individually. Only metabolites with at least 10 non-missing observations across subjects and timepoints were included, ensuring sufficient degrees of freedom for model stability.
For each metabolite, the following model was fit:

$$Y_{ij}=\beta_0+\beta_1(\text{Time}_{ij})+\beta_2(\text{StudyArm}_{ij})+\beta_3(\text{Time}\times\text{StudyArm}_{ij})+\beta_4(\text{AgeCriteria}_{ij})+ u_i+\epsilon_{ij}$$

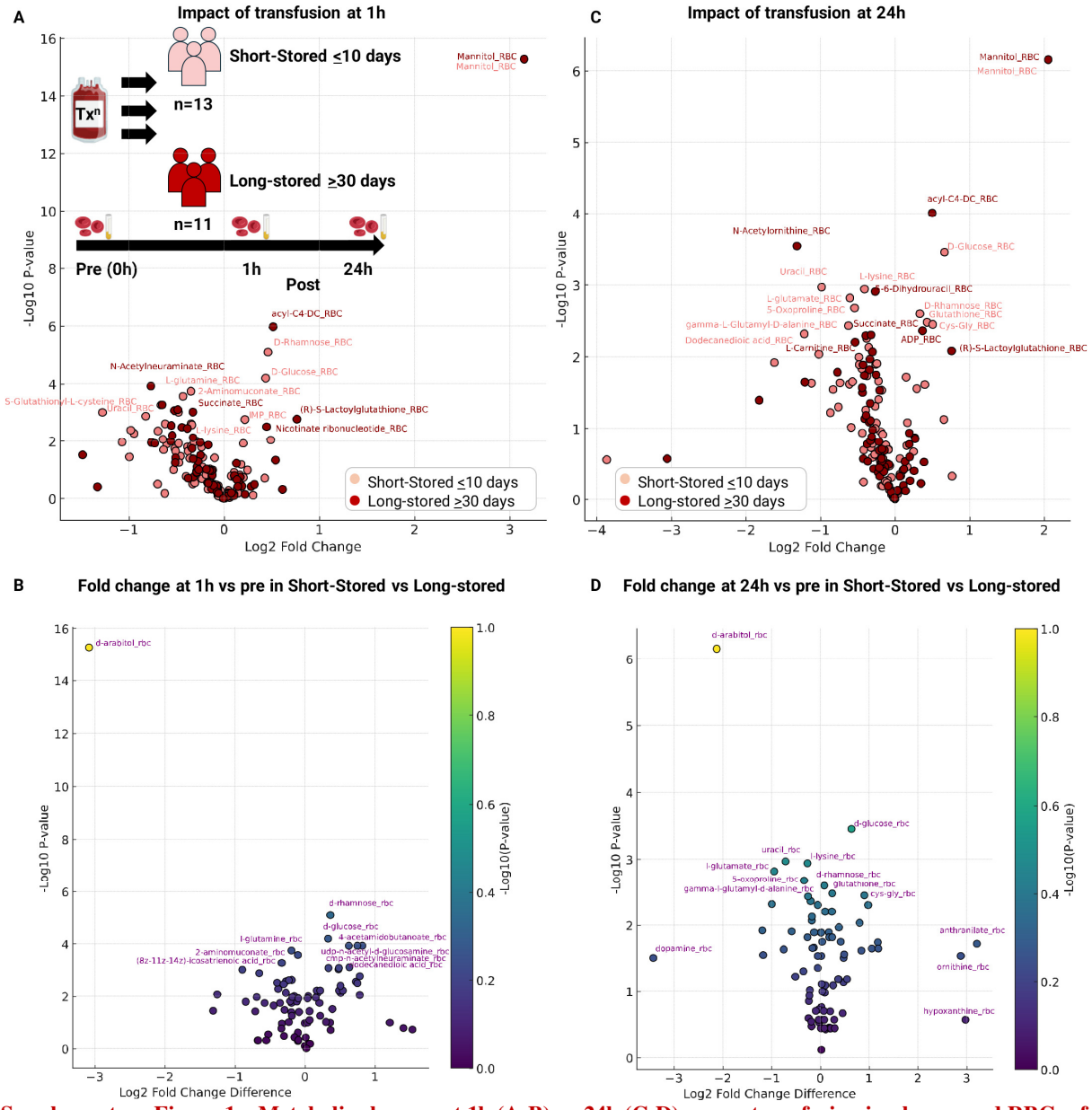
where:

