TRANSFUSION MEDICINE

*Review*

# The Red Blood Cell-Improving Transfusions for Chronically Transfused Recipients (RBC-IMPACT) study: protocol description of an international multi-site observational clinical study

Matthew S. Karafin[1*], Shannon Kelly[2*], Kathleen M. Chapman[3], Lisa Baumann Kreuziger[4], John P. Manis[5], Carla Dinardo[6], Cassandra D. Josephson[7], Mars Stone[8,9], Nareg H. Roubinian[8,9,10], Brian Branchford[4], Bruce S. Sachais[11], Benyam Hailu[12], Ester C. Sabino[13], Eldad A. Hod[14], Brian Custer[8,9] for the National Heart, Lung, and Blood Institute (NHLBI) Recipient Epidemiology and Donor Evaluation Study-IV-Pediatric (REDS-IV-P)[†]

*co-primary Authors
†A complete list of study group members appears in the Acknowledgments

[1]*Department of Pathology and Laboratory Medicine, University of North Carolina School of Medicine, Chapel Hill, NC, USA;*
[2]*Department of Pediatrics, University of California, San Francisco Benioff Children's Hospital Oakland, Oakland, CA, USA;*
[3]*Westat, Rockville, MD, USA;*
[4]*Versiti Blood Research Institute, Versiti, Milwaukee, WI, USA;*
[5]*Department of Pathology, Harvard Medical School and Boston Children's Hospital, Boston, MA, USA;*
[6]*Division of Immunohematology, Faculdade de Medicina da Universidade de São Paulo and Fundacao Pro Sangue, São Paulo, Brazil;*
[7]*Departments of Oncology, Pediatrics, Pathology, Johns Hopkins University School of Medicine and Johns Hopkins All Children's Hospital, Baltimore, MD, and St. Petersburg, FL, USA;*
[8]*Vitalant Research Institute, San Francisco, CA, USA;*
[9]*Department of Laboratory Medicine, University of California San Francisco, San Francisco, CA, USA;*
[10]*Kaiser Permanente Division of Research, Pleasanton, CA, USA;*
[11]*New York Blood Center Enterprises, New York, NY, USA;*
[12]*National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA;*
[13]*University of São Paulo, São Paulo, Brazil;*
[14]*Department of Pathology & Cell Biology, Columbia University Irving Medical Center, New York, NY, USA*

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**Correspondence:** Brian Custer
e-mail: bcuster@vitalant.org

**Background** - Variability in blood donors, components, and recipients are known to affect transfusion outcomes, yet the combined effects of these factors remains unclear.

**Materials and methods** - The Red Blood Cell - Improving Transfusions for Chronically Transfused Recipients (RBC-IMPACT) study was a multi-center longitudinal study conducted in the United States (US) and Brazil over two years to investigate RBC survival after transfusion (Aim 1) and acute increase in iron post transfusion (Aim 2) (see https://clinicaltrials.gov/study/NCT05255445). The US RBC-IMPACT study included patients with thalassemia and sickle cell disease (SCD) and, in Aim 2 only, children with hematology-oncology diseases with a hypoproliferative bone marrow. In Brazil, the study was conducted within an established SCD cohort. Blood samples were collected immediately before and after RBC transfusion to measure hemoglobin (Hb), hemoglobin A (HbA) in SCD, and markers of iron and hemolysis. Samples were collected two hours post transfusion in a subset of participants receiving primarily single unit transfusions for Aim 2. Transfusate samples were collected from transfused units. Single nucleotide polymorphism array typing of donors and recipients to measure genetic variants including those associated with increased *in vitro* hemolysis of stored RBCs was conducted. Comprehensive information regarding donors, components and some recipient data were linked to key clinical data extracted from recipients' medical records to assess factors associated with RBC transfusion effectiveness.

**Results** - The outcomes for Aim 1 were RBC survival between successive transfusions, calculated as ΔHbA per day for SCD and by ΔHb per day for thalassemia, and Δbilirubin for both patient groups. The primary outcome for Aim 2 was change in serum iron from before to 2 hours after transfusion.

**Discussion** - This study will be the most detailed and granular evaluation of the predictive variables that may optimize RBC effectiveness and safety in these chronically transfused patient populations.

**Keywords:** *red blood cells, sickle cell disease, thalassemia, chronic transfusion, hemoglobin increment.*





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Patients with thalassemia and sickle cell disease (SCD) often require life-long RBC transfusion therapy. The primary hematologic goal of chronic transfusion therapy for thalassemia is suppression of ineffective endogenous erythropoiesis. For SCD, the goal is to decrease the percentage of sickle hemoglobin (HbS), thereby preventing complications of RBC sickling. Variability in the blood donor population and manufacturing process is known to affect blood component quality, and understanding the impact on transfusion recipient outcomes would allow optimization of transfusion[1-3]. The clearance of transfused RBC varies[4-10], between transfused individuals and among sequential transfusions within the same patient, suggesting that recipient, manufacturing, and donor unit characteristics can influence transfused RBC survival[11-17].

Transfusion to those who are chronically-transfused requires additional considerations, such as the selection of units matched for particular antigens to decrease the risk of alloimmunization[18,19]. A consequence of antigen-matching is the risk of receiving units that have genetic traits linked to the matched antigens. For example, patients with SCD are more likely to receive units from donors with glucose-6-phosphate dehydrogenase (G6PD) deficiency or hemoglobin variants other than HbS[20,21]. These units may be associated with decreased RBC transfusion effectiveness, as evidenced by increased clearance of HbA post-transfusion in patients with SCD[7,8]. Genome wide association studies from the Recipient Epidemiology and Donor Evaluation Study (REDS)-III identified multiple *loci* in blood donors associated with increased *in vitro* hemolysis[15,23]. The clinical consequence to patients receiving units from donors carrying these variants (e.g., polymorphisms in *G6PD*, *SEC14L4*, *HBA2*, *MYO9B*, *LPCAT3*, *ACSL4*, *STEAP3*, *TP53*, *SLC7A5*, *ARG1* and *ABCG2*) may lead to less effective transfusions[23-31].

Receiving less effective blood products over time results in increased transfusion to achieve the same clinical endpoint, contributing to iron overload. Excess iron is toxic to the heart, liver, pituitary, pancreas, and gonads; and liver fibrosis, cardiomyopathy, and endocrinopathies may develop over time. Cardiac iron overload resulting in heart failure is the leading cause of death in transfusion-dependent thalassemia patients[32].

