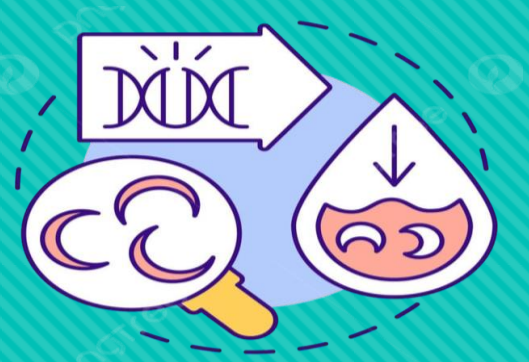

In vivo measurement of RBC survival in patients with sickle cell disease before or after hematopoietic stem cell transplantation

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Supervised by: Dr Mirzaee

CLINICAL TRIALS AND OBSERVATIONS | APRIL 9, 2024

In vivo measurement of RBC survival in patients with sickle cell disease before or after hematopoietic stem cell transplantation

Clinical Trials & Observations

Alexis K. Leonard, Dana Furstenau, Zaina Inam, Christina Luckett, Rebecca Chu, Selami Demirci, Khaled Essawi, Bjorg Gudmundsdottir, Malikiya Hinds, Julia DiNicola, Quan Li, William A. Eaton, Troy Cellmer, Xunde Wang, Swee Lay Thein, Elizabeth R. Macari, Sara VanNest, Matthew M. Hsieh, Melissa Bonner, Francis J. Piercley, John F. Tisdale

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Connected Content

A related article has been published: Long live the red blood cell: biotin tagging in SCD

- Biotin labeling of RBCs is a safe and feasible methodology to evaluate RBC survival in patients with SCD before and after HSCT.
- Understanding differences in RBC survival may inform hemoglobin composition thresholds required to reverse the phenotype after gene therapy.

Volume 8, Issue 7
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Potential Articles of Interest

Long live the red blood cell: biotin tagging in SCD
Sarah McCuskee, Blood Advances, 2024

At Least 20% Donor Myeloid Chimerism Is Necessary to Reverse the Sickle Phenotype after Allogeneic Hematopoietic Stem Cell Transplantation
Fitzhugh, Blood, 2016

At least 20% donor myeloid chimerism is necessary to reverse the sickle phenotype after allogeneic HSCT
Courtney D. Fitzhugh, Blood, 2017

Red blood cell transfusion in sickle cell disease
Kelsey Uminski, CMAJ, 2023

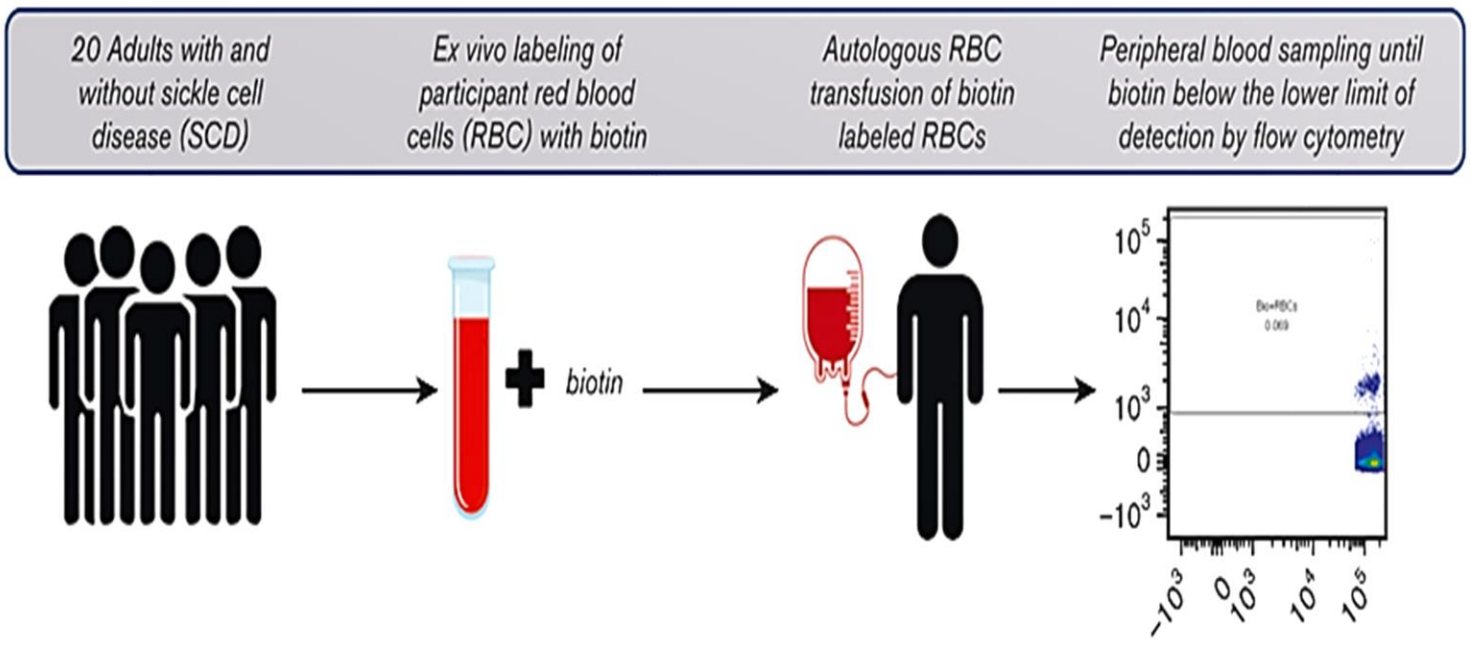
Altered Collection Interfaces in Hematopoietic Progenitor Collection of Sickle Cell Patients
Jonathan Tsai, American Journal of Clinical Pathology, 2019

Title	Subject Category	Publisher/ Holder	IF	IF Quartile	CiteScore	CiteScore Quartile
Blood Advances ISSN/ISBN: 2473-9529, 2473-9537	10%	Other	7.400	Q1	12.70	Q1

Introduction

- Sickle cell disease (SCD) is a severe form of hemolytic anemia in which an inherited mutation in the β-globin gene results in HbS polymerization in RBCs.
- Several biochemical markers correlate with RBC survival in patients with SCD including: ARC, a-globin gene number, and percentage of HbF
- The only current curative treatment for SCD is allogeneic HSCT.
- 20% stable, mixed-donor–recipient chimerism after allogeneic HSCT, is sufficient for phenotypic disease reversal, and results from differences in donor/recipient RBC survival.
- Understanding the variability and predictors of red cell survival among patients with SCD vs individuals with SCT and healthy donors is critical for gene therapy approaches.

