

REDS III International Program - Brazil

**Establishing a Brazilian Sickle Cell Disease Cohort, Identifying
Molecular Determinants of Response to Transfusions and Genetic
Determinants of Alloimmunization**

Protocol Team Members

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Concept Synopsis

Sickle cell disease (SCD) is the most prevalent clinically-significant inherited blood disorder worldwide, with more than 13 million people affected by the disease[1]. While caused by a single nucleotide substitution in the beta globin chain of hemoglobin the pathophysiology of SCD is complex. It involves a combination of vaso-occlusion, hemolysis, endothelial dysfunction and inflammation, and patients with SCD suffer severe complications of virtually every organ system. The Cooperative Study of Sickle Cell Disease followed a large cohort of SCD patients beginning in the late 1970s to describe the epidemiology of these complications, baseline laboratory values and causes of mortality in the US population. This study was instrumental in guiding clinical management and research in SCD for decades. However, there have been no studies to investigate this epidemiology in the Brazilian SCD population and few large contemporary cohort studies world-wide to continue to re-evaluate the epidemiology and mortality of SCD in the era of increased chronic transfusion therapy, hydroxyurea use and current supportive care practices.

The four REDS III Brazilian Hemocenters are blood banks which provide outpatient care and transfusion therapy for almost 10,000 SCD patients. This provides a unique opportunity to study the epidemiology and transfusion outcomes of a large number of SCD patients in a research network that has previously been connected through REDS II. This study will aim to establish a Brazilian SCD cohort and subsequently perform targeted studies focused on transfusion outcomes in SCD patients.

Aim A: Establish a cohort of approximately 3000 SCD patients seen at the four REDS III Hemocenters in Brazil. We will develop a comprehensive electronic database to centralize detailed clinical, laboratory and transfusion information. We will also establish a biospecimen repository of blood samples from all enrolled patients to support biological studies relevant to SCD pathogenesis and transfusion complications.

This cohort will help define the current epidemiology of SCD as well as characterize blood utilization patterns and transfusion outcomes in the Brazilian SCD population. Allogeneic blood transfusion was one of the earliest therapies shown to alleviate SCD complications. Blood transfusion can be life saving in acute illness and chronic transfusion therapy can prevent many complications of SCD such as stroke [2]. However, there is a lack of consensus regarding transfusion guidelines for many other manifestations of SCD and large-scale studies that characterize blood utilization and transfusion complications in these patients. These will be goals of the Brazilian cohort.

Finally, the cohort will provide the infrastructure to efficiently identify subpopulations for future studies in SCD. Initial planned studies will investigate the impact of blood transfusion on the inflammatory pathophysiology of SCD and the genetic basis of alloimmunization.

Aim B: Characterize changes in markers of inflammation in response to transfusion by analyzing chemokine/cytokine panels in serial post transfusion specimens.

Despite the acknowledged clinical benefit of transfusion in SCD, the underlying mechanisms by which this occurs remain incompletely understood. Transfusion is presumed to improve SCD symptoms by a combination of dilution of cells containing hemoglobin S and improved rheology and oxygen carrying capacity[3]. However, the dramatic clinical response observed following transfusion in the treatment of specific manifestations such as acute chest syndrome or stroke is only partially explained by these mechanisms. Inflammation is now recognized to play a critical role in the pathophysiology of SCD[4, 5] and transfusion is known to modulate inflammation in

other populations[6]. We will measure a panel of chemo/cytokines before and serially after transfusion to characterize the impact of transfusion on the inflammatory pathophysiology of SCD.

Aim C: Identify particular single nucleotide polymorphisms (SNPs) that contribute to the risk of red blood cell alloimmunization in SCD by performing a genome-wide association study (GWAS) in transfused SCD patients.

Approximately 30% of SCD patients develop at least one antibody to transfused red blood cells (RBC) and almost 10% will form more than one RBC antibody[7] [8]. Alloimmunization can have a profound impact on transfusion management for SCD patients. Alloantibodies can result in serious sequelae, including life threatening hemolytic transfusion reactions, in addition to incurring significant delays in the procurement of compatible blood for transfusion [9]. Prospective phenotypic matching has been shown to decrease, but not eliminate, alloimmunization [10] and adds significantly to the cost and workflow burden of transfusing SCD patients. Therefore, identification of those patients at risk for alloimmunization would allow for more targeted phenotypic matching transfusion strategies. We will employ whole genome single nucleotide polymorphisms (SNP) typing to identify particular SNPs associated with risk of alloimmunization through a GWAS.

Objectives

Aim A: Develop a comprehensive electronic database to centralize clinical, laboratory and transfusion information for a cohort of 3000 sickle cell patients followed by the four REDS III Hemocenters in Brazil. This will allow the following descriptive analyses in the study population:

1. Prevalence of SCD complications
2. Epidemiology of blood utilization
3. Prevalence of HIV and Hepatitis B/C
4. Rates of transfusion transmitted infections (including HIV and Hepatitis B/C)
5. Rates and correlates of alloimmunization
6. Impact of chronic transfusion therapy on clinical outcomes
7. Characterization of baseline hematologic and hemolytic laboratory values and correlation with clinical outcomes
8. Rates and causes of mortality

Secondary Objective

Build a well-characterized phenotype database and biospecimen repository to provide the infrastructure for future SCD research.

Aim B: Characterize changes in markers of inflammation in response to transfusion by analyzing chemokine/cytokine panels in serial post transfusion specimens.

- 100 patients with a minimum of 1 year of chronic transfusions
- 150 non-transfused control patients.
 - 75 treated with hydroxyurea
 - 75 not treated with hydroxyurea

Aim C: Identify particular single nucleotide polymorphisms (SNPs) that contribute to the risk of red blood cell alloimmunization in SCD by performing a genome-wide association study (GWAS) to compare patients with red blood cell antibodies to patients without red blood cell antibodies

Significance

The well-characterized cohort, which will centralize the clinical/transfusion data of approximately 3000 patients seen at REDS III Hemocenters, will allow a characterization of the epidemiology of SCD, description of blood utilization and a comprehensive evaluation of the therapeutic and adverse impact of transfusion therapy in the Brazilian SCD population. In addition, the combined database and biospecimen repository will provide a foundation for designing future genotype-phenotype studies relevant to SCD pathophysiology. The initial targeted studies to be conducted will be of paramount importance for sickle cell patients. Identification of patients with increased risk of alloimmunization would allow for more targeted use of extensively matched blood and have implications for cost management and appropriate resource allocation for transfusing this population. Characterization of the impact of transfusion on the inflammatory pathophysiology of SCD may shed light on the mechanisms by which transfusion provides clinical benefit in SCD and potentially identify new therapeutic targets. Therefore we anticipate this study will not only impact the management of the Brazilian SCD population but ultimately the management of SCD patients worldwide.