We hypothesized that by identifying the factors impacting RBC transfusion effectiveness, we could establish clinical evidence to support more optimal RBC transfusion practice. The goal of the RBC-IMPACT study was to assess donor, manufacturing, and recipient factors that affect RBC survival and iron parameters to improve patient safety by enhancing transfusion effectiveness. Here, we describe the protocol and specific aims of this international multi-site observational clinical study.

## MATERIALS AND METHODS

### REDS-IV-Pediatric (REDS-IV-P) program

REDS-IV-P is the fourth iteration of the US National Institutes of Health, National Heart, Lung, and Blood Institute (NHLBI) REDS programs and focuses on improving transfusion recipient outcomes across the lifespan (see Josephson *et al.*[33]). The US program built a vein-to-vein (V2V) database, linking information collected from blood donors, their donations, the resulting manufactured components and data extracts from hospital electronic medical records of transfused and non-transfused patients[34]. The Brazil program built a database of donors, donations and components collected and manufactured at participating hemocenters. Alongside these databases, the Brazil program has a large prospective cohort of patients with SCD[35,36]. These databases serve as the backbone for multiple observational studies in transfusion epidemiology and recipient outcomes in both the US and Brazil programs, including RBC-IMPACT which was implemented by both programs (see https://clinicaltrials.gov/study/NCT05255445).

### Study aims and hypotheses

***Aim 1:*** the primary hypothesis was that specific genetic and non-genetic donor factors, manufacturing factors, and recipient factors significantly predict RBC survival. RBC survival was defined by change in hemoglobin (thalassemia) or hemoglobin A (SCD) per day between transfusion visits. The predictors evaluated include donor (e.g., sex, age, race/ethnicity, smoking), component (e.g., storage duration, storage solution, irradiation), recipient data (**Table I**), and donor and recipient data obtained from a single nucleotide polymorphism (SNP) array (referred to as the Precision Transfusion Medicine Array [PTMA]). (**Table II**). Secondary hypotheses included the association of RBC survival with a) recipient hemolysis pre-transfusion, b) hemoglobin increment (immediate

**Table I** - *Key donor, component, and recipient variables collected as part of RBC-IMPACT*

| Variable | Variable type and categories |
|---|---|
| **Donor factors** | |
| **Sex** | Categorical (M/F) |
| **Age** | Continuous |
| **ABO** | Categorical (A, B, AB, O) |
| **Rh** | Categorical (−, +) |
| **Race/ancestry/skin color** | Categorical - based on USA census definitions for US sites and Brazilian census definitions for Brazil sites |
| **Ethnicity** | Categorical (self-reported: Hispanic, not Hispanic, unknown/unreported) |
| **Donor fingerstick hemoglobin** | Continuous (g/dL) |
| **Hub/hemocenter** | Categorical |
| **No. of RBC-containing donations in past 2 years** | Continuous (double RBC counts as 2) |
| **No. of PLT donations in past 2 years** | Continuous |
| **30-day smoking** | Categorical (Y/N) |
| **30-day smoking intensity** | Categorical 0 = 0 per day; 1 = less than one per day; 2 = 1 cigarette per day; 3 = 2-5 cigarettes per day; 4 = 6-15 cigarettes per day; 5 = 16-25 cigarettes per day; 6 = 26-35 cigarettes per day; 7 = 2 packs or more per day; 9 = unknown |
| **E-cigarettes or vaping** | Categorical (everyday, some days, not at all) |
| **BMI** | Continuous |
| **Currently taking sex hormones** | Categorical (Y/N) |
| **Type of hormone being taken** | Categorical (female, male, both) |
| **Use of iron supplementation or multivitamin containing iron** | Categorical (Y/N) |
| **Blood unit factors** | |
| **Gamma irradiation** | Categorical (Y/N) |
| **Time from gamma irradiation to transfusion** | Continuous (days) |
| **Storage duration** | Continuous (days from collection) |
| **Storage duration maximum for multiple unit transfusion events** | Continuous (maximum age of unit per transfusion event) |
| **Total hb transfused** | Continuous (Hb concentration in transfusate x volume transfused) |
| **Collection method** | Categorical (whole blood/apheresis) |
| **Additive solution** | Categorical (CPD, CPDA-1, AS-1, AS-3, AS-5, SAG-M, Other) |
| **Leukoreduction** | Categorical (Y/N) |
| **Storage duration × additive solution** | To test interaction (i.e., is the effect of storage different in different storage solutions) |
| **Other RBC modification** | Categorical (washed, frozen, volume reduced) |
| **Recipient factors** | |
| **Sex** | Categorical (M/F) |
| **Age** | Continuous |
| **ABO** | Categorical (A, B, AB, O) |
| **Rh** | Categorical (−, +) |
| **ABO-identical transfusion** | Categorical (Y/N; yes only if A−>A, B−>B, O−>O, AB−>AB) |
| **BMI** | Continuous |
| **Disease** | Categorical (SCD, Thal, Heme/Onc) |
| **Disease genotype, cancer diagnosis** | SCD categorical (SS, SC, $SB^0$Thal, etc) Thal categorical (aa/a−, aa/−− or a−/a−, etc); cancer categorical |
| **History of respiratory complications** | Categorical (complicated-, uncomplicated-ACS, None) |
| **Comorbidities** | Categorical (from medical record abstraction) |
| **Taking prescription hormones** | Categorical (No., female, male, both) |
| **Total blood volume** | Continuous |
| **Pre-transfusion hemoglobin** | Continuous |
| **Inpatient (for Heme/Onc)** | Categorical (Y/N) |
| **Hospital** | Categorical (random 3-digit number) |
| **Year** | Categorical |
| **Number of RBC units in this transfusion event** | Categorical |
| **Time from last RBC transfusion event** | Continuous (days) |
| **Count of RBC units from last transfusion event** | Continuous |
| **History of alloimmunization** | Categorical (Y/N) |
| **Splenectomy** | Categorical (Y/N) |
| **Splenomegaly** | Categorical (Y/N) |
| **Elixhauser Comorbidity Index (USA sites only)*** | Continuous |

*Note in the Brazil IMPACT study, some variables differed to reflect the SCD-only study population in addition to using categories appropriate in that setting. The Elixhauser Comorbidity Index derived from ICD-10 codes in the Vein-to-Vein database is not available in the Brazil SCD cohort database.