Methods


20 Adults with and without sickle cell disease (SCD)
Ex vivo labeling of participant red blood cells (RBC) with biotin
Autologous RBC transfusion of biotin labeled RBCs
Peripheral blood sampling until biotin below the lower limit of detection by flow cytometry
biotin
10^5
10^4
10^3
0
-10^3
-10^3
0
10^3
10^4
10^5

Participant Selection

- Patients with SCD, patients with SCD who have undergone HSCT, individuals with SCT, and healthy donors **=>** determine and compare RBC survival
- Participants aged ≥18 years were eligible if:
  - Confirmed diagnosis of SCD (HbSS, HbSC, HbSβ+, and HbSβ0)
  - SCT (HbAS)
  - Healthy volunteer (HbAA)
- With:
  - Normal renal function (creatinine <1.5 mg/dL)
  - Negative DAT
  - Ability to provide informed consent
- Participants were excluded if:
  - Any uncontrolled chronic illness other than SCD
  - Active infection
  - Consumption of biotin supplements within 30 days
  - Blood loss >540 mL within the previous 8 weeks
  - Pregnancy
  - Preexisting antibodies against biotin
- All participants were evaluated with a medical history, physical examination, screening labs, and venous assessment.

Biotinylation of RBCs

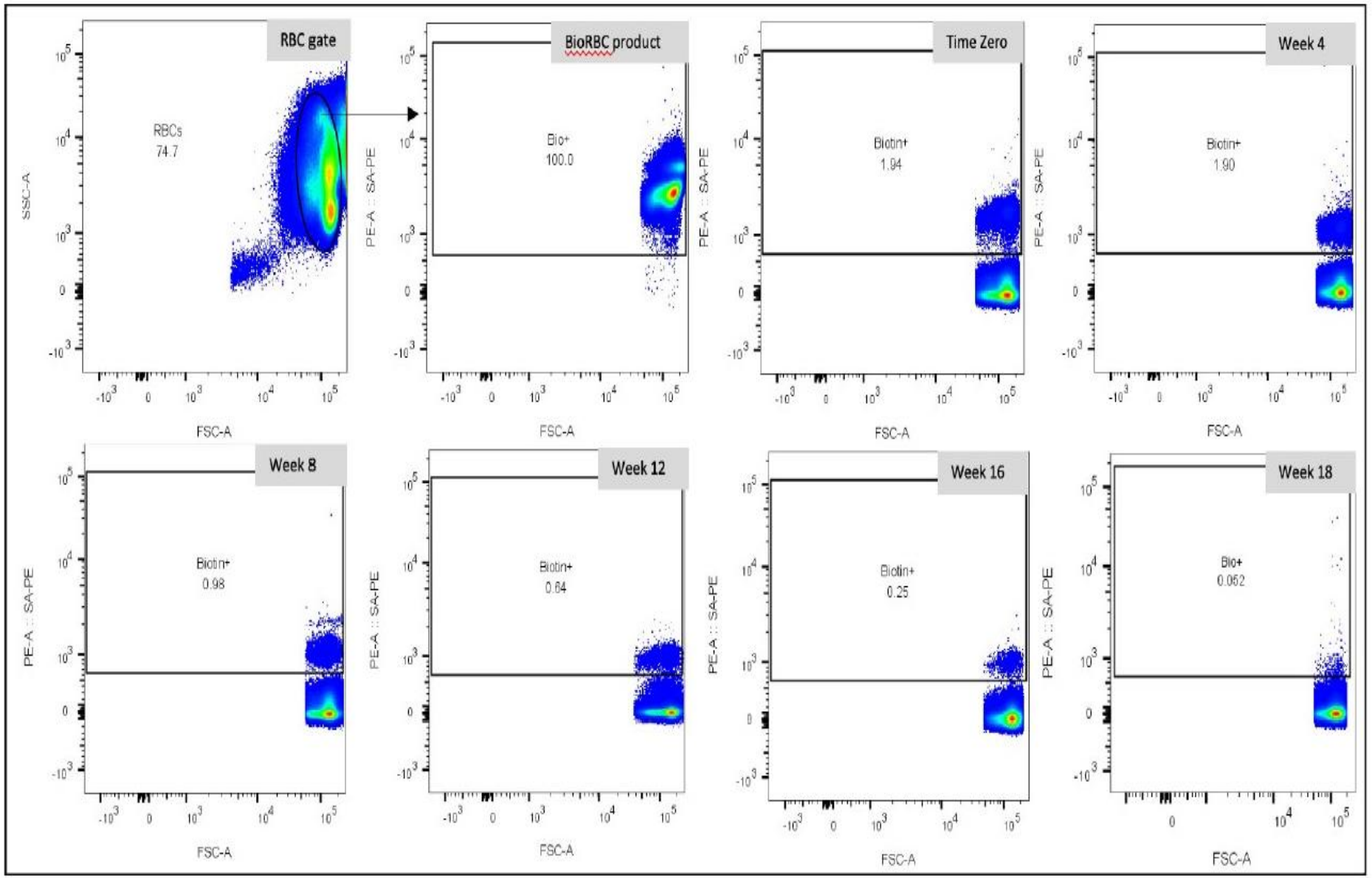
- 100 mL of venous blood was collected in acid-citrate-dextrose anticoagulant:
  - to obtain >15 mL of packed RBCs for biotinylation of RBCs.
  - routine labs
  - a-globin genotyping
- Whole blood was centrifuged **=>** The plasma layer was aspirated and saved **=>** RBCs were resuspended to a 25% suspension of RBCs **=>** biotin solution was added (final concentration of biotin =18 µg/mL)
- Cells were incubated at room temperature for 30 minutes, washed, and resuspended **=>** The washed biotinylated RBCs (BioRBCs) were resuspended in autologous plasma at a Hct of 50%.
- Before release of the cellular product, samples were removed for:
  - complete blood count
  - biotin labeling efficiency determination via flow cytometry
  - safety assays (sterility and endotoxin)

Infusion of Product and Sample Collection

- BioRBCs were reinfused via an 18- or 20-gauge peripheral or central IV (The time at 20 minutes after the end of the infusion was designated time 0)
- 5 mL of venous blood was collected at 20 minutes after infusion (time 0) in all participants.
- Participants with SCD who did not undergo transplantation:
  - samples collected bi-weekly for the first 2 weeks
  - then weekly until the percentage of BioRBCs decreased to the lower limit of detection (less than ~0.06%)
- All other participants:
  - samples drawn weekly for the first 4 weeks
  - then every other week until the percentage of BioRBCs decreased to the lower limit of detection (less than ~0.06%)
- All participants had antibiotin antibody testing (using gel card antibody detection) done at screening, at 12 weeks after infusion, and at 6 months after infusion.