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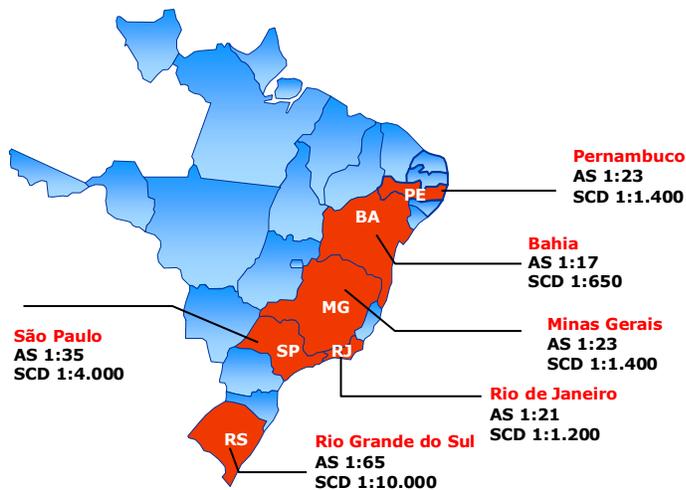
Aim A Establish a cohort of SCD patients seen at the four REDSIII Hemocenters in Brazil. Develop a centralized, comprehensive electronic database of clinical, laboratory and transfusion information and biospecimen repository of blood samples from all enrolled patients to support biological studies relevant to SCD pathogenesis and transfusion complications.

1. Background

1.1 History and Current Status of SCD Prevalence & Treatment in Brazil

The genes of Sickle Cell Disease and its related disorders were first brought to Brazil in the colonial era after 1500. Sickle cell, alpha thalassemia and beta thalassemia genes were introduced by African and European populations, which ultimately mixed with each other and the Brazilian native population. Brazil currently has ~25,000– 30,000 people with SCD and ~7 million carriers, leading to the birth of ~3,500 new babies with SCD each year. The prevalence varies markedly across the country (see figure 1). Medical surveillance and assistance varies significantly by state, hence many SCD cases previously went undiagnosed or inadequately treated. To help redress this situation, the Brazilian Government implemented a series of new regulations in 2001 and 2005 requiring infant screening and comprehensive care of SCD. The Ministry of Health (MoH) has also issued treatment protocols, including guidelines for transfusion management of sickle cell patients. However there is still no national database or organized network for research in SCD. There have been no large-scale multi-center studies to define the prevalence of SCD complications in Brazilian SCD patients, characterize blood utilization patterns to determine compliance with MoH transfusion guidelines or evaluate the therapeutic and adverse impacts of transfusion therapy in this population.

Figure 1: Prevalence of SCD and sickle cell trait (AS) by state



1.2 SCD Patient Populations and Facilities of REDSIII Hemocenters

The four REDS III Hemocenters care for approximately 9,700 SCD patients. Table 1 summarizes the number of SCD patients registered at each of the Hemocenters.

Table 1: Sickle Cell Patient Populations at REDS III Hemocenters

Hemocentro	Hemominas/UFMG			Hemope	Hemorio	Icr-ITACI	Total
City	Belo Horizonte	Montes Claros	Juiz de Fora	Recife	Rio de Janeiro	Sao Paulo (Children)	
Total	2934	903	483	1365	3830	157	9672
Number of newborn cases/year	120	30	11	110	104	5	380

The sites chosen for the REDS III SCD study perform newborn screening and approximately 380 new cases of SCD will be detected by the Hemocenters each year. Only the pediatric center in Sao Paulo at the Instituto da criança – Instituto de Tratamento do Cancer Infantil (Icr-ITACI) is participating in the study. Hemorio in Rio de Janeiro and Hemope Recife are in smaller states where patient treatment is concentrated in these cities. Minas Gerais, by contrast, is roughly the size of France and has 13 locations for the treatment of 6000 SCD patients. Here, we have chosen three of the main sites (Belo Horizonte, Montes Claros and Juiz de Fora) that care for 75% of the patients. Currently, most of the clinical data of SCD patients seen at the Hemocenters is in hand-written charts, requiring significant effort to capture and organize into an electronic database under standardized definitions, which is a major objective of this project. The study will not dictate changes in clinical care, but will centralize clinical, laboratory and transfusion data collected as part of patients' routine care in the electronic database.

1.3 Development of a Comprehensive Sickle Cell Database in the US

The use of centralized databases was established in the United States SCD population with the creation of the US Cooperative Study of Sickle Cell Disease (CSSCD) cohort in 1978[11]. This cohort helped define the clinical course of SCD in the US sickle cell population and provided the foundation for decades of seminal research which continues to guide the management of SCD today[12-24]. Based on this success, the Comprehensive Sickle Cell Centers (CSCC) launched the CSCC Collaborative Data Project (CSCC C-Data) under the direction of the NHLBI in 2004. This database involved an exhaustive evaluation of the clinical complications and laboratory parameters of all enrolled patients. An ancillary protocol to collect and store biologic samples, from patients enrolled on C-Data was developed in 2005. The protocols and case report forms from the CSCC Collaborative Data Project and the related genotype-phenotype study will serve as templates for establishing the database and biospecimen repository for the SCD population in Brazil. These forms will be modified to take into account more recent scientific developments and care paradigms as well as the unique population being studied.

1.4 Standardized Definitions

One of the difficulties in organizing clinical data into a comprehensive research database is the lack of standardized definitions for classification of clinical outcomes. To help address this issue in sickle cell disease, members of the C-Data Protocol Committee were charged with drafting specific definitions of SCD manifestations. The resulting definitions of the phenotypic manifestations of SCD were published in the American Journal of Hematology in 2010[25]. Definitions from this publication were approved by the Brazilian SCD co-investigators to guide classification of clinical events entered into the database for this project (see appendix 1 for complete list of definitions).

1.5 Goal of the Electronic Database

Given the dearth of epidemiologic data for Brazilian populations with SCD and need for updated data for the SCD population worldwide, the database will allow investigators to begin to define the prevalence of SCD phenotypes, mortality, transfusion rates and associated complications. The database will also provide the infrastructure to identify subpopulations for future studies. The initial targeted studies to be performed are detailed in Aims B and C.