**Table II** - *Donor and recipient genetic factors from SNP typing using the PTMA*

| Variable, factor or SNP (nearest gene) | Chr | Hemolysis type | GWA significant populations[23] | Comments |
|---|---|---|---|---|
| ***ANK1*** | 8 | Osmotic | All, Caucasian | Categorical (Y/N) |
| ***SPTA1*** | 1 | Osmotic | All, Caucasian | Categorical (Y/N) |
| ***G6PD*** | 23 | Oxidative | All, African American | Categorical (Y/N) |
| ***MYO9B*** | 19 | Osmotic | All, Caucasian | Categorical (Y/N) |
| ***HBA2*** | 16 | Osmotic | All, Caucasian, African American | Categorical (Y/N) |
| ***GPX4*** | 19 | Oxidative | All | Categorical (Y/N) |
| ***PIEZO1*** | 16 | Osmotic | All, Caucasian | Categorical (Y/N) |
| ***ATAD2B/MFSD2B*** | 2 | Osmotic | All, Caucasian | Categorical (Y/N) |
| ***BRAP*/many** | 12 | Osmotic | All, Caucasian | Categorical (Y/N) |
| ***HBA2*** | 16 | Osmotic | East Asian | Categorical (Y/N) |
| ***GLRX*** | 5 | Oxidative | All, Caucasian | Categorical (Y/N) |
| ***HK1*** | 10 | Osmotic | All, Caucasian | Categorical (Y/N) |
| ***SWAP70*** | 11 | Osmotic | All, Caucasian | Categorical (Y/N) |
| ***HBB*** | 11 | Osmotic | African American | Categorical (Y/N) |
| ***AQP1*** | 7 | Osmotic | All, Caucasian | Categorical (Y/N) |
| ***MIR4289*** | 9 | Osmotic | All, Caucasian | Categorical (Y/N) |
| ***SEC14L4*** | 22 | Oxidative | All | Categorical (Y/N) |
| ***EYS*** | 6 | Osmotic | Hispanic 2 | Categorical (Y/N) |
| ***CNTN5/ARHGAP42*** | 11 | Osmotic | All, Caucasian | Categorical (Y/N) |
| **Nothing** | 13 | Osmotic | Hispanic 2 | Categorical (Y/N) |
| **NA** | 3 | Osmotic | African American | Categorical (Y/N) |
| ***TMC8*; *TMC6*** | 17 | Storage | All | Categorical (Y/N) |
| ***IKZF1*** | 7 | Osmotic | All, Caucasian | Categorical (Y/N) |
| | 8 | Storage | All, Caucasian | Categorical (Y/N) |
| | 1 | Oxidative | Hispanic 2 | Categorical (Y/N) |
| ***SLC4A1* (Band 3)** | 17 | Osmotic | All, Caucasian | Categorical (Y/N) |
| ***TFB2M*** | 1 | Osmotic | East Asian | Categorical (Y/N) |
| ***HK1*** | 10 | | | Categorical (Y/N) |
| ***G6PD* deficiency ratio for transfusion event** | | | | Categorical (0 [no deficient units], 0.25 [1 of 4 units deficient], 0.5 [1 of 2 units deficient], 0.75 [3 of 4 units deficient], or 1.0 [all units deficient]) |
| **Polygenic risk score (PRS) for hemolysis markers** | | | | Continuous |
| **Race, genetic** | | | | To be defined from SNP array typing: categorical based on Principal Components Analysis (PCA) |



post-pre hemoglobin), and c) hemolysis parameter increments immediately following transfusion.

*Aim 2:* this aim sought to measure markers of hemolysis and iron parameters from before to two hours after the transfusion in single RBC-unit transfusion events. In the US program, transfused patients with SCD, thalassemia, and patients with pediatric hematologic/oncological diagnoses with hypoproliferative bone marrow were included. Because the pathophysiology of the disease and therefore the effects of hemolysis and iron handling may be different when comparing patients with thalassemia (ineffective erythropoiesis), SCD (chronic hemolysis), or hypoproliferative bone marrow (absent sink for iron in the bone marrow), the primary hypothesis of this aim in the

US study was that the increase in iron post transfusion differs by disease.

As only patients with SCD were included in the Brazil program, the Aim 2 hypothesis was that storage duration of units impacts iron exposure in this population. In healthy adult volunteers, the storage duration before transfusion correlates with an increase in serum iron 2 hours after transfusion[37], but this has not been confirmed in patient populations. Secondary hypotheses included the relationship between storage age and increase in markers of hemolysis and that increased non transferrin bound iron (NTBI) or transferrin saturation following transfusion is associated with increased risk of clinical adverse effects (e.g., SCD complications).

## Study design and setting

Both the US and Brazil studies included prospective data and blood sample collection combined with retrospective data acquisition using the REDS-IV-P V2V database (see Birch *et al.*[35]) in the US study, and data collected from the REDS-IV-P Brazil SCD cohort and donor/donation/components databases.

Participating hospitals and blood centers included Columbia University Irving Medical Center/Morgan Stanley Children's Hospital of New York, Weill Cornell Medical Center/Komansky Children's Hospital, New York Blood Center Enterprises (NYBCe), Boston Children's Hospital, Froedtert & Medical College of Wisconsin/Children's Hospital of Wisconsin, Versiti, University of California San Francisco, Benioff Children's Hospital Oakland, and Vitalant in the US. Participating hemocenter sites for Brazil included the Children's Institute and Adult Clinics at Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo - HCFMUSP (São Paulo), Fundaçao Pro-Sangue (São Paulo), HEMOAM (Manaus), HEMOMINAS (Belo Horizonte), HEMOPE (Recipe) and HEMORIO (Rio de Janeiro). A data coordinating center (DCC, Westat, Rockville, MD) managed activities in both studies.

## Recipient population

### *Aim 1*

*Inclusion criteria:*

1. well-characterized transfusion-dependent form of SCD (US & Brazil) or thalassemia (US) on chronic transfusion therapy;
2. on a regular simple or partial manual exchange RBC transfusion schedule (i.e., 1-3 units scheduled every 2-6 weeks for a minimum period of 6-months before enrollment); for partial manual exchange, phlebotomy must be completed before transfusion is started without alternating cycles of phlebotomy and transfusion;
3. enrollment in the REDS-IV-P Brazil SCD Cohort (Brazil).

*Exclusion criteria:*

1. patient institutionalized, imprisoned or in foster care;
2. weight <11 kg.