Determination of BioRBC Survival, F-cells, and Hb content

- Samples were analyzed for:
  - Percent survival of BioRBCs by flow cytometry
  - F-cell percentage using anti-HbF antibody
- Hb content was determined using high performance liquid chromatography on:
  - Whole-blood samples
  - The biotin-positive fraction after isolation by flow cytometry
  - Reticulocytes
- Reticulocytes were isolated at least once on every participant using magnetic bead separation.



Supplemental Figure S1. Flow cytometry plots of an example participant.

Samples were analyzed in real time for percent biotinylated RBCs remaining in circulation. Samples were analyzed at specified intervals by flow cytometry using a streptavidin-conjugate fluorochrome until the percentage of biotinylated RBCs decreased to the lower limit of detection (approximately less than 0.06%).

Metabolite and Sickling Measurements

- Whole blood levels of ATP, 2,3-DPG and pyruvate kinase protein were measured. (using liquid chromatography tandem mass spectrometry)
- For measurement of sickled cells at the end of deoxygenation:
  - Whole-blood samples were collected and diluted into pbs solution.
  - 10 µL of the cell suspension was added to each well of a 384-well plate.
  - The plate was inserted into the 37◦C humidified chamber of a BioTek automated microscope.
  - Deoxygenated using the BioTek O2 gas controller with 100% N2 to 5% O2 to induce sickling.
  - Images of cells were collected every 15 minutes for 9 hours.
  - Sickling times for each cell were determined at 15 minute intervals with an image analysis software.
  - The output of an experiment is a plot of the fraction of sickled cells vs time.

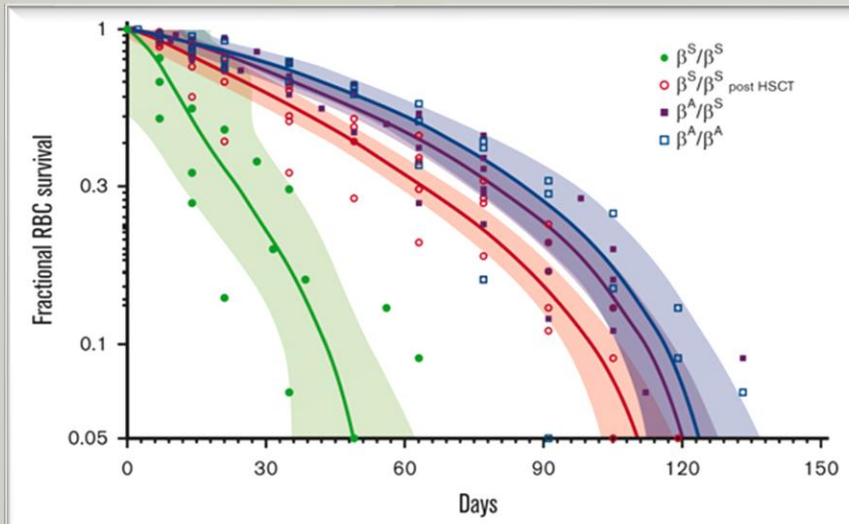
Single cell Western

- To determine the proportion of RBCs that contained HbA, HbS, or both in whole-blood or biotin enriched fractions:
  - ~50 000 RBCs were loaded onto a small single-cell Western chip
  - After washing, gel electrophoresis separation is performed
  - The chip is probed with: an anti-HbA, a secondary antibody, and an anti-HbS.
  - Analyzed using the Scout software (to determine the proportion of occupied wells that were positive for HbA, HbS, or both)

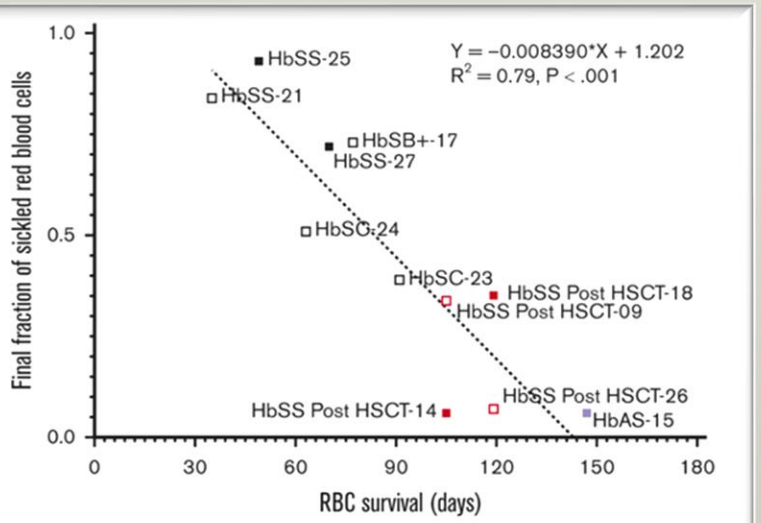
Data and Statistical Analysis

- RBC survival was determined by plotting the percentage of BioRBCs in circulation vs time after transfusion.
- Average lifespan among the groups studied was compared using means and standard deviations.
- Correlation of mean RBC survival with various markers of red-cell survival was performed using Pearson Correlation. (A P-value of <0.05 was considered statistically significant)

Results



Biotin labeling of RBCs is a safe and feasible methodology to evaluate RBC survival in patients with SCD before and after HSCT



RBC lifespan strongly correlates to the final fraction of sickled RBCs after deoxygenation and separates symptomatic from asymptomatic individuals