2. Objectives

2.1 Primary Objective

Develop a comprehensive electronic database to centralize clinical, laboratory and transfusion information for a cohort of 3000 sickle cell patients followed by the four REDS III Hemocenters in Brazil. This will allow the following descriptive analyses in the study population:

1. Prevalence of SCD complications
2. Epidemiology of blood utilization
3. Prevalence of HIV and Hepatitis B/C
4. Rates of transfusion transmitted infections (including HIV and Hepatitis B/C)
5. Rates and correlates of alloimmunization
6. Impact of chronic transfusion programs on clinical outcomes
7. Characterization of baseline hematologic and hemolytic laboratory values and correlation with clinical outcomes
8. Rates and causes of mortality

2.2 Secondary Objective

Build a well-characterized phenotype database and biospecimen repository to provide the infrastructure for future SCD research.

3. Study Populations

Adult (≥ 18 years) and pediatric patients (< 18 years) with a diagnosis of SS, SC, $S\beta^0$ thalassemia or $S\beta^+$ thalassemia who are currently active patients at REDSIII Hemocenters and expected to return regularly represent the accessible population. Active patients will be defined as having a clinic visit at the Hemocenter within the last 36 months prior to start of enrollment. Date of most recent clinic visit and basic demographic data was extracted from Hemocenters' electronic records to determine the total number of active, eligible patients and define stratified enrollment goals (results in Table 2). Age, gender and SCD genotype are summarized only for active patients.

Table 2: Percent Active Patients and Demographics of Active Patients per Hemocenter

Hemocenter City	Hemominas			Hemope	Hemorio	ITACI	Total
	Belo Horizonte	Juiz de Fora	Montes Claros				
Total Number of Patients	2934	483	903	1365	3830	157	9672
Active Patients (% of Total)	2348 (80%)	413 (86%)	786 (87%)	1185 (87%)	2992 (78%)	135 (86%)	7850 (81%)
Demographics of Active Patients							
Age (mean, range, years)	22.1 (1-78.9)	20.5 (0.7-64.1)	18.4 (0.7-77.3)	20.7 (0.8-75.1)	19.8 (0.7-95.7)	12.2 (2-19.5)	20.2 (0.7-95.7)

Number Adult/Pediatric ‡ Patients (%) pediatric	1182/1166 (50%)	204/209 (51%)	337/449 (57%)	592/593 (50%)	1329/1663 (56%)	13/121 (90%)	
Female (%)	1262 (54%)	216 (52%)	405 (52%)	620 (52%)	1555 (52%)	60 (45%)	4118 (52%)
SS	1342 (57%)	265 (64%)	500 (64%)	940 (79%)	**SS/Sβ ⁰ 2262 (75.6%)	90 (67%)	
SC	917 (39%)	123 (30%)	265 (34%)	166 (14%)	580 (19.%)	23 (17%)	
Sβ ⁺	Sβ ⁺ / Sβ ^{0*}	Sβ ⁺ / Sβ ^{0*}	Sβ ⁺ / Sβ ^{0*}	62 (5%)	150 (5%)	4 (3%)	
Sβ ⁰	89 (3%)	25 (6%)	13 (2%)	17 (1%)	**	17 (13%)	

* Note Sβ⁺ and Sβ⁰ patients were classified together in Hemominas

** Note SS and Sβ⁰ patients were classified together in Hemorio

‡ Adult defined as ≥18 years and pediatric defined as <18 years

4. Study Enrollment

4.1 Screening and Recruitment

To limit volunteer bias in the cohort and aim to enroll a sample of the available patient population which is representative of the entire range of clinical severity in the Brazilian SCD population, a random sampling of the 7859 active patients will be recruited into the study. Based on estimates of Brazilian investigators familiar with the study population, we anticipate enrollment of ~70% of recruited patients. Therefore a list of all patients seen within the last 3 years at each site will be generated and 4289 patients will be randomly selected to be eligible for the study in order to achieve an enrollment goal of 3000. Patients designated eligible by the random sampling will be recruited at routine Hemocenter visits by participating sickle cell physicians or clinical research assistants. The research assistant at each center will review scheduled appointments 1 week in advance to identify dates of scheduled routine visits for eligible patients. The assistant will call eligible patients prior to scheduled visit to remind patients of routine visit.

4.2 Stratification of Enrollment

The enrollment goals will be stratified by Hemocenter to approximately reflect the percentage of total active patients represented by each Hemocenter (see enrollment goals table 3). The number of enrolled patients at the smallest centers, Sao Paulo and Juiz de Fora, will be a larger percentage than actual percentage of active population to maximize use of resources/personnel at these sites. Within each Hemocenter, enrollment will also be stratified based on age, gender and SCD genotype to ensure distribution of pediatric/adult, male/female and SCD types of enrolled subjects reflect overall demographics of the Hemocenter.

Table 3: Enrollment Goals per Hemocenter

Hemocenter	Active Patients	Enrollment Goals
Belo	2348	850
Juiz de Fora	413	250
Montes Claros	786	350

Recife	1185	550
Rio	2992	900
Sao Paulo	135	100
Total	7859	3000 (38% of active population)

5. Timeline

Phase 1 Activities

Study phase	Phase 1							
Calendar Year	2011			2012			13	
Quarter	2	3	4	1	2	3	4	1
Sickle Cell Project								
Aim A: Establishing a SCD Cohort/Biorepository								
Design of CRFs and central data warehouse								
Sickle cell protocol development								
Sickle cell IRB approvals [US and Brazil Aims A-C)								
Pilot test electronic database each Hemocenter								
OMB clinical exemption								

Phase 2 and 3 Activities

Study phase	Phase 2															
Calendar Year	2013				2014				2015				2016			
Quarter	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	
Sickle Cell Project																
Aim A: Establishing a SCD Cohort/Biorepository																
Manual of procedures																
Training all study personnel																
Limited enrollment into cohort																
Full enrollment into cohort																
Follow-up visits																
Analysis and reporting																

6. Schedule of Measurements

6.1 Schedule of Study Visits

A total of 4 study visits will occur: 1 enrollment visit to obtain informed consent, enter detailed demographic, clinical, laboratory and transfusion history into electronic database and collect blood samples for the biorepository. Patients will then have three follow up visits to update all data collected during enrollment visit. Blood samples will only be collected at enrollment, follow up 2 and follow up 3.

6.2 Demographic, Clinical, Laboratory and Transfusion Data

The centralized electronic database is organized into 15 forms. See appendix 2 for complete paper version of questionnaire and overview of data in Table 4. During the follow up visits (not enrollment visit), supplemental forms will be collected for the 17 SCD manifestations identified in Table 4. Upon death, withdrawal or loss to follow up the termination form will be completed. A subject will be classified as lost to follow up after six months past date of study visit and no ability to contact subject despite a minimum of 6 attempts by the research assistant.