- $Y_{ij}$ is the metabolite concentration for subject $i$ at timepoint $j$,
- **Time** represents the transfusion event timepoint,
- **StudyArm** denotes randomization to <10-day vs >30-day stored RBC units,
- **Time × StudyArm** captures the interaction between transfusion timing and storage age,
- **AgeCriteria** is a binary covariate adjusting for whether all transfused units met the intended storage duration (1 = yes, 2 = no),
- $u_i$ is a random intercept accounting for repeated measures within subjects,
- $\epsilon_{ij}$ is the residual error.

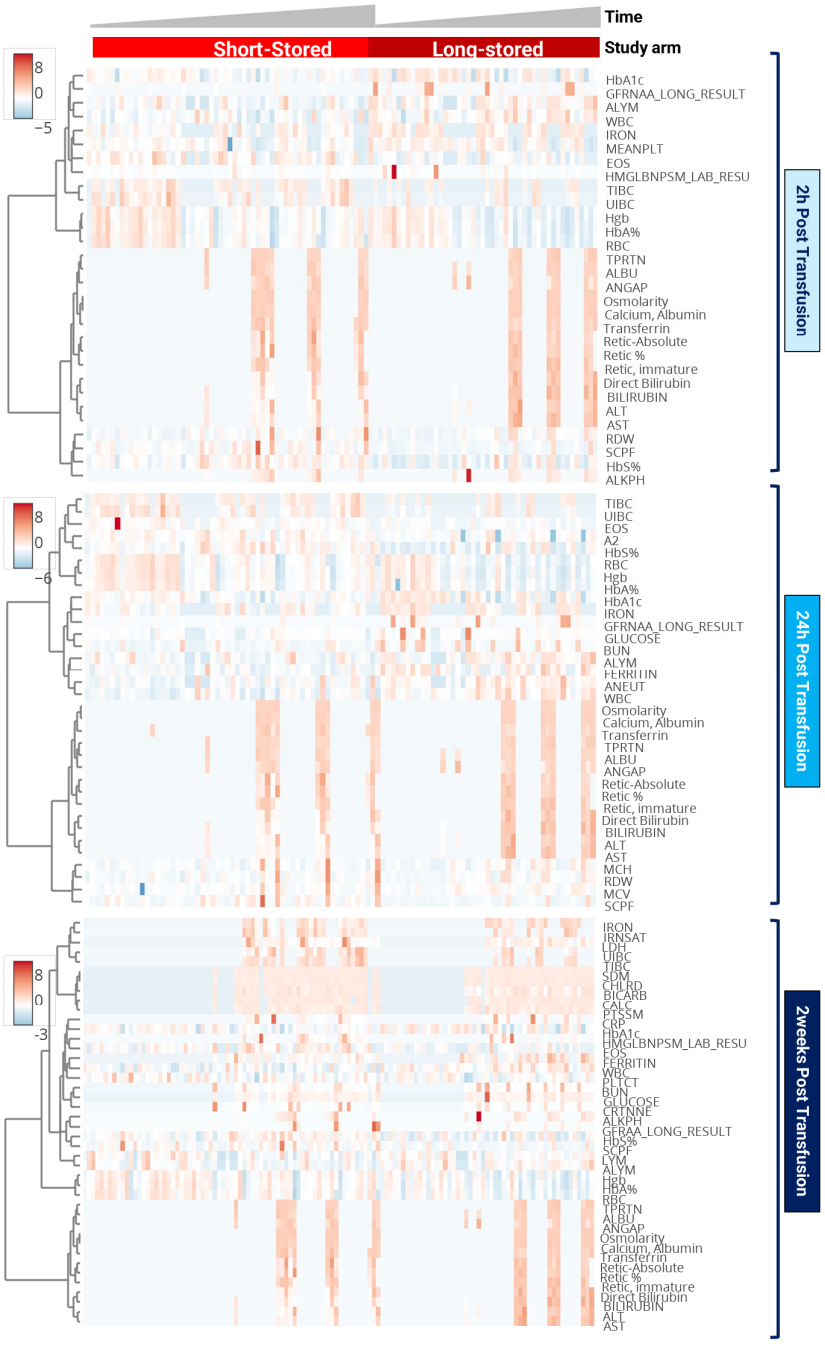
Models were fitted using the statsmodels package (v0.14.0) in Python, using the mixedlm function with maximum likelihood estimation.
P-values were extracted for fixed effects (Time, Study Arm, Time × Arm interaction, and Age Criteria adjustment).
Results were reported only for metabolites meeting data completeness thresholds to ensure model validity.