Table 1. Participant characteristics

Clinical parameters	N = 20 (%)
Sex	
Male	7 (35%)
Female	13 (65%)
Age median (range)	43.5 (19-62)
Genotype before HSCT	
AA	3 (15%)
AS	6 (30%)
SS	3 (15%)
$S\beta^+$	1 (5%)
SC	2 (10%)
Genotype SCD after HSCT (N = 5), SS	5 (100%)
Alpha globin gene mutation	
Single mutation	10 (50%)
No mutation detected	10 (50%)

Hematologic data at time of enrollment (mean, SD)	
WBC (K/mcL)	6.6 ± 3.1
RBC (M/mcL)	4.0 ± 0.9
Hemoglobin (g/dL)	11.5 ± 2.3
Hematocrit (%)	34.2 ± 7.5
MCV (fL)	85.9 ± 7.6
Platelet count (K/mcL)	308.2 ± 116.4
ARC (K/mcL)	125.8 ± 82.2
HbF (%)	3.2 ± 6.4
HbA (%)	48.2 ± 32.9
HbA2 (%)	3.2 ± 0.5
HbS (%)	41.2 ± 24.1
HbC (%)	43.2 ± 0
AST (U/L)	22.6 ± 8.8
Total bilirubin (mg/dL)	1.0 ± 0.9
LDH (U/L)	254.2 ± 111.2
Study duration mean number of days (range)	103 (35-147)

Table 2. Characteristics of participants with SCD

	Pre-HSCT (SCD)	Post-HSCT
Total N (%)	6	5
Sex		
Male	2 (33%)	4 (80%)
Female	4 (67%)	1 (20%)
Age (median, range)	41.5 (26-61)	31 (19-54)
Hydroxyrea use (at time of study)	4 (67%)	0
Type of transplant		
Matched related donor	NA	3 (60%)
Haploidentitical	NA	2 (40%)
Stem cell donor genotype		
AS	NA	4 (80%)
AA	NA	1 (20%)
Chimerism at time of enrollment		
Full DMC	NA	3 (60%)
Mixed DMC	NA	2 (40%)
Hematologic data, average		
WBC (K/mcL)	8.9	6.9
RBC (M/mcL)	3	4.1
★ Hemoglobin (g/dL)	8.9	12.4
★ Hematocrit (%)	25.2	36.6
MCV (fL)	86	90.2
Platelet count (K/mcL)	406.4	277
◎ ARC (K/mcL)	191.6	175
★ HbF (%)	10.2	1
★ HbA (%)	0	56.8
HbA2 (%)	3.7	3.1
★ HbS (%)	68.8	39.1
HbC (%)	43.3	NA
◎ AST (U/L)	26.3	27.5
◎ Total bilirubin (mg/dL)	1.7	1.2
◎ LDH (U/L)	324.9	271.5
Time from transplant mean ± SD (mo)	NA	29.8 ± 17.3

Biotin labeling of RBCs: Safe and Feasible

- There were no AEs attributable to the use of biotin or the infusion of BioRBCs during this study.
- No antibodies to biotin were detected throughout the study.
- Hct was measured before and after biotin labeling:

  => No differences were observed between healthy volunteers (HbAA) and those with SCD or SCT

  => suggesting that RBC loss during processing was minimal.

RBC survival
(Figure 1)

- The average RBC lifespan was significantly shorter ($P < .001$) for participants with SCD (HbSS) before HSCT (51.3 days, range 35-70) compared with:
  - SCD after HSCT (113.4 days, range 105-119)
  - HbAS (126.0 days, range 119-147)
  - HbAA (123.7 days, range 91-147)
- There were no statistically significant differences in lifespan in:
  - The different genotypes of participants with SCD before HSCT (HbSS 51.3 days vs HbSC/HbSβ+ 77.0 days)
  - Participants with SCD after HSCT with full vs mixed donor chimerism (114.3 days vs 112.0 days)