Table 4: Forms of the Electronic Database

Form #	Form Name	Data Collected
Enrollment and Annual Follow Up Forms		
1	Interview with Patient/Family 1	Vital sign measurements, documentation of participation in other studies
2	Interview with Patient/Family 2	Race, employment/school status; marital status; income; transfusion received at other institutions, unscheduled visits due to SCD; missed school/work due to SCD-related pain; tobacco/alcohol use; pregnancy history
3	Hospital Admissions	Discharge diagnoses and dates for all hospital admission in previous 12 months
4	Surgical History	Date, type of surgeries
5	Stem cell transplantation	Date, type, indication of transplantation
6	Medication History	Medications used in the previous 12 months
7	Selected Screening or Diagnostic Tests	Results of TCD, MRI/A, Echo, PFTs in previous 12 months
8	Infectious Disease Test Results	Results of Hepatitis, HIV, syphilis, HTLV testing in previous 12 months
9	Baseline labs	CBC, Reticulocyte, HbF, LDH (most recent in previous 12 months recorded in outpatient, stable baseline state)
10	Iron Overload status	Ferritin, liver biopsy, MRI
11	Blood bank/transfusion data	ABO/RBC phenotype, antibody history
12	Transfusions History	Number of units transfused in patients life, age at first transfusion, details of any previous treatment with chronic transfusion
13	Transfusions last year	Indication, transfusion method and associated transfusion reactions for all units transfused in previous 12 months.
14	Data of units transfused last year	Phenotype, age, leukoreduction status of all units transfused in previous 12 months
15	Medical History	SCD complications
Supplemental Forms		
1	Aplastic episode	Nadir hemoglobin, results of parvovirus testing, treatment
2	Splenic sequestration	Nadir hemoglobin, reticulocyte count, platelet count, treatment
3	Infarctive stroke	Region of infarct (MRI), treatment
4	Silent infarct	Region of infarct (MRI)
5	Hemorrhagic stroke	Region of infarct (MRI), Other diagnostic tests, treatment
6	Seizures	Etiology, diagnostic tests
7	Hepatic sequestration	Nadir hemoglobin, liver function tests, treatment
8	Pulmonary embolism	Diagnostic tests, treatment
9	Acute chest syndrome	Use of mechanical ventilation, treatment
10	Pulmonary hypertension by catheterization	Results of catheterization, treatment

11	Pulmonary hypertension by echocardiogram	Results of echo, treatment
12	Avascular necrosis	Diagnostic tests, grade of necrosis, treatment
13	Priapism	Frequency/duration of priapism, treatment
14	Proteinuria	Proteinuria quantified
15	Chronic renal failure	Etiology, treatment
16	Acute renal failure	Etiology, treatment
17	Serious infection	Culture results bacteremia, urinary tract infection, meningitis
1	Termination Form	Date/Cause of death or date of termination/transfer

7. Specimen Procurement and Storage

7.1 Specimen Acquisition

Blood will be collected upon enrollment and two subsequent study visits. Approximately 11 mL whole blood will be collected from all patients with the exception of pediatric patients weighing <8kg who will have 7mL of blood collected. No more than a maximum of 2.5% total blood volume of blood will be collected from any subject.

7.1.1 Sample Collection at Enrollment and Follow up 2 Visit

Whole blood will be collected in two 4mL anticoagulated EDTA tubes or 1 4mL EDTA tube for patients <8kg. Tubes will centrifuged at each Hemocenter within 6 hours of collection to separate whole blood into plasma and packed cell components. Plasma and cellular components will each be divided into 1cc aliquots and aliquots will be stored in the repository. A 3mL Tempus tube for RNA stabilization will also be collected for the biorepository.

7.1.2 Sample Collection at Follow up 3 Visit

Whole blood will be collected in one 4mL anticoagulated EDTA tubes and 1 serum tube for patients. Tubes will centrifuged at each Hemocenter within 6 hours of collection to separate whole blood into plasma and packed cell components. Plasma and cellular components will each be divided into 1cc aliquots and aliquots will be stored in the repository. A 3mL Tempus tube for RNA stabilization will also be collected for the biorepository.

7.2 Specimen Storage

All aliquots will be sent quarterly in batch shipments to the biorepository at the Instituto de Medicina Tropical (IMT) in Sao Paulo for permanent storage at -80C.

8. Planned analyses

Descriptive statistics will be used for the following analyses.

- Prevalence of specific SCD complications identified at enrollment
- Incidence of specific SCD complications identified over follow up period
- Prevalence of HIV and Hepatitis B/C
- Prevalence of alloimmunization identified at enrollment and alloimmunization rate identified over follow up period

- Correlates of alloimmunization status to be analyzed: age at first transfusion, phenotypic matching, total number of units transfused
- Blood utilization patterns
 - Indications for acute and chronic transfusions
 - Number of units transfused in age-stratified groups
 - <10 years
 - 10-20years
 - >20 <60 years
 - >60 years
 - Mean age of transfused units
- Impact of chronic transfusion program on clinical endpoints
- Rates of transfusion transmitted infections and other transfusion adverse effects
- Rates/causes of mortality

9. Statistical Considerations

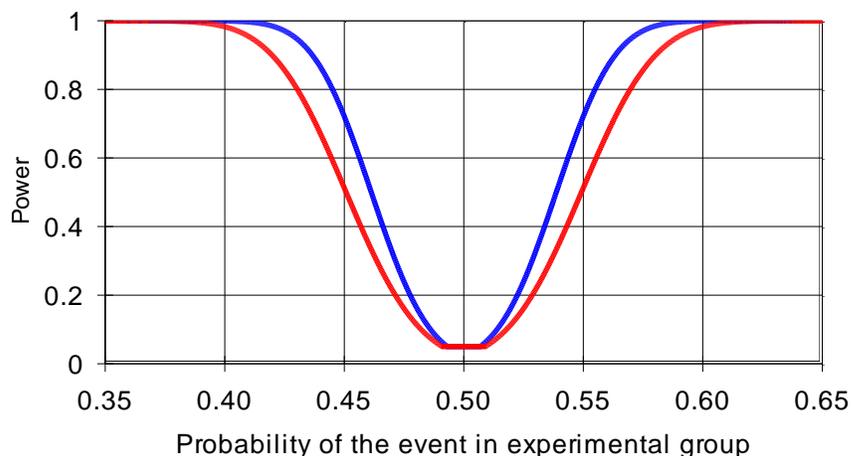
9.1 Sample size justification

The sample size of 3000 was selected as maximum feasible size of the cohort based on available budget and personnel resources. This will represent approximately one-third of the entire Hemocenters' SCD population and 38% of the active SCD population, therefore we anticipate this will reflect an adequate representation of the accessible population.