## SUPPLEMENTARY REFERENCES

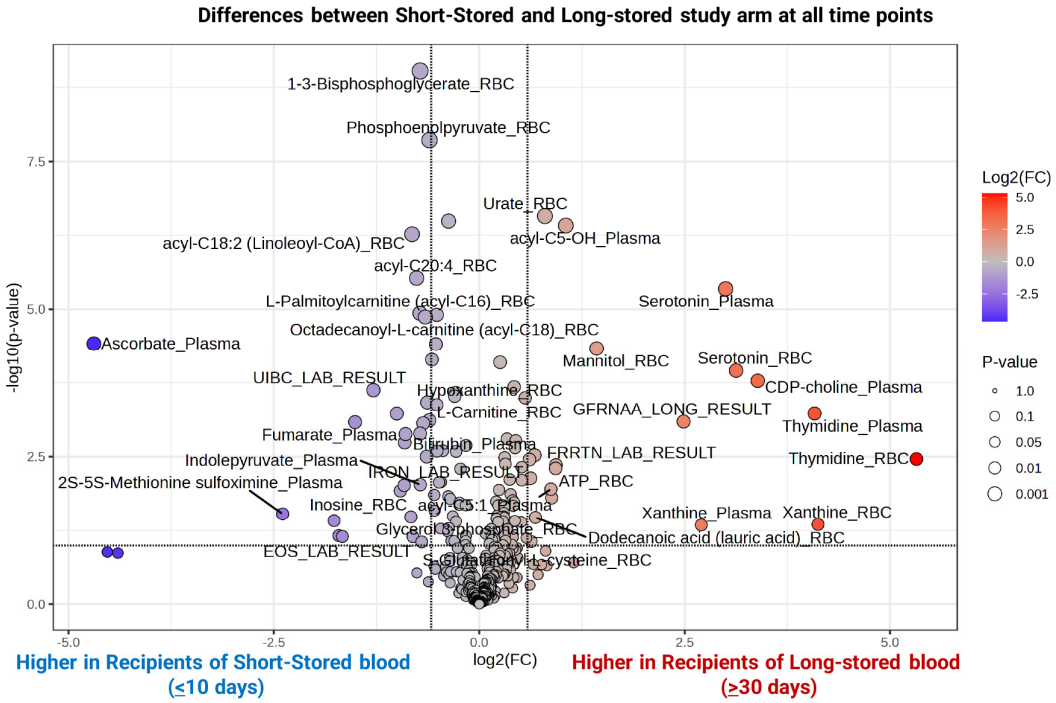

1. Stefanoni D, Shin HKH, Baek JH, Champagne DP, Nemkov T, Thomas T, et al. Red blood cell metabolism in Rhesus macaques and humans: comparative biology of blood storage. *Haematologica.* 2020;105(8):2174-86.
2. Nemkov T, Yoshida T, Nikulina M, and D'Alessandro A. High-Throughput Metabolomics Platform for the Rapid Data-Driven Development of Novel Additive Solutions for Blood Storage. *Front Physiol.* 2022;13:833242.
3. Nemkov T, Reisz JA, Gehrke S, Hansen KC, and D'Alessandro A. High-Throughput Metabolomics: Isocratic and Gradient Mass Spectrometry-Based Methods. *Methods Mol Biol.* 2019;1978:13-26.
4. Nemkov T, Hansen KC, and D'Alessandro A. A three-minute method for high-throughput quantitative metabolomics and quantitative tracing experiments of central carbon and nitrogen pathways. *Rapid communications in mass spectrometry : RCM.* 2017;31(8):663-73.