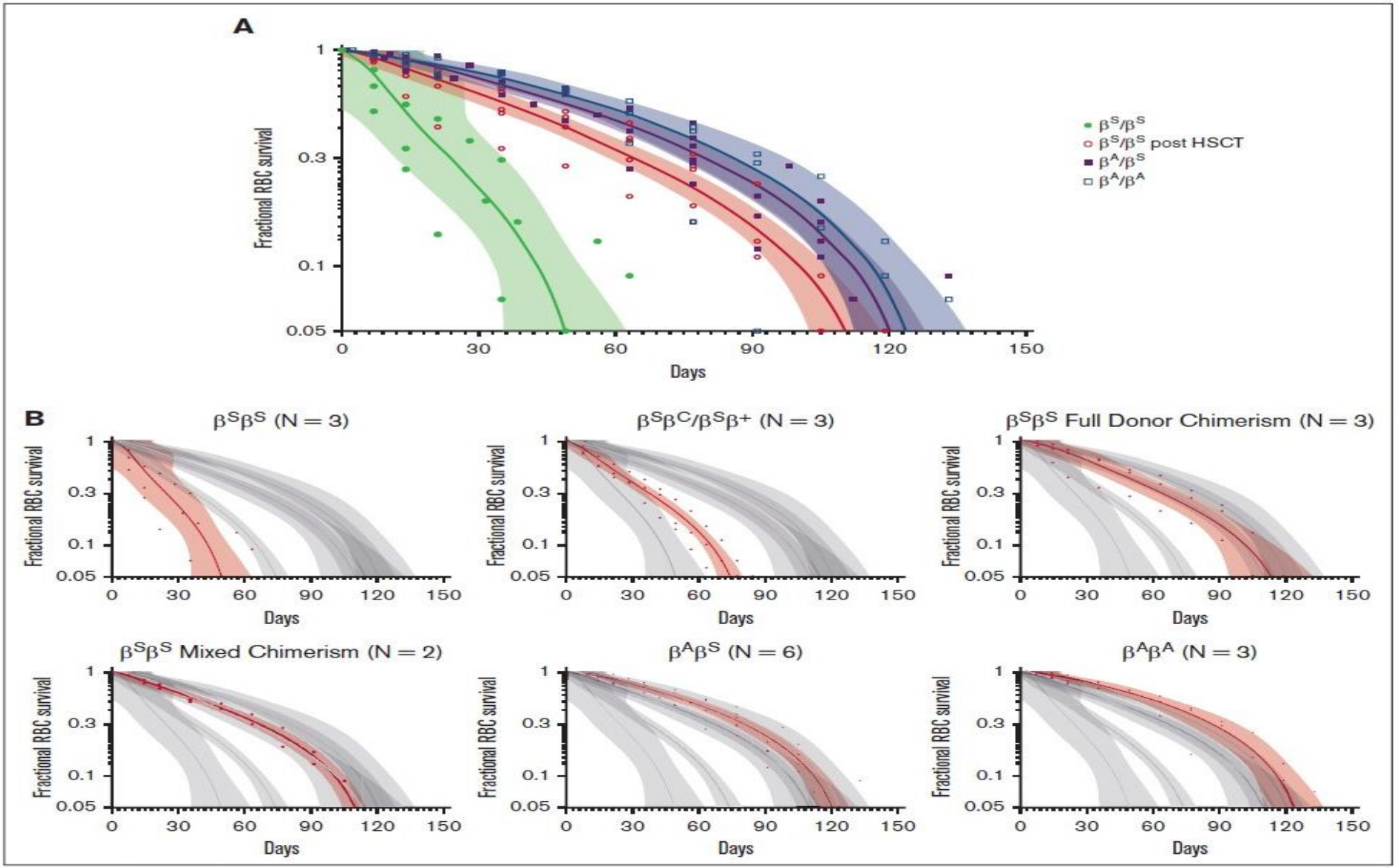


Figure 1. Fractional RBC Survival Peripheral blood samples were collected at regular intervals in all participants. Samples were analyzed in real time for percent survival BioRBCs by flow cytometry using a streptavidin-conjugate fluorochrome (streptavidin-phycoerythrin; S-866; Molecular Probes) until the percentage of BioRBCs decreased to the lower limit of detection (approximately <0.06%). Values are represented as the RBC fractional survival given absolute differences in the starting percent biotin fraction among participants. Dots represent individual participant BioRBC fractional survival, shown with a nonlinear regression line and 99% confidence interval bands. (A) Fractional RBC survival curves for participants with HbSS pre- and posttransplant, HbAS, and HbAA. (B) Fractional RBC survival curves for each cohort as compared to the larger group are highlighted in red, including HbSS vs non-HbSS genotypes (HbSC and HbSβ+) in the pre-HSCT cohort, and full DMC vs mixed chimerism in the post-HSCT cohort.

RBC survival
(Figure 2)

- RBC survival in participants with HbSS before and after HSCT (N = 8) was positively correlated with:
  - RBC count (P < .01)
  - Hb (P < .01)
  - Hct (P < .01)
  - %HbA (P < .01)
- And negatively correlated with:
  - %HbS (P < .01)
  - AST (P = .04)
  - total bilirubin (P < .01)
  - LDH (P = .03)
  - ARC (P = .11)

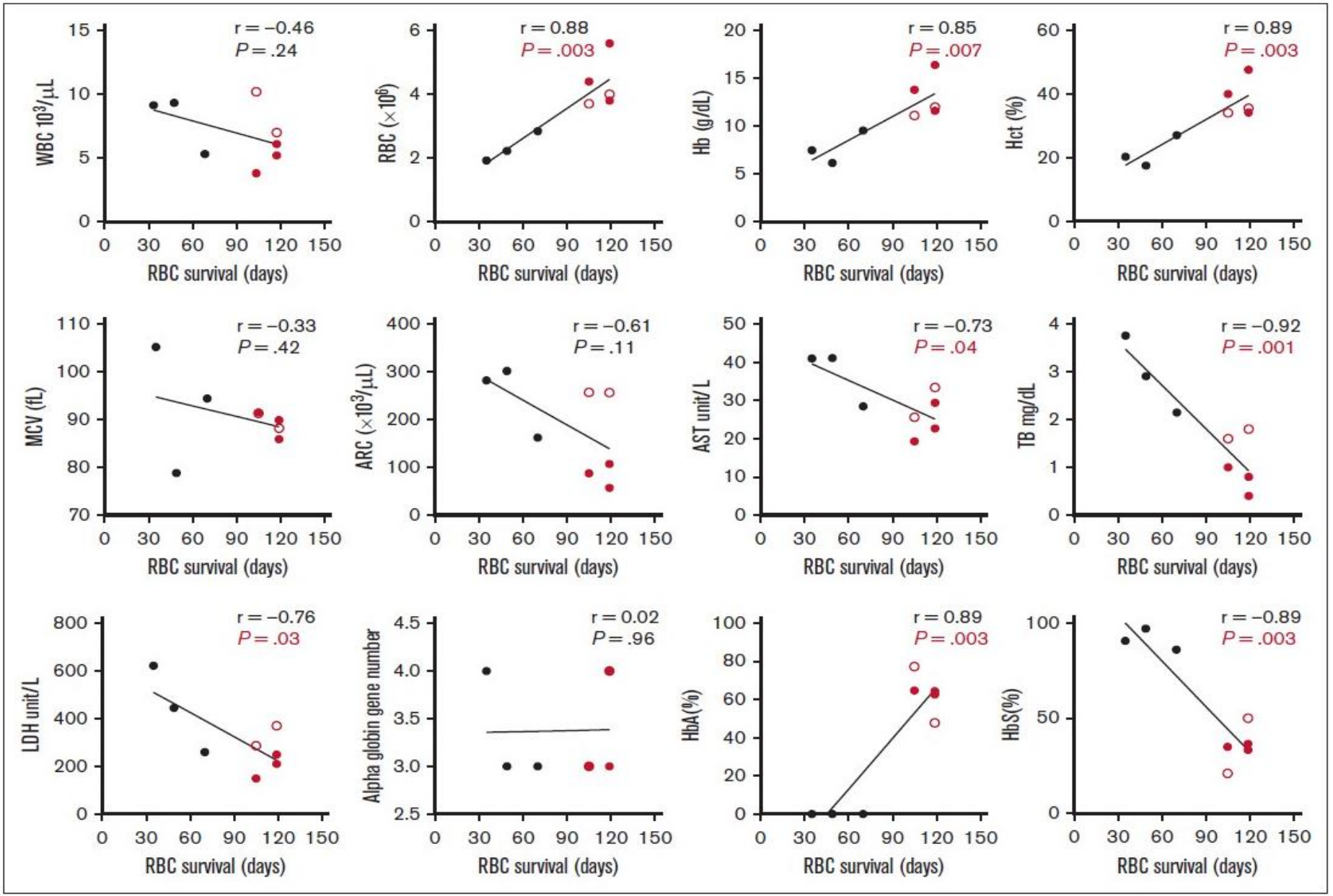


Figure 2. Correlation of RBC survival to measurable hematologic parameters in participants with HbSS pre- vs posttransplant. Hematologic parameters were measured with each sample throughout the study and correlated to the final RBC survival (days). AST, aspartate transaminase; Hb, hemoglobin; Hct, hematocrit; HbA, adult hemoglobin; HbS, sickle hemoglobin; MCV, mean corpuscular volume; TB, total bilirubin; WBC, white blood cell count. Black, HbSS pretransplant; Red, HbSS posttransplant (○mixed chimerism; ●full-donor chimerism).