9.2 Power

Power is considered to detect differences in the rates of exposure between various subgroups of the SCD patients. When the outcome of interest, e.g. alloimmunization, is present in 1/6th of the patients and exposure is 50%, we are able to detect significant differences in the rate of exposure of 7.4% (50% vs 42.6%). Power is greater when the outcomes are more common, and less when exposure is rarer.

Figure 2: Power estimation according to probability of event



10. Database Management

The Brazil Central Data Coordinating Center (B-DCC) has the primary responsibility for programming and hosting the web-based data collection and study management systems. The REDS-III DCC (RTI) and participating organizations will collaboratively influence the design and structure of these systems to ensure all needs are met and that the resulting data will be of the quantity and quality necessary to address the study aims. Data extracted from both systems will be uploaded to the REDS-III DCC (RTI) on a regular basis (weekly or monthly, as deemed appropriate by the study team). Quality control will occur at multiple levels for this study: 1) at data abstraction/capture; 2) at data entry; 3) pre-upload to DCC; and 4) at the DCC. The team will develop automated processes to achieve a high level of quality for this data.

Further, the DCC will provide the sites with a data entry system to track specimen collection (BSI) along with details on any aliquots made, along with detail on the location (freezer, box, and position) for all specimens collected on this protocol. Aliquot related data will be entered directly into the BSI system, using a study specific template to be designed by the DCC, BSRI, and the Brazil central lab at Fundação Pró-Sangue in Sao Paulo. These data will be accessible to all participating organizations, but maintained primarily by the DCC.

Aim B: Characterize changes in markers of inflammation in response to transfusion by analyzing chemokine/cytokine panels in serial post transfusion specimens

11. Background

11.1 Role of Transfusion Therapy in Sickle Cell Disease

The clinical benefits of blood transfusion for sickle cell patients have been recognized since the early 1950s, although the associated complications initially limited its use. With significant improvements in blood safety over the past few decades and several landmark studies demonstrating the efficacy of blood transfusion in SCD, blood use in these patients has increased[26]. Transfusion can reverse or improve acute clinical complications of SCD manifestations, chronic transfusion can prevent some major complications[2, 27, 28], and routine preoperative transfusion can decrease perioperative morbidity[29]. Transfusion presumably improves SCD symptoms by a combination of dilution of cells containing mostly hemoglobin S and improved rheology and oxygen carrying capacity[3]. However, these mechanisms are insufficient to explain the degree of clinical benefit observed with transfusion in treatment of specific manifestations such as ACS or stroke.

Sickle cell is now recognized to also be a disease of vasculopathy and chronic inflammation [4, 5] and transfusion is known to impact inflammation in other populations[6]. Therefore, in order to improve our understanding of how transfusion impacts the inflammatory pathophysiology of SCD, panels of cytokines will be measured in serial post transfusion samples. In addition, blood will be collected into PAXgene tubes, which stabilize mRNA, before and after transfusion. These tubes will be stored in the biorepository and available for future analysis of changes in gene expression in response to transfusion.

11.2 Inflammation in SCD

Although the original view of SCD pathophysiology focused on polymerization, hemolysis and vaso-occlusion, the critical role of inflammation has been increasingly recognized over the last decade. An inflammatory response is provoked by the damage caused by interaction of the rigid sickle RBC with vascular endothelium as well as the hypoxia-reperfusion injury associated with poor tissue perfusion[5]. In addition, hemolysis contributes to reduced nitric oxide bioavailability, which ultimately results in increased endothelial adhesion molecule expression

and activation of white blood cells and platelets[30]. SCD patients, even at baseline, have elevated levels of multiple inflammatory markers including C reactive protein, tumor necrosis factor, interleukins and white blood cells[4, 31-35]. Elevated WBC counts have been correlated with specific SCD manifestations and shown to be predictive of severe disease and mortality[19]. Despite recognition that SCD is a disease of inflammation and endothelial activation, there have been few studies to characterize how this inflammatory milieu shifts with transfusion. Sakhalkar et al reported soluble vascular cell adhesion molecule 1 (sVCAM-1) levels were significantly lower in chronically transfused patients compared to “asymptomatic” SCD patients, but other markers were not measured and the comparison was performed at a single point in time[36]. We plan to measure a comprehensive panel of cytokines and inflammatory markers in serial samples after transfusion. The panels consist of markers of inflammation or endothelial activation that have been identified as important in SCD pathophysiology and are also included on standard panels measured in the immunology core lab at BSRI (see complete list in table 5 of methods section).

The multianalyte cytokine detection approach is a relatively new screening approach for a wide array of systemic immunological changes. Our group has recently published several examples of successful use of these assays to define the pathophysiology of acute infectious diseases[37] and even predict disease outcome[38]. While previous reports have shown substantial heterogeneity in cytokine levels among SCD patients, the best method to control for this heterogeneity is to use patients as their own control. To that end, serial samples will be drawn on all patients including baseline pre-transfusion samples.

Using this methodology in transfused trauma patients we were able to provide a detailed characterization of the major shift in the immunologic environment in response to trauma and transfusion. We demonstrated an initial significant anti-inflammatory response to transfusion followed later by a response dominated by proteins involved in wound healing and homeostasis (Fig. 2)[6].