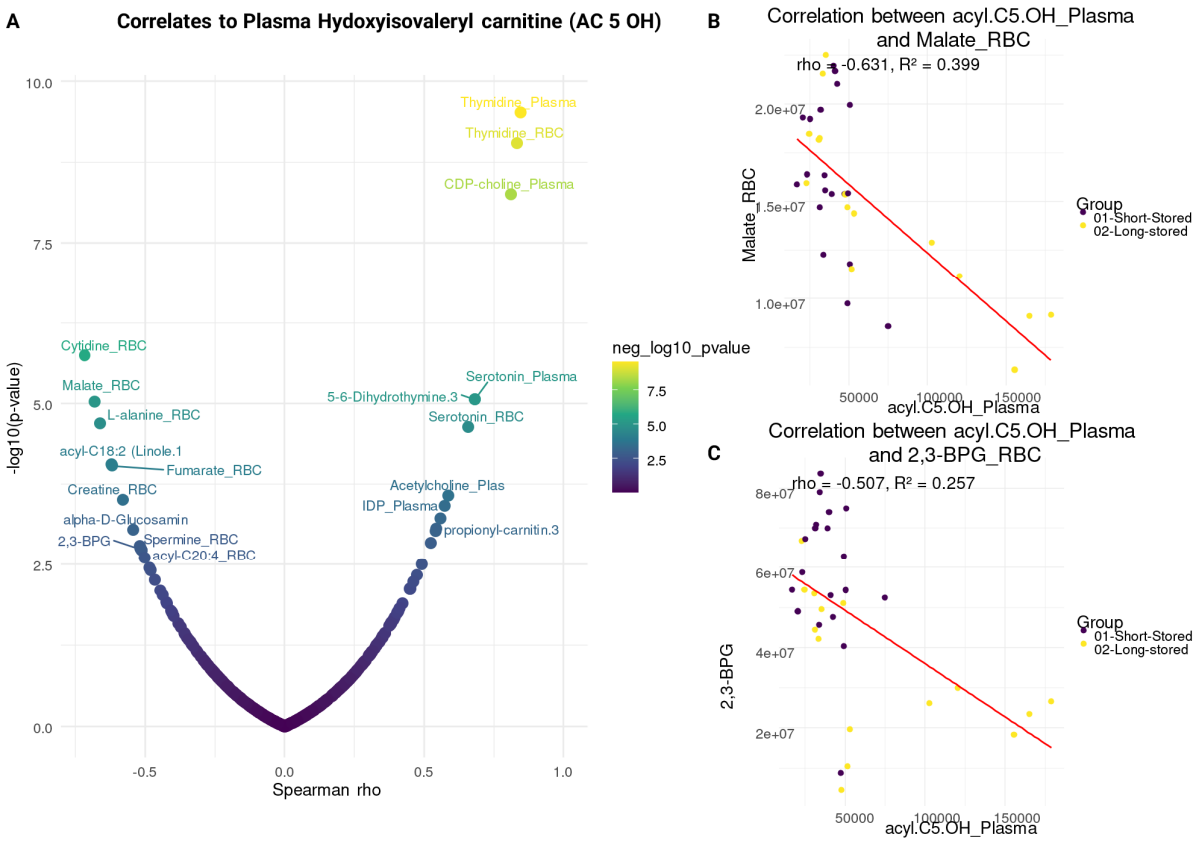

**Supplementary Figure 1 – Metabolic changes at 1h (A-B) or 24h (C-D) vs pre-transfusion in plasma and RBCs of recipients with sickle cell disease.** Results are either presented as volcano plots of pre- vs post-transfusion (**A-C**) or log2 fold change of post to pre-transfusion ratios (**B-D**).