### *Aim 2*

*Inclusion criteria:*

1. either included in Aim 1 (US and Brazil) or patients with pediatric oncologic diagnoses with anemia due to chemotherapy or primary/secondary hypo-proliferative bone marrow likely to require an RBC transfusion, including patients with hematopoietic stem cell transplants (US only);
2. age ≤21 years old (US);
3. planned single unit RBC transfusion.

*Exclusion criteria:*

1. patient institutionalized, imprisoned or in foster care;
2. weight <18 kg;
3. active auto-immune hemolytic anemia based on positive direct antiglobulin test (DAT) with laboratory evidence of hemolysis and increased transfusion requirement;
4. active microangiopathic hemolytic anemia.

### *Screening process for eligible participants*

Study staff (1) used database extractions of patient populations to identify eligible participants if available, (2) met with primary clinical teams at regular intervals to review patient lists or (3) reviewed medical records of patients with scheduled transfusion visits to determine eligibility. Participation in Aim 2 (which required remaining for 2 hours after transfusion for another sample) was discussed with SCD and thalassemia participants likely to receive 1 unit at the initial consent visit. Pediatric oncology participants (who were only eligible for Aim 2 in the US) were identified by meeting with inpatient oncology services at various intervals to identify eligible participants. Once eligibility was confirmed for Aim 1 or 2, the primary clinical team was asked to introduce the study

personnel to potential participants prior to discussion of informed consent with research staff.

Participants in Brazil were recruited from the REDS-IV-P Brazil SCD cohort. An extraction of cohort study data was used to identify eligible participants who were recruited by research staff at routine transfusion visits. Consent for both Aim 1 and Aim 2 (if participant was interested and likely to receive only 1 unit per transfusion) was sought.

### Research ethics

All aspects of RBC-IMPACT were conducted according to the defined protocol, US FDA regulations, relevant Brazil regulations, ICH-GCP Guidelines, and HIPAA for protection of human subjects under local and central institutional review board (IRB) oversight. In the US, a single IRB process was used (Westat). Each site relied on this single IRB with additional local IRB context determinations. In Brazil, the study was approved by CONEP (*Comissão Nacional de Ética em Pesquisa* [National Research Ethics Committee]) and by the local Ethics Committees (*Comissão Ética em Pesquisa* - CEP [Research Ethics Committee]) of each participating hemocenter. For the Brazil cohort, IRB approval was also obtained from UCSF, IRB of record for Vitalant (VRI), and from the Westat IRB.

For potential participants <18 years of age, the parent(s)/guardian(s) was/were consented for the study. If consent was obtained, the pediatric patients were approached by research staff for assent to participate in the study using age-appropriate assent forms. In the US, pediatric patients were approached by research staff for assent to participate in the study using an assent form for patients 6-18 years of age. In Brazil, patient assent forms were different for patients between 6-12 years of age, and between 13-17 years of age. Children turning 13 during the study received a copy of the assent form designated for those over 12 years. Patients under the age of 6 were included in the study with only parental/guardian consent. However, children turning 6 during the study were assented.

### Sample collection

During each study visit, blood specimens were obtained from participants immediately before and after each transfusion for approximately two years of observation (**Figure 1**). Samples from each RBC unit transfused (transfusate samples) were also obtained along with the volume of blood transfused and, in the case of a manual exchange transfusion, the volume removed. Whole blood specimens were obtained from donors either by recovering retention tubes routinely collected from blood donors in the US, or by a sample from the donation or an additional research visit and phlebotomy in Brazil. An additional blood specimen was obtained 2 hours after transfusion for those participating in Aim 2 of the study.

### Biospecimen handling and testing

Several laboratory/clinical parameters were measured before and after each transfusion event (**Figure 2, Table III**). Testing strategies for some analytes varied between the US and Brazil study sites. Specifically, hemoglobin and hemoglobin variant testing were conducted on frozen samples at the central laboratory (Center for Transfusion Laboratory Science, CTLS, at VRI) for US study sites. In Brazil, hemoglobin (as part of complete blood counts) and hemoglobin variant testing were conducted on fresh samples in clinical laboratories in each city (Diagnosticos Brasil) and total hemoglobin was also measured on transfusate samples in the quality control laboratory of each hemocenter. All other laboratory testing was conducted using frozen samples shipped to the CTLS for US and Brazil sites. Laboratories with specialized expertise in testing certain analytes conducted those tests for all study sites. At US sites, all sample testing was conducted in laboratories in San Francisco, CA (CTLS, VRI), Oakland, CA (Benioff Children's Hospital), and New York, NY (Columbia University Medical Center).

The PTMA is a further refined and content-targeted immunohematology array developed by ThermoFisher (Waltham, MA, USA) in partnership with the Blood Groups Consortium (BGC)[38]. REDS-IV-P participated in the array development by including genetic marker content on hemoglobin variants, hemolysis, alloimmunization, and heritage based on our findings from REDS-III[39,40]. All DNA extraction and array testing for RBC-IMPACT was conducted at the NYBCe, National Center for Blood Group Genomics (NCBGG) in Kansas City, MO, USA.

### Blood donor population

A blood sample from each donor whose unit was transfused into a consented participant was required for genetic marker testing. A waiver of written informed consent was IRB approved for obtaining