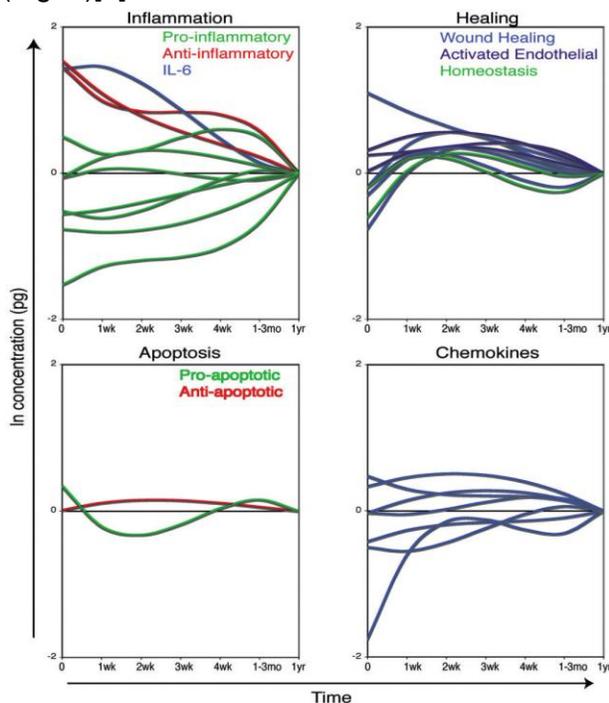


Figure 3: Kinetics of immune response to trauma. Overlays of the models' prediction of the influence of time since trauma controlling for the other covariates are plotted by protein type. Predicted values at 1 year after trauma are set as the baseline (0) for each cytokine to show elevation or depression relative to this value. The inflammation plot includes the proinflammatory cytokines IL-1 α , IL-5, IL-9, IL-17, TNF α , TNF β , MIF; the anti-inflammatory cytokines IL-1Ra and IL-10; and IL-6, which has both pro and anti-inflammatory properties. The healing plot includes the wound healing proteins EGF, FGF-2, VEGF, MMP-9 and tPAI-1; the activated endothelial markers sE-selectin, sICAM-a and sVCAM-1; and the homeostasis cytokines IL-7 and IL15. The apoptosis plot includes the proapoptotic sFasL and the antiapoptotic sFas. The chemokine plot includes IP-10, IL-8, MIP-1 α , eotaxin, fractalkine.

We will measure the presumed immunosuppressive profile in SCD transfusion recipients and expect the parameters to be measured will capture the complexity of the immune perturbation

seen after transfusion and contribute to a fundamental understanding of how transfusion affects SCD pathophysiology. We will measure the panels pre and post transfusion in patients on chronic transfusion therapy as well as non transfused controls to characterize the impact of transfusion on the inflammatory pathophysiology of SCD. Because some research has shown hydroxyurea may impact inflammation, non transfused controls will be stratified into patients treated and not treated with chronic transfusion.

11.3 Chronic Transfusion

Stroke prevention was one of the first indications for chronic transfusion therapy and remains the most common reason for patients to be placed on a chronic transfusion program. Russell et al initially reported a 75 fold decrease in recurrence of stroke with transfusions [39, 40], and Pegelow showed significantly lower rates of recurrent stroke compared to non transfused historical controls [41]. The Stroke Prevention Trial in Sickle Cell Anemia (STOP) then investigated the ability of chronic transfusions implemented based on abnormal transcranial Doppler (TCD) flow to prevent primary stroke and demonstrated a 92% reduction in stroke in those patients randomized to transfusion[2]. Subsequently, the STOP II trial showed that children randomized to discontinuation of therapy after 30 months of chronic transfusions resulted in a high rate of reversion to abnormal TCD and stroke[42]. These results led to premature closure of the study and effectively committed patients with stroke risk to indefinite transfusion therapy.

In addition to preventing stroke, chronic transfusions have been shown to significantly decrease the rate of both vaso-occlusive pain events and acute chest syndrome hospitalizations[27, 28] and the Brazilian MoH recommends chronic transfusion therapy for patients with recurrent episodes of ACS or intense and frequent episodes of vaso-occlusive pain. Other indications for chronic transfusion such as recurrent priapism and pulmonary hypertension have remained controversial.

Patients receiving their transfusions as part of a chronic program receive blood at routine Hemocenter visits during their baseline state every 3 to 4 weeks, most commonly for stroke prevention. Patients with at least 1 year of chronic transfusion therapy will be enrolled to determine the impact of chronic transfusions on the inflammatory pathophysiology of SCD. Patients who have not been transfused in 3 months will serve as controls, stratified on treatment with hydroxyurea.

12. Study Populations and Specimen Procurement

12.1. Eligibility

Enrollment into Aim B is not contingent upon enrollment into Aim A. For patients enrolled into Aim B who are not already enrolled in Aim A, the 15 Enrollment Forms of Aim A will be completed during enrollment into Aim B to collect detailed sickle cell and transfusion history. Patients enrolled into Aim B will only be included in Aim B analyses as the Aim A analyses will be limited to patients identified from a random sampling of the accessible population. Patients in the transfusion and non transfusion group must meet the below eligibility criteria.

1. 100 patients with a minimum of 1 year of chronic transfusion therapy

Inclusion

- Patients on chronic transfusion for at least 1 year with no more than 2 missed transfusions over the last 12 months. Chronic transfusion program is defined as regular transfusions every 3-5 weeks, initiated for any indication.

Exclusion

- Any acute illness in the past 30 days. Acute illness will be defined as any event requiring hospitalization, ER visit, unscheduled acute care visit, fever at home or pain crisis requiring treatment with pain medication.

2. 75 non-transfused control SS/SB0 patients treated with HU

Exclusion

- Any acute illness within the past 30 days. Acute illness will be defined as any event requiring hospitalization, ER visit, unscheduled acute care visit, fever at home or pain crisis requiring treatment with pain medication.
- Transfusion in past 3 months
- Current use of anti-inflammatory medication
- Co-existing autoimmune or inflammatory disorder
- Treated with HU for at least previous three months

3. 75 non-transfused control patients NOT treated with HU

Exclusion

- Any acute illness within the past 30 days. Acute illness will be defined as any event requiring hospitalization, ER visit, unscheduled acute care visit, fever at home or pain crisis requiring treatment with pain medication.
- Transfusion in past 3 months
- Current use of anti-inflammatory medication
- Co-existing autoimmune or inflammatory disorder
- Not Treated with HU at any time during the last 1 year

12.2 Screening and Recruitment

1. 100 patients with a minimum of 1 year of chronic transfusion therapy

We anticipate there will be approximately 600 chronically transfused patients with a one-year history of transfusion therapy based on Hemocenters' estimates. We will consecutively recruit chronic transfusion patients during routine transfusion visits.

2. 150 non transfused controls

Patients seen for routine visits during enrollment period will be screened by consecutive sampling. All patients who meet eligibility criteria will be recruited consecutively until target enrollment goal of 75 HU and 75 non HU controls is achieved.

12.3 Specimen Procurement and Storage

12.3.1 Cytokine Panels

- Timing of sample collection:
 - All patients will have a pre-transfusion sample (Time 0) collected.
 - Follow up samples will be collected at 1-6 hours, 1 week, 3 weeks, 6 weeks
 - For patients on chronic transfusion therapy, the 3 and 6 week samples will be collected immediately prior to their scheduled transfusion.
- Blood samples:
 - Two 4cc EDTA tubes will be drawn

- Blood will be centrifuged then separated into plasma and cellular components. Components will then be divided into 1cc plasma and 1cc cellular aliquots for storage
- All aliquots will be stored at -80°C at the Hemocenter
- Aliquots will be batch shipped quarterly from Hemocenters to Sao Paulo
- After samples are collected on all enrolled subjects, 1 plasma aliquot on each subject will be shipped to BSRI in 3 shipments for analysis.