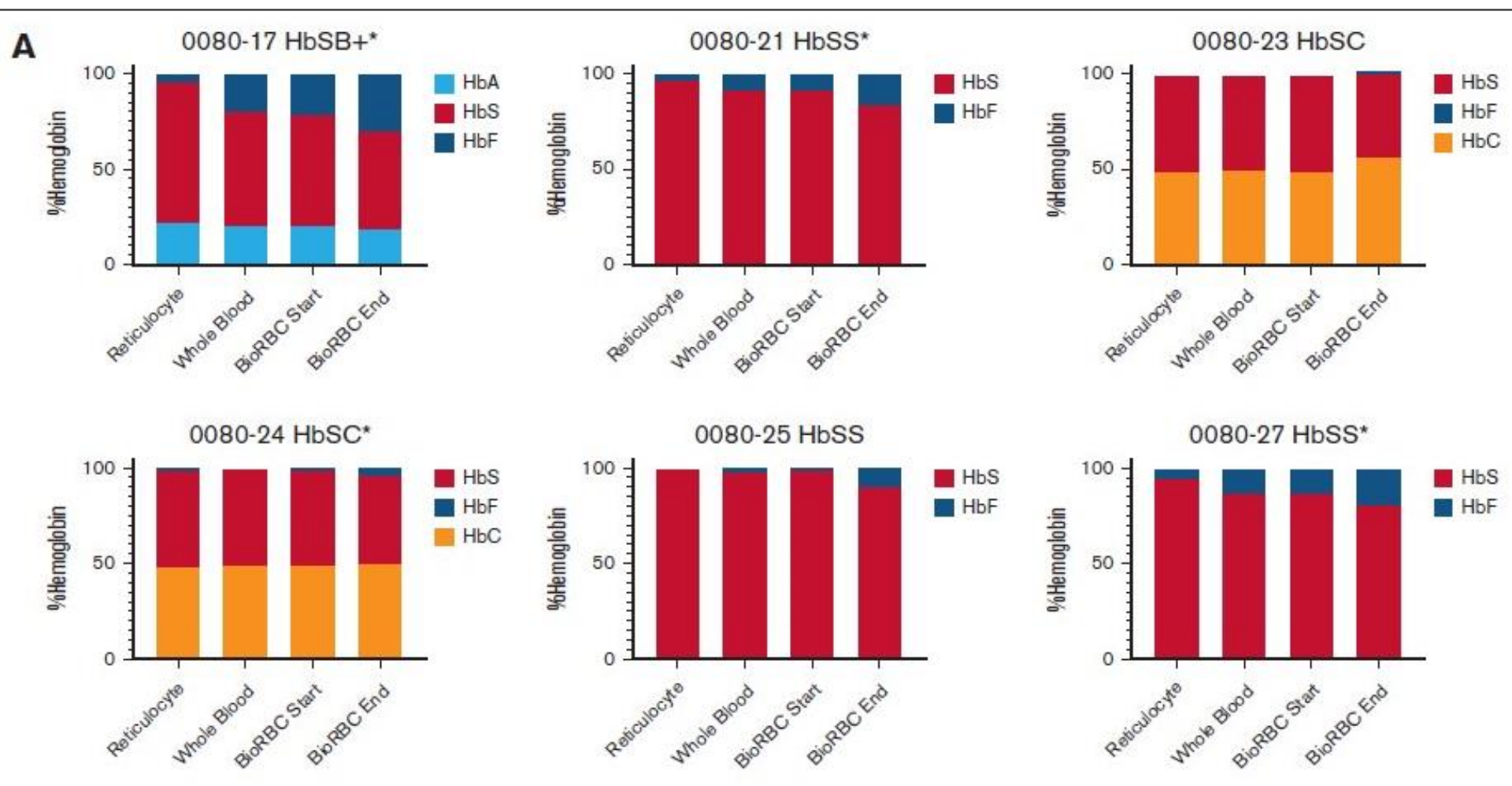


Figure 3. Change in hemoglobin content in surviving BioRBCs compared with whole blood.

(A) Change in % sickle, fetal, or C hemoglobin as performed by high performance liquid chromatography in the participants with SCD pretransplant in reticulocytes, whole blood, and the biotin-positive fraction sorted by flow cytometry at the time of product infusion and near the end of the RBC lifespan.
*Participants on HU.