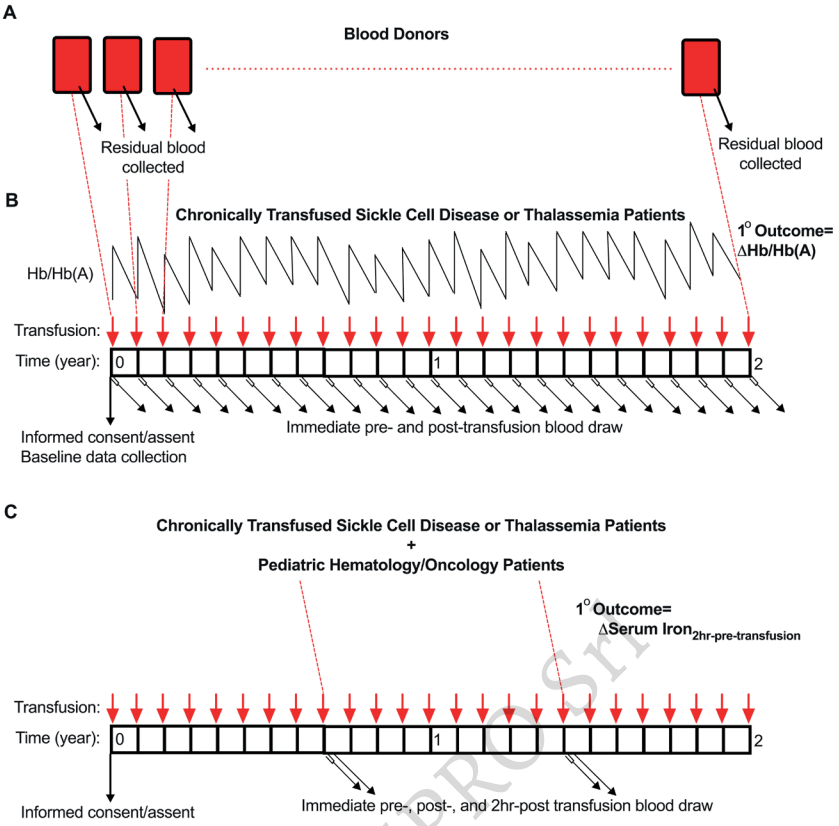


**Figure 1 - Study scheme**
(**A**) Residual blood obtained from standard RBC donor units for determining genetic variant/marker status. (**B**) In Aim 1, chronically transfused participants consented to obtain clinical data and additional blood samples immediately pre- and post-transfusion from every transfusion episode over a 2-year study period. (**C**) In Aim 2, participants from Aim 1 and an additional cohort of pediatric hematology/oncology subjects with hypo-proliferative bone marrow recruited to obtain the blood samples collected in Aim 1 plus an additional blood draw 2-hours post-transfusion. Only single RBC unit transfusion events were enrolled and participation in Aim 2 was optional for SCD and thalassemia patients at each scheduled transfusion. Note only participants with SCD were included in the Brazil study.



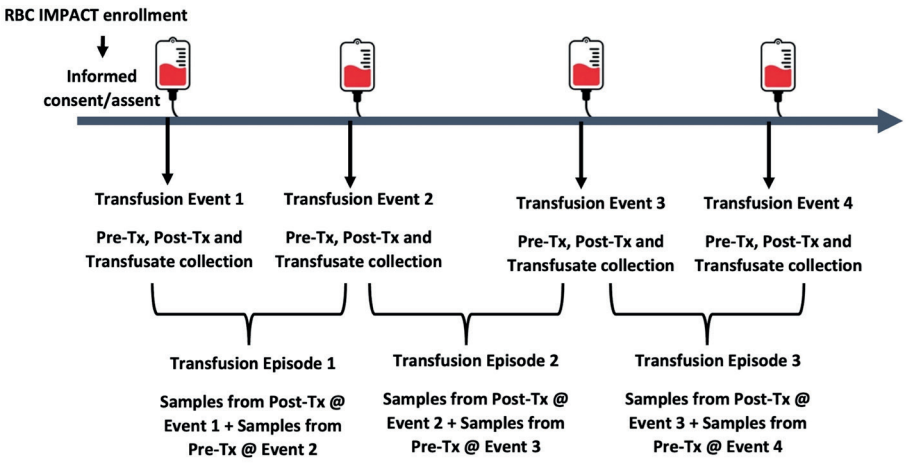


**Figure 2 - Study episodes and events**
The transfusion episode, or study episode, includes the collection of post-transfusion samples for one transfusion paired with the pre-transfusion samples from the next transfusion. The transfusion event, or study event, refers to the actual visit: the time point at which the participant comes to the clinic for their scheduled transfusion and the study coordinator collects samples pre- and post-transfusion and associated data.

**Table III** - *Laboratory testing for recipients and donors*

| Analyte | Testing location and lab | Recipient | | | RBC unit donor | |
|---|---|---|---|---|---|---|
| | | Recipient pre-transfusion | Immediately post-transfusion | 2 hr post | Transfusate | Donation |
| **Hemoglobin (g/dL)** | *, ** see below | 1, 2*** | 1, 2 | 2 | 1,2 | |
| **Hemoglobin variant quantification (% each variant)** | * see below | 1, 2 | 1, 2 | 2 | 1,2 | |
| **Serum iron (mg/dL)** | CTLS | 1, 2 | 1, 2 | 2 | | |
| **Ferritin (ng/mL)** | | 1, 2 | 1, 2 | 2 | | |
| **Indirect bilirubin (mg/dL)** | | 1, 2 | 1, 2 | 2 | | |
| **Total iron binding capacity (µg/dL)** | | 1, 2 | 1, 2 | 2 | | |
| **Non-transferrin-bound iron (µM)** | Columbia | 2 | 2 | 2 | | |
| **Hepcidin (ng/mL)** | CTLS | 1, 2 | 2 | 2 | | |
| **Plasma free hemoglobin (mg/dL)** | | 1, 2 | 1, 2 | 2 | | |
| **Malondialdehyde (MDA)** | | 1, 2 | 2 | 2 | | |
| **Cytokine panel (e.g., IL-6, IFN-g)** | CTLS | 1, 2 | | 2 | | |
| **MxA protein assay (type I interferon)*** ** | CTLS | 1, 2 | | | | |
| **Precision transfusion medicine array** | NCBGG | 1,2 | | | | 1,2 |

*Hemoglobin and hemoglobin variants were batch tested using frozen stored samples in central laboratories for the US study and measured as part of a complete blood count (CBC) and variant testing on fresh samples in clinical laboratories in each Brazil study site.
**CBC data may be available in the vein-to-vein database for the US study.
***1: testing planned under Aim 1 of the protocol; 2: testing planned under Aim 2.



and testing all US donor samples. Three of the four blood centers participating in the US study (NYBCe, Versiti, Vitalant) have IRB approved protocols which allow broad use of donor samples for research purposes. The fourth blood center serves one hospital (Boston Children's), and the local IRB required a study-specific donor information form to allow donors to opt-out of the study.

Initially, donors of units transfused to participants in Brazil were recruited to return for a research visit to obtain informed consent and a blood sample. However, contacting and scheduling donors was challenging, and a low proportion of samples were obtained by mid-study. Therefore, approval for waiver of donor consent was also obtained from Brazil Ethics Committees to allow testing of residual samples from the donation or unit transfused to the consented participant.