12.3.2 Tubes for Gene Expression Analysis

- Timing of sample collection:
 - Patients will have a pre-transfusion and a single post-transfusion sample collected in a PAXgene tubes
 - The post transfusion sample will occur at 1-6 hours after transfusion
 - At each time point, 3 mL will be drawn into PAXgene tubes
 - Only a 1-6 hour sample will be collected because changes in mRNA expression should be rapid compared to protein changes seen in cytokine panels.
- Blood samples:
 - Samples will be kept at room temperature for a minimum of 2 and maximum of 24 hour prior to storage at -80C.
 - PAXgene tubes will be batch shipped quarterly to the Central Laboratory in Sao Paulo for storage in the biorepository

13. Timeline

Study phase	Phase 1				Phase 2																Phase 3				
	2013				2014				2015				2016				2017				2018				
Calendar Year	2013				2014				2015				2016				2017				2018				
Quarter	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1				
Aim B: Post-transfusion immune modulation																									
Protocol Development																									
Manual of operations																									
Training all study personnel																									
Identifying/recruiting subjects at each center																									
Clinical data capture & sample collection																									
Testing of samples (BSRI)																									
Analysis and reporting																									

14. Measurements

14.1 Cytokine analysis

Cytokines included in panels are summarized in Table 5. Panels will be measured according to manufacturer instructions.

Table 5

Cardiovascular-associated	Growth factors	Pro-inflammatory	Anti-inflammatory
MMP-9 ^b	PDGF-AA	IL-1 β	IL-4
MPO	PDGF-AB/BB	IL-2	IL-5
PAI-1 (total)	VEGF	IL-6	IL-10
sE-Selectin	EGF	IL-7	IL-13
sICAM-1	GRO	IL-8	
sVCAM-1		IL-12 (p70)	
		IFN- γ	
		GM-CSF	
		TNF- α	

^bMMP, matrix metalloproteinase; MPO, myeloperoxidase; PAI, plasminogen activator inhibitor; sICAM, soluble intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; EGF epidermal growth factor; GRO, growth related oncogene.

15. Statistical Considerations

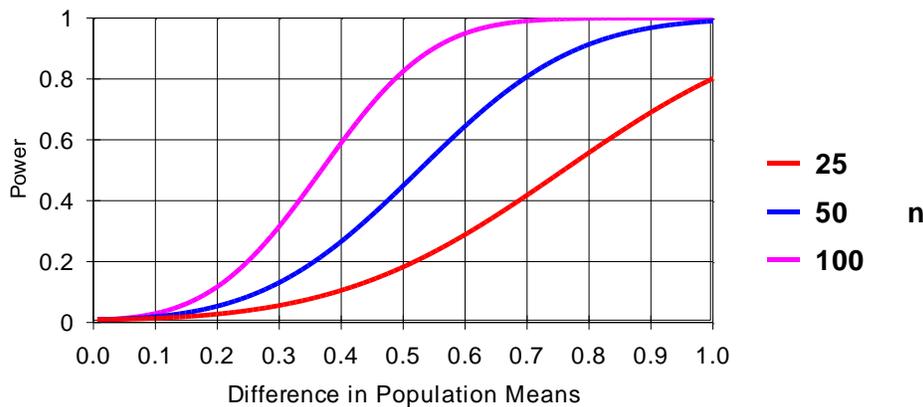
15.1 Planned Analysis

Cytokine values are often right and left censored, as a result we will use quantile normalization to generate normally distributed data. Cytokines will be compared using a linear model adjusted for appropriate covariates. Cytokine significance will be adjusted using FDR based methods.

15.2 Power calculations

Power for cytokines is based upon an alpha < 0.01 to moderately control for multiple testing. In a sample size of 100 per group there is good power (> 80%) to detect a difference in population means between the groups of 0.47 σ .

Figure 4 Power estimation for cytokine analysis



Aim C: Identify particular single nucleotide polymorphisms (SNPs) that contribute to the risk of red blood cell alloimmunization in SCD

16. Background and Significance

Early studies of SCD reported alloimmunization in approximately 30% of transfused SCD patients and up to 10% of patients will form more than one antibody [43, 44] [8] [7]. Alloantibodies can have a profound impact on transfusion management by contributing to

significant delays and increased cost in the procurement of compatible blood as well as potentially causing life-threatening hemolytic reactions [9].

Alloimmunization is a complex process which depends upon multiple factors including differences in RBC phenotypes between recipients and donors[43], age at first transfusion [8], total unit exposure [8] [29] and possibly baseline inflammatory status of the recipient [45] [46] [47]. SCD patients have a higher rate of alloimmunization than most other transfused populations, due in part to the disparity of RBC antigen frequencies between SCD patients and the blood donor population in countries where these two populations have divergent genetic ancestry [7, 43].

Vichinsky et al showed that prospective phenotypic matching could decrease, but not eliminate, alloimmunization. The blood transfused during the STOP trial was matched for a minimum of C, E and Kell antigens and compared to historical controls, the alloimmunization rate decreased from 3% per unit to 0.5% per unit and hemolytic transfusion reactions by 90% [10]. Despite these data, there remains controversy over the most appropriate transfusion protocol in SCD as locating antigen negative blood can be difficult, expensive and time consuming [48]. Identification of those patients with significant alloimmunization risk would allow for more targeted transfusion strategies.

It has been noted since the early SCD literature that some patients form multiple antibodies even with limited RBC antigen exposure and other patients never become alloimmunized despite repeated transfusions, suggesting a genetic influence on the process. We intend to investigate the potential genetic basis for this variation in risk of alloimmunization. In a study of SCD patients using high resolution HLA genotyping, the HLA-DRB1*1503 allele was associated with an increased risk ($p = 0.039$), while HLA-DRB1*0901 conferred protection from alloimmunization ($p = 0.008$) [49]. We will perform whole genome SNP typing in 500 antibody positive patients and 500 antibody negative patients to identify polymorphisms in HLA or other alleles associated with alloimmunization risk.

17. Study Populations

We plan to compare alloimmunized and non-alloimmunized subjects within the cohort. We estimate the alloimmunization prevalence will be 15 – 20% based on data from local Hemocenter blood banks, therefore anticipate enrollment of ~500 antibody subjects. Power calculations will be based on this estimate.

17.1 Definition of cases (Antibody Positive Patients)

Positive alloantibody status will be defined as a positive indirect antiglobulin test and at least one distinct antibody with defined specificity identified on panel investigation. All testing is performed as part of routine clinical care using local blood bank techniques to identify antibodies. The supervising immunohematologist at each Hemocenter will review blood bank records to confirm antibody positive status of all enrolled patients. Central review of blood bank records by an immunohematologist at BSRI will occur on a subset of 20% of patients classified as antibody positive.