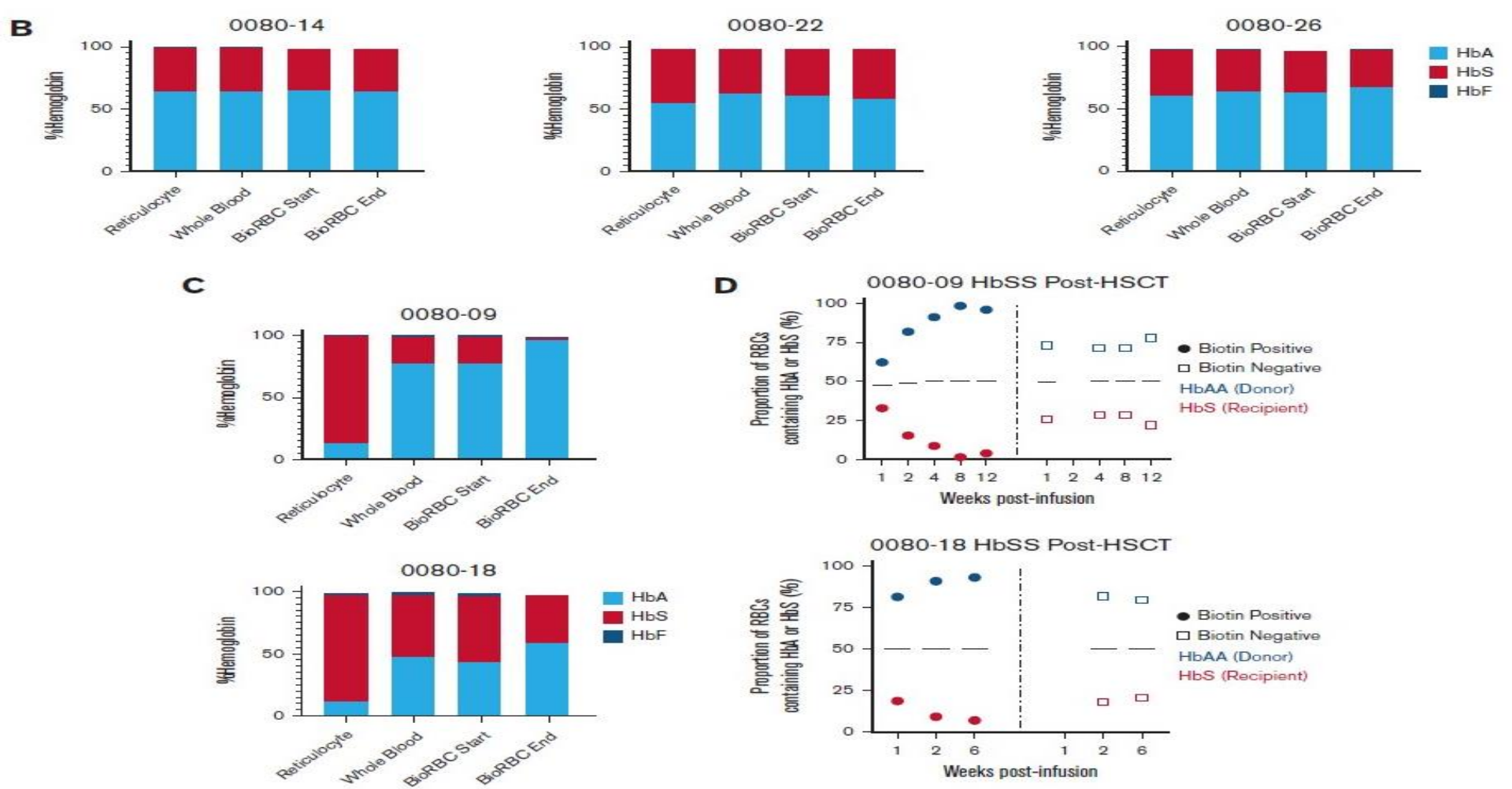


Figure 3. Change in hemoglobin content in surviving BioRBCs compared with whole blood.

(B-C) Change in % sickle or adult hemoglobin in the participants with SCD who underwent HSCT and had full DMC **(B)** or mixed chimerism **(C)**.

(D) Single cell Western analysis demonstrating single cell proportion of HbA to HbS of the biotin-positive fraction after sorting compared to the biotin negative fraction on the 2 participants with HbSS post-HSCT with mixed donor chimerism.