## Study endpoints

### *Primary outcome measures*

The primary outcome measure in Aim 1 was RBC survival, calculated as ΔHbA per day for SCD and by ΔHb per day for thalassemia from pre and post transfusion samples:

$$\frac{\Delta \text{Hb(A)}}{\text{day}} = \frac{\text{Post-transfusion Hb(A) visit}_{i+1} - \text{Pre-transfusion Hb(A) visit}_{i+1}}{\text{Days between visit}_{i+1} \text{ and visit}_{i}}$$

where *i* represented the visit number and Hb(A) referred to the measured hemoglobin level (thalassemia) or hemoglobin A level (SCD). Participants receiving simple transfusion, but not partial manual exchange, were also assessed using the following formula:

$$\frac{\Delta \text{Hb(A)}}{\text{day}_i} = \frac{\text{PreHb(A)}_i + \left(\frac{\text{Hb}_{\text{RBC}} V}{\text{TBV}}\right) - \text{PreHb(A)}_{i+1}}{D_i}$$

where $i$ is the visit number, PreHb(A) is the pre-transfusion hemoglobin or hemoglobin A value, $\text{Hb}_{\text{RBC}}$ is the hemoglobin value for the RBC unit transfused at visit $i$, $V$ is the volume transfused, $D_i$ is the number of days between visit $i$ and visit $i+1$, and $TBV$ is the total blood volume at visit $i$ estimated using Nadler's equation for adults and 70 mL/kg for children. The primary outcome measure in Aim 2 was the change in serum iron measured from before to two-hours after transfusion.

### Secondary outcome measures

Secondary outcome measures for Aim 1 included using the primary RBC survival outcome measure, and comparing it to measures of a) recipient hemolysis pre-transfusion (e.g., indirect bilirubin, serum iron, plasma free hemoglobin), b) hemoglobin increment (Hb(A)(post-transfusion)$_{visit(i)}$ – Hb(A)(pre-transfusion)$_{visit(i)}$), and c) changes in hemolysis parameters (i.e., serum iron, indirect bilirubin, plasma free hemoglobin). In the Brazil study, where detailed data were collected as part of the REDS-IV-P Brazil SCD cohort, RBC survival was also compared to the rate of SCD complications such as acute vaso-occlusive pain episodes and acute chest syndrome.

Secondary outcome measures for Aim 2 included markers of hemolysis 2 hours after transfusion (i.e., transferrin saturation, indirect bilirubin, NTBI, plasma free hemoglobin) and hepcidin. Additional markers including cytokines, malondialdehyde, and MxA protein were measured to further characterize recipients prior to transfusion.

### Adverse events and serious adverse events

Adverse events (AE) and serious adverse events (SAE) that occurred during the study were documented and investigated. The most common anticipated acute adverse reaction from the study phlebotomies was vasovagal reaction in recipients. Witnessed or un-witnessed vasovagal reactions were considered an AE, unless resulting in hospitalization, in which case the event was considered an SAE. Bruising and pain at the site of needle insertion specific to study procecures that resulted in a participant complaint was considered an AE, and permanent or persistent nerve damage from a study phlebotomy was considered an SAE. Transfusion reactions were also captured and reported as AEs or SAEs, however given that it was very unlikely that these events would be determined by the medical monitor to be directly related to the observational study protocol, the study members reported these events every 6 months to the Observational Study Monitoring Review Board (OSMB).

There was no additional blood removed, or additional needle sticks required, for any donors participating in the US study and most in the Brazil study. Thus, the only risk to most donors was that of loss of confidentiality. The same AEs/SAEs related to phlebotomy discussed for recipients applied for the minority of Brazilian donors who provided an additional research sample.

### Sample size and power

*Patient/recipients*

In the US study, before study launch, 83 pediatric and 157 adult patients with thalassemia, and 71 pediatric and 75 adult patients with SCD were identified as eligible for inclusion in Aim 1. For patients with pediatric hematology/oncology diagnoses with hypo-proliferative bone marrow (relevant to Aim 2) we expected to consent a total of 100.

In the Brazil study, about 100 SCD cohort participants were estimated to be treated with primarily simple chronic transfusion therapy with the expectation that at least 50% would consent to participate given their membership in the SCD cohort.

*Power calculation*

Aim 1 was powered to be able to determine significant differences in donor SNPs based on preliminary data in a previous study of persons with SCD demonstrating ΔHbA per day was greater when comparing transfusion episodes with ≥1 G6PD-deficient units to transfusion episodes with no G6PD-deficient units (means 0.098 *vs* 0.086 g/dL/day, p=0.033)[7]. In the US, over 1,700 SCD and over 4,000 thalassemia transfusion episodes were anticipated for Aim 1. We estimated 90% power (alpha = 0.05) to determine an effect size of 0.005 at nearly a 10% allele frequency for patients with SCD, and an effect size of 0.005 at a 5% allele frequency for patients with thalassemia for the US study. For the Brazil study alone, the estimated 1,200 transfusion episodes for the SCD participants would achieve 80% power using the same criteria (*Online Supplementary Content*, **Table SI**).

Aim 2 of the US study was powered to detect a significant difference in serum iron in the 100 anticipated patients. We estimated we had 84% power to detect a minimum detectable difference of 1.5 μg/dL between the 3 patient groups. Aim 2 of the Brazil study was powered to detect serum iron differences between RBC units aged 1 to 3 weeks. We anticipated approximately 20 participants might be willing to stay for 2 hours to participate in Aim 2 approximately 10 times over the 2-year study. Using preliminary data measuring increase in iron in healthy volunteers after transfusion of autologous units stored for

1-3 weeks, we estimated >80% power to detect the change of at least 1.5 μg/dL between 1 and 3-week-old RBC units (*Online Supplementary Content*, **Table SII**).

### *Data management*

The DCC developed systems for collecting data from all participating sites into an integrated database for the protocol that would merge the separate data sources into unified analytical files. The system was configured by the DCC for the US study with several data entry modules to capture information on enrollment and consent; relevant patient, medical and specimen information at each eligible transfusion event by patient query and medical record abstraction; and key medical history information abstracted from electronic medical records at the beginning and end of the study period. A transfusion event module captured details related to each eligible study transfusion event or episode (**Figure 2**), specifically those data elements that would otherwise be difficult to capture from the electronic medical record, such as the actual volume of RBCs transfused. Linkages between the specimens collected during the transfusion event and the subject ID also occurred in the transfusion event data module. Parallel approaches were implemented in the Brazilian study management system to track all RBC-IMPACT research activities.