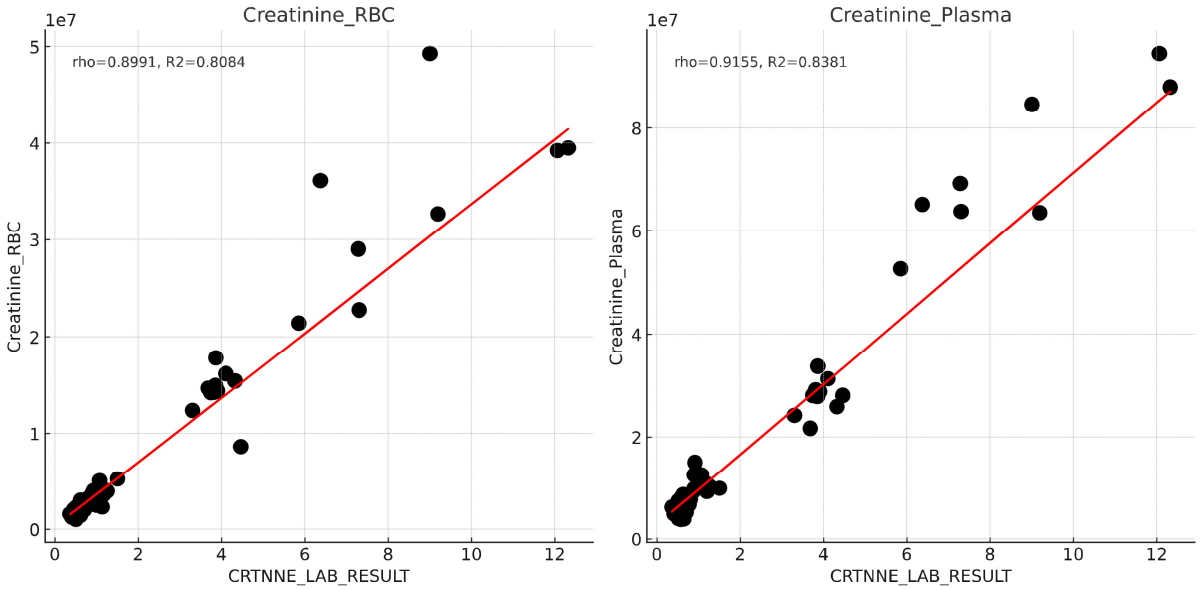

**Supplementary Figure 2 – Heat map of the clinical impact of transfusion of pRBCs stored ≤ 10 days (fresh) or ≥30 days (long-stored) on the clinical chemistry and complete blood counts of recipients with sickle cell disease.**