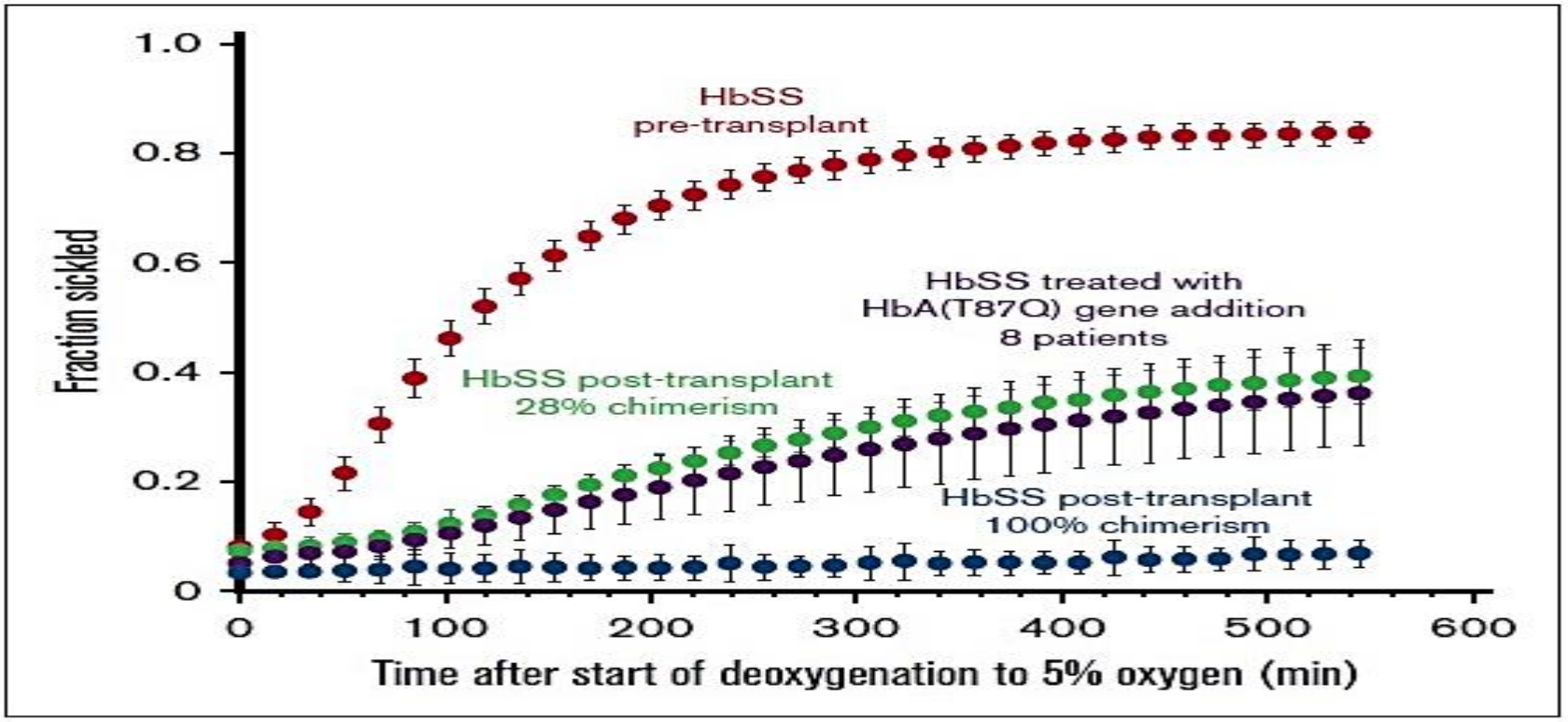


Figure 4. Representative sickling curves. The fraction sickled vs time after the start of deoxygenation with nitrogen to 5% oxygen is shown for blood samples from: 1 participant with HbSS pretransplant (red points) (0080-27), 1 participant with HbSS posttransplant with 28% donor chimerism (green points) (0080-18), 1 participant with HbSS posttransplant with 100% donor chimerism (blue points) (0080-26), and the average for 8 participants treated with HbA^{T87Q} globin addition (purple points) as previously published.[17] The error bars represent 1 standard deviation from the average value at each time point for the 8 patients and are primarily due to differences in chimerism among the patients and are not the result of error in the experimental measurements or variation of the sickling curves for the individual patients. Only curves for the patients following 6 months or more after transplant were included, because it requires 6 months for the hemoglobin composition of the whole blood to stabilize.

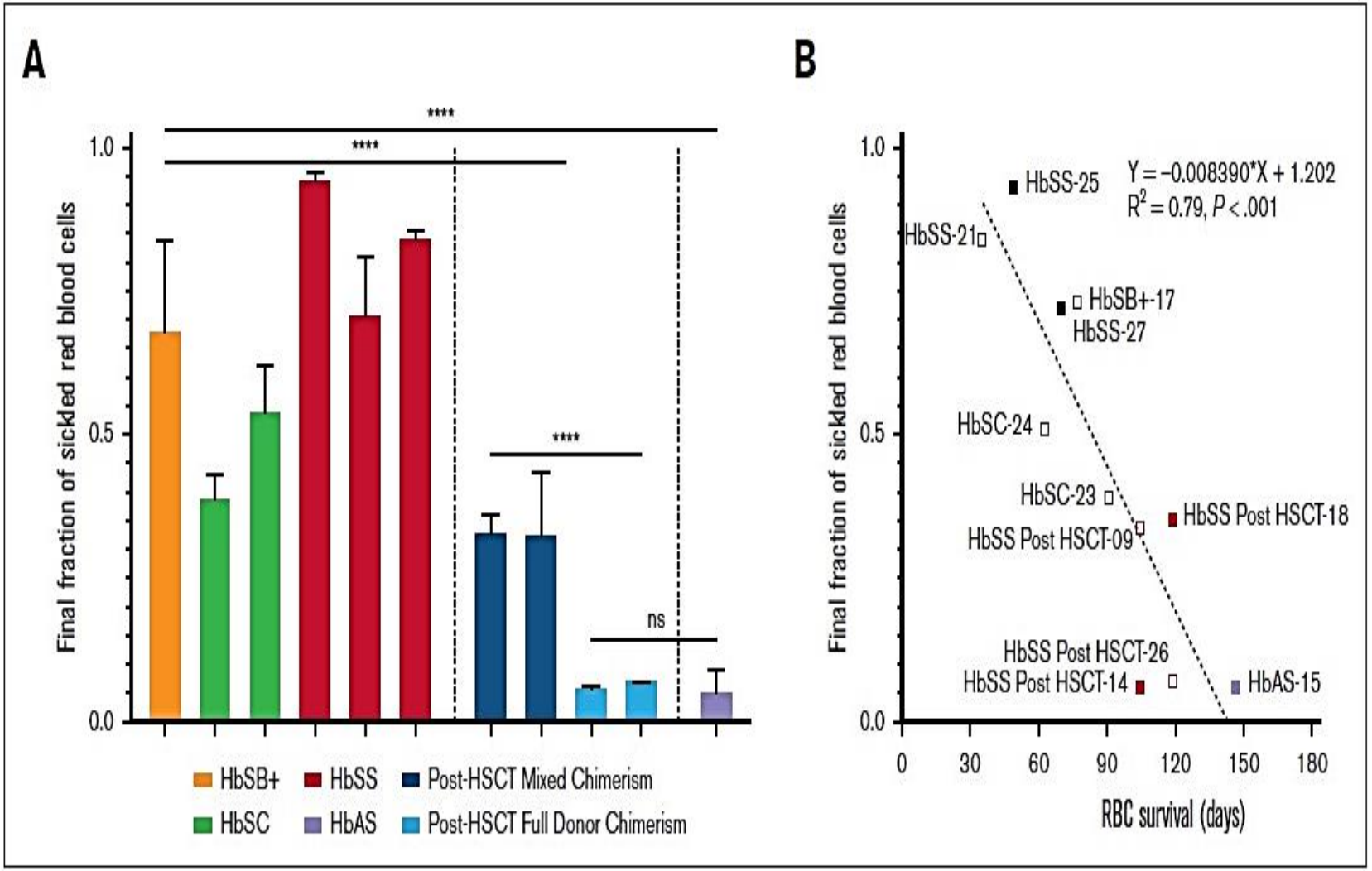


Figure 5. Fraction of sickle RBCs at the end of deoxygenation. (A) The average final fraction of sickled RBCs based on genotype is shown. Whole-blood samples from participants with SCD pre- and posttransplantation were deoxygenated with nitrogen to 5% oxygen. (B) The average final fraction of sickled RBCs from whole blood is compared with the measured RBC survival in days. Samples from 1 posttransplant participant with full DMC only underwent deoxygenation to 0% oxygen and are not included. Samples from 1 participant with HbAS that underwent deoxygenation with 5% oxygen is included. Open symbols represent participants with single alpha globin gene deletion.

Discussion

- Biotin labeling of RBCs is a safe and feasible methodology to evaluate RBC survival in patients with SCD before and after HSCT. (novel method for patients with SCD with genotypes other than homozygous HbSS, SCD after HSCT, and SCT)
- This method can directly measure RBC survival in order to characterize how RBC populations and Hb fractions may differ after transplantation, in which DMC may differ. these characterization data could inform minimally sufficient gene modification rates for gene therapies.
- After gene therapy, this method can be used to answer questions surrounding RBC lifespan after genetic modification of HSCs, including if the therapy results in pancellular or heterocellular distribution of therapeutic protein (HbF induction) and, if heterocellular, what threshold will have an adequate reduction in sickling after deoxygenation to reverse clinical phenotype.

Thank You for Your Attention!