Recipient blood samples were prepared at the hospitals/hemocenters and labeled with a barcoded ID label provided by the DCC that included an embedded subject number, time point (e.g., pre-transfusion, immediately post-transfusion, two-hours post-transfusion), sample type (e.g., whole blood, transfusate, plasma, or serum), and aliquot number. Each series of samples collected at a single transfusion event had the same base (or core) ID sequence, and the study coordinators linked this core sequence and the subject ID in the appropriate data entry module. The coordinator also entered each sample and aliquot in a unified specimen tracking system to initiate the workflow and tracking of the lifecycle of each specimen. This system governed the routine shipment of specimens to the CTLS, initiated comprehensive tracking for each sample, as well as the outcome/status of the testing.

Donor and component data were obtained from the V2V database in the US and the donor/donation database in Brazil. These data were collected using rigorous QC procedures determined by those protocols[35]. The DCC was responsible for harmonizing all RBC-IMPACT study specific data, including standardizing data formats and developing systems for routinely and securely transferring data to the DCC.

### *Statistical analyses*

For Aim 1, the association of ΔHb/HbA per day with donor, manufacturing, and recipient variables will be assessed per patient in thalassemia and SCD patient groups. Least-square means (ls-means) will be used to compare ΔHb/HbA per day in transfusion episodes with 1 RBC unit transfused to >1 RBC units transfused. All factors will be assessed for quality, including missingness, prior to analysis. Univariable associations of ΔHb/HbA per day with donor, manufacturing, and recipient factors will be determined by linear mixed effects modeling. Variables associated with ΔHb/HbA will be used to identify candidate covariates for inclusion in the multivariable model. Multivariable linear mixed effects modeling will be used to examine the effects of the different factors on ΔHb/HbA per day, accounting for fixed effects of donor, manufacturing, and recipient factors per patient and the random effects due to repeated transfusions per patient. To account for differing number of units per transfusion episode (cumulative effect), the number of units per episode will be included as a covariate. To adjust for the timing of multiple transfusions, time-since-last-transfusion will be included as a continuous covariate. A model selection approach (e.g., stepwise or lasso) will be used to identify covariates that contribute significantly to predicting ΔHb/HbA in the presence of other factors.

The genotype data from the SNP array (PTMA) will be quality controlled and filtered (e.g., on the mean allele frequency [MAF], Hardy-Weinberg Equilibrium [HWE], and missingness of both SNPs and subjects) prior to analysis. To adjust for the number of units that are homozygous minor (or hemizygous minor) for a particular allele from the SNP array typing, the ratio of specific alleles of interest in units to total units per episode will be calculated. For instance, the G6PD-deficient ratio will be calculated as previously described by Sagiv *et al.*[7].

Data from SCD participants included in Aim 1 of the US and Brazil studies will be carefully reviewed to determine the presence of any systematic differences due to differences in the underlying populations, and a pooled analysis will be performed if appropriate. Key differences between the US and Brazil studies are summarized (**Table IV**).

**Table IV** - *Commonalities and key differences between US and Brazil RBC-IMPACT*

| | US RBC-IMPACT | Brazil RBC-IMPACT |
|---|---|---|
| **Aim 1** | | |
| **Hypothesis** | There are specific donor genetic and non-genetic factors, manufacturing factors and recipient factors that predict the RBC survival of transfused units. | |
| **Study population** | Chronically transfused individuals with SCD or thalassemia | Chronically transfused individuals with SCD enrolled in the REDS-IV-P Brazil SCD Cohort |
| **Primary outcome** | Change in hemoglobin/day (thalassemia) or hemoglobin A (SCD) between successive transfusions | Change in hemoglobin A/day between successive transfusions |
| **Aim 2** | | |
| **Hypothesis** | Increase in serum iron 2 hours after transfusion differs by disease status due to differences in iron metabolism in thalassemia (ineffective erythropoiesis), SCD (chronic hemolysis) and hematology/oncology diseases with hypoproliferative marrow (absent sink for iron in bone marrow) | There is a relationship between RBC unit storage age and increase in serum iron 2 hours after transfusion |
| **Study population** | SCD and thalassemia participants included in Aim 1 and patients with hematology/oncology diseases with hypoproliferative marrow who receive 1 RBC unit | SCD participants included in Aim 1 who received primarily 1 RBC unit transfusions |
| **Primary outcome** | Change in serum iron from pre to 2 hours post transfusion | |
| **Data source for recipient information** | Limited data asked of participants at study visits | |
| | Abstraction of key clinical outcomes from medical record by research staff | Abstraction of comprehensive clinical outcomes performed as part of REDS-IV-P cohort, variables required for IMPACT extracted from REDS-IV-P cohort database |
| | Extraction of some clinical outcomes from Vein-to-Vein database files | |
| **Collection of donor samples** | Waiver of donor consent approved from beginning of study to test retention tubes from donation | Initially donors recruited to return for informed consent and blood sample collection. Waiver of donor consent approved mid study to allow testing on residual samples from donation or transfused unit |
| **Data source for donor and component manufacturing information** | Vein-to-Vein database | Donor/donation/components database |



Aim 2 will be analyzed separately for the Brazil and US studies due to different predictors/hypotheses. For Aim 2 of the US study, the association of Δiron (measured as 2-hour post-transfusion iron - pre-transfusion iron) with donor, manufacturing, and recipient factors will be assessed utilizing mixed modeling to account for repeated measures per patient. The primary hypothesis that the slope (i.e., beta coefficient) of the relationship between storage duration and Δiron is affected by the underlying disease (i.e., SCD, thalassemia, or pediatric heme/onc) will be tested by incorporating an interaction term composed of the underlying disease and storage duration. A significant beta coefficient for this interaction term would suggest that the relationship between storage duration and Δiron is affected by the underlying disease. For Aim 2 of the Brazil study, the primary hypothesis was that the relationship between Δiron varies over storage duration (i.e., the beta coefficients are not equal to zero). This will be tested by including storage duration as a covariate in the model. A significant beta coefficient for this term would suggest that there is a significant difference in delta serum iron levels between 1 and 3-week-old RBC units.

For all analyses, a level of significance (alpha) of 0.05 will be used unless otherwise stated. Estimates and 95% confidence intervals will be reported for mixed models. Where appropriate, results will be adjusted for multiple comparisons using the FDR method of correcting p-values[41] or confidence intervals[42] and/or the Bonferroni method of correcting for multiple comparisons[43].