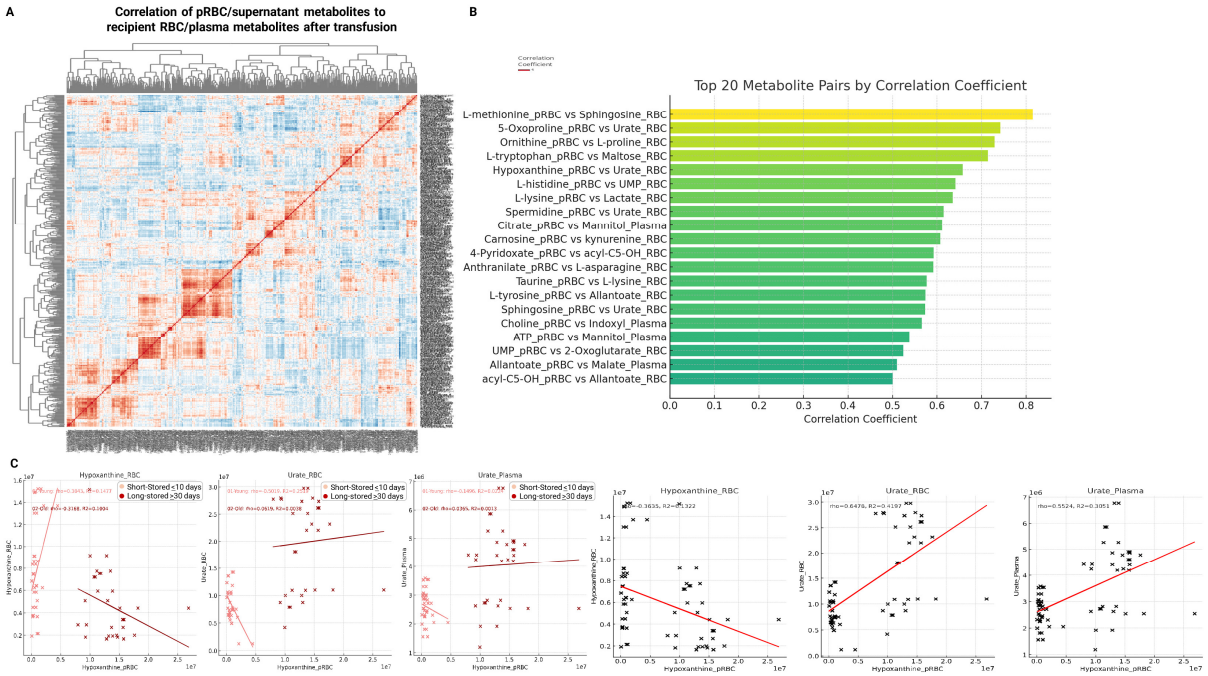

**Supplementary Figure 3 – Metabolic and clinical differences in RBC and plasma of recipients of pRBCs stored ≤10 days or ≥30 days.**



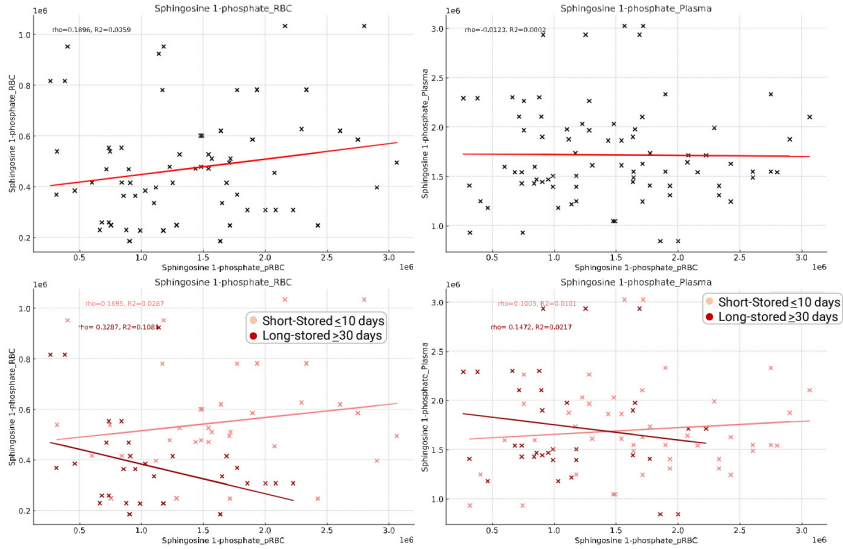

**Supplementary Figure 4 – Example of the interactive ShinyApp for data analysis of the results presented in this study, with a focus on hydroxyisovaleryl-carnitine – a product of branched-chain amino acid metabolism.**




**Supplementary Figure 5 – Correlation between RBC and plasma creatinine levels – as measured by mass spectrometry or CLIA-certified clinical chemistry assays.**




**Supplementary Figure 6 – Correlation between metabolites in the blood bag that was transfused (pRBC or supernatants) and metabolites in the post-transfusion RBC and plasma in recipients with sickle cell disease.** In **A-C**, results are shown as correlation matrix, bar plots of the top 20 strongest correlations, and related scatter plots, broken down by young vs old pRBC transfusion (light vs dark red, respectively).




**Supplementary Figure 7 – Levels of markers of hypoxia, like sphingosine 1-phosphate, poorly correlated between pRBC and recipients' RBC post-transfusion, or pRBC supernatants and recipients' plasma – independently of the storage age of transfused products.**