### Study monitoring

Regular reports were provided to the NHLBI REDS-IV-P OSMB, whose responsibility was to oversee study conduct and to ensure participant safety and adherence to planned study procedures. The OSMB was independently charged with reviewing the study protocol, informed consent forms, and data reporting templates. The specimen collection plan for this study was deemed minimal risk as collections occurred as part of routine chronic transfusion therapy (while the IV is inserted) and donor risks were minimal. A designated medical monitor in the US study and a different medical monitor in the Brazil study reviewed all AE/SAEs. Reports submitted to the OSMB included study progress, including recruitment/enrollment rates, attrition, and demographics, and summary outcome data tables were also sent to the OSMB for review. Lastly, the OSMB received and monitored all reported AE/SAEs. Upon review, the OSMB provided recommendations for review and approval by NHLBI. Annual reports were also submitted to study IRBs.

## RESULTS

Patient enrollment began in January 2022 and follow-up and donor sample acquisition was completed in March 2024 in the US and August 2024 in Brazil. Laboratory testing was completed in April 2025, with analysis and reporting of the results expected through August 2026. Summary recipient and donor enrollment in the US and Brazil are provided (**Table V**). For participants with thalassemia, there were 1,504 complete transfusion events on study and 1,482 evaluable transfusion episodes, and for participants with SCD there were 1,354 complete transfusion events on study and 1,232 evaluable transfusion episodes. Specific clinical hypotheses will be tested using subsets of the data restricted to thalassemia and SCD patients. In addition, overall assessment of donor and component modification characteristics associated with hemolysis will be evaluated using the full study datasets.

**Table V** - *Overall summary of blood recipient and donor enrollment in the US and Brazil*

| Study group | United States | Brazil | Total |
|---|---|---|---|
| **Recipients** | | | |
| Thalassemia | 93 | - | 93 |
| Sickle cell disease | 47 | 72 | 119 |
| Pediatric oncology | 17 | - | 17 |
| **Donors** | | | |
| RBC units on study | 5,070 | 1,732 | 6,802 |
| Unique donor samples obtained | 3,769 | 1,393 | 5,162 |

A subset of the thalassemia and SCD recipients and all pediatric oncology recipients participated in Aim 2.

## DISCUSSION

To our knowledge, the REDS-IV-P RBC-IMPACT study represents the largest and most granular study to date on RBC quality and short-term outcomes for patients receiving chronic transfusion therapy. Previous studies have characterized donor factors, including genetic factors associated post-transfusion hemoglobin, and storage hemolysis using linked datasets to assess the effectiveness of RBC transfusion[7,11-15]. These previous studies have not collected specific peritransfusion data in a standardized manner in multiple lcoations for all studied transfusion episodes to assess RBC effectiveness. In addition, the previous studies did not have access to transfusate samples to directly characterize total hemoglobin and hemoglobin variants in each transfused unit. These features of RBC-IMPACT will allow for new insights into the factors associated with RBC survival in chronically transfused patients.

Despite the potential of this study to improve transfusion quality for these patient populations, study limitations exist. Automated RBC exchange transfusions are becoming more common for patients with SCD. It is unclear whether the findings of chronic simple or partial manual exchange transfusions will translate to those who receive a mixture of more RBC units per procedure using an apheresis device. While systems were developed to identify and track donors who were transfused into study participants, obtaining blood samples from all donors, especially those whose blood was obtained from outside the REDS-IV-P study blood centers, was logistically challenging, and so some studied transfusion episodes will not have donor samples from which to compare the key genetic marker information in Aim 1.

Primary analyses for Aim 1 will focus on the donor, component, and recipient clinical factors associated with markers of RBC transfusion effectiveness, including event-level hemoglobin increment, episode-level hemoglobin decrement, and event-level and episode-level

changes in bilirubin. Additional analyses will focus on the donor genetic SNPs associated with hemolysis markers and their relationships with RBC survival in patients. In addition, the surrogate or intermediate factors which define the "in the bag" transfusate hemoglobin level compared to RBC effectiveness in transfused patients will be assessed. Planned other papers will report on validation of the PTMA and SNP frequencies in donor and recipient populations. Primary analyses for Aim 2 will assess the changes in hemoglobin increment immediately and two hours after transfusion among those participants with these data.

### Study close-out and access to data

At study close out, the de-identified analytic files, documentation and all codes used in processing will be archived in a way that will allow replication of the results. De-identified public use datasets are planned to be created and delivered to the NHLBI at the end of the study and will be made available for future analyses. Data that are not included in the de-identified public use dataset will be archived for three years after the end of the REDS-IV-P contract (June 30, 2029). Each organization affiliated with the study, through subcontract or otherwise, will manage their RBC-IMPACT protocol specific data according to research records retention policies in the US and Brazil. Biospecimens will be retained at the REDS-IV-P central laboratory (VRI) or a public repository such as BioData Catalyst in the US and at the biobank at the University of Sao Paulo in Brazil.



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**US Hubs**

- *A.E. Mast* and *L. Baumann* Kreuziger, Versiti Blood Research Institute, Milwaukee, WI, USA;
- *E.A. Hod*, Columbia University Medical Center, New York, NY and *B.S. Sachais*, New York Blood Center, New York, NY, USA;
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**Brazil**

- *B. Custer*, Vitalant Research Institute, San Francisco, CA, USA;
- *E.C. Sabino*, University of Sao Paulo, Sao Paulo, SP, Brazil;
- *C. Dinardo*, Fundacao Pro-Sangue, Sao Paulo, SP, Brazil;
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- *L. Amorim*, Hemorio, Rio de Janeiro, RJ, Brazil;
- *A. Ferraz*, Hemope, Recife, PE, Brazil;
- *N. Fraiji*, Hemoam, Manaus, AM, Brazil.

**Data Coordinating Center**

- *K.M. Chapman*, Westat, Rockville, MD, USA;
- *D.B. Bookwalter*, Westat, Rockville, MD, USA;
- *N.L. Luban*, Children's National Medical Center, Washington, D.C., USA.

**Central Laboratory**

- *P.J. Norris* and *M. Stone*, Vitalant Research Institute, San Francisco, CA, USA.

**Publications Committee Chair**

- *P.M. Ness*, Johns Hopkins University, Baltimore, MD, USA.

**Steering Committee Chairpersons**

- *C.D. Josephson*, Johns Hopkins University School of Medicine and Johns Hopkins All Children's Hospital, Baltimore MD and St. Petersburg, FL, USA;
- *S.H. Kleinman*, University of British Columbia, Victoria, BC, Canada.

**National Institute of Child Health and Human Development (NICHD)**

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## AUTHORS' CONTRIBUTIONS

MK and SK wrote the manuscript. EH and BC developed the study protocol design and contributed to all components of the study and this manuscript. All co-Authors contributed to the performance of the study and reviewed the manuscript.

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