17.2 Definition of controls (Antibody Negative Patients)

For each identified case, all patients within the REDS III Cohort who have not formed an alloantibody will be counted as a control. The supervising immunohematologist at each Hemocenter will review blood bank records to confirm antibody negative status of all controls.

18. Study Enrollment and Specimen Procurement

18.1 Screening and Recruitment

A list of all alloantibody positive patients in the Aim A Cohort will be generated to be compared to antibody negative patients. All details of sickle cell and transfusion history will have been collected as part of Aim A enrollment & blood sample will be stored in biorepository, therefore no additional visits will be required for patients from the REDS III Aim A Cohort who are enrolled into Aim C.

18.2 Specimen Procurement

Samples from the Aim A cohort will be used for Aim C genotyping. Samples will be batch shipped to BSRI in three shipments for analysis.

19. Timeline

Study phase	1	Phase 2																Phase 3				
Calendar Year	2013	2013			2014				2015				2016				2017	2017				18
Quarter	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	
Aim C: Red blood cell allo-immunization																						
Protocol Development																						
Manual of operations																						
Training all study personnel																						
Selecting antibody positive patients from cohort																						
Recruiting additional antibody positive																						
Selecting antibody negative controls from cohort																						
Testing samples																						
Analysis and reporting																						

20. Measurements

20.1 SNP Typing

We will use the Affymetrix Axiom genotyping platform at the Institute of Human Genetics at UCSF for the whole genome SNP typing. The current version of the array accurately interrogates 567,096 polymorphisms, and captures >85% of the CEU HapMap reference genome of variants at 5% minor allele frequency or greater. Approximately 250 ng of genomic DNA from blood will be used for genotyping. Samples will be processed according to manufacturer’s protocols. Clustering and genotype calling will be performed using the proprietary Axiom GT1 genotyping console software on each plate of 96 samples.

20.2 Clinical and Transfusion Data

All details of sickle cell and transfusion history will be captured using enrollment case report forms described in Aim A. The below data will be extracted for detailed description of and comparison between alloantibody positive and negative patients:

1. Sickle cell severity as defined by prevalence of specific SCD complications (stroke, number of acute chest syndrome admissions previous 2 years, number of vaso-occlusive pain crisis admissions past two years, pulmonary hypertension)

2. Total number of units transfused over patients' life
3. Red blood cell phenotype
4. Formation of co-existing autoantibodies
5. Co-existing autoimmune disorders
6. Date of formation of alloantibodies (categorized as before or after phenotypic matching policies in place at Hemocenter)
7. Age at first transfusion

21. Statistical Considerations

21.1 Planned Analysis

An initial screen to test for association across the whole genome will be performed by examining allele and genotype frequency distributions in the dataset of allo-antibody positive and negative individuals. We will initially generate allele frequencies for each SNP, perform quality control including Hardy-Weinberg Equilibrium testing, detection of cryptic relatives, and removal of poorly performed SNPs and individuals. We will impute up to ~5-10 million SNPs using IMPUTE 2 and a cosmopolitan reference population. We will perform association tests for allele counts in cases and controls using logistic regression and an additive model. We will also calculate odds ratios (ORs) and 95% confidence intervals (CI). P-values will be determined both asymptotically and empirically, for comparison and interpretation using permutation. We have previously used PLINK (<http://pngu.mgh.harvard.edu/~purcell/plink>) and HelixTree software packages (<http://www.goldenhelix.com>) to successfully perform these statistical analyses. Both software packages were written specifically to manage large SNP datasets and perform these statistical tests. All results will be plotted graphically (all comparisons; p-values (inverse) x chromosome/map position) for interpretation according to criteria for significance. We will further analyze the data using pathway based methods and rare variant/burden testing such as VEGAS and SCID.

21.2 Sample Size Justification

Statistical power was estimated using CaTS software, which is designed for genome-wide association case-control studies[50]. Sample size n=500 per arm and minor allele frequency (MAF) of 0.1 was assumed. For genome-wide association tests, type I error rate is controlled at 1E-8. It can be observed that power is sufficient when OR is close or greater than 1.56 for GWAS with n=500 samples.

Table 6: Power calculation for genome-wide association tests (n=500)

	OR 1.4	OR 1.5	OR 1.75	OR 2.0
Power achieved assuming MAF =0.1 Prev = 0.5 $\alpha < 10^{-8}$	0.23	0.67	1.00	1.00

22. Future additional GWAS

The initial GWAS will focus on RBC alloimmunization as outlined in the protocol. However, as all subjects will undergo whole genome SNP typing, we will have the genetic data available for additional future GWAS using other SCD phenotypes to define case status. As one example, ischemic stroke is one of the most devastating complications of SCD and occurs in 5-10% of

patients. Although studies in SCD sibling pairs has suggested a genetic contribution to stroke risk and some candidate gene studies have identified SNPs associated with stroke, most of this research has not been replicated and SNPs, haplotypes or specific alleles have not been verified in other cohorts.

The most recent and largest study to investigate this association utilized data and blood samples collected from patients in 5 separate trials to identify a group of 177 patients with stroke and 335 controls without stroke[51]. The group identified 139 SNPs associated with stroke ($p < 0.0001$), but none reached genome-wide significance threshold. They next performed whole exome sequencing (WES) to identify variants associated with stroke in a discovery phase, then tested specific candidates in a validation step by randomly dividing patients into a discovery cohort (120 stroke and 104 nonstroke controls) and a validation cohort (57 stroke and 231 non stroke controls). Using the SNP array and WES results in combination, there were two variants (one in GOLGB1 and another in ENPP1) confirmed to have significant association for a decreased risk of stroke. The REDS-III Brazil SCD Cohort would provide 1) an independent validation cohort to test identified candidate genes and 2) a larger study population with additional power to detect genetic determinants of stroke.

Other large SCD studies have shown 5-10% of patients will have an ischemic stroke. If approximately 8% of the REDS cohort have a history of stroke, power to detect genetic determinants of stroke are presented in table 7. Power is presented for an additive model, $\alpha = 5 \times 10^{-8}$, prevalence in this population, and a minor allele frequency of 0.2. With this presumed stroke prevalence, there is good power to detect a locus with an odds ratio 2.23 for stroke. In the future, this study can be combined with others in a formal meta analyses to improve power.

Table 7 Power Achieved for Detection of SNPs Associated with Stroke within REDS Cohort

Phenotype	Odds Ratio								OR with 80% power
	1.3	1.4	1.6	1.8	2	2.2	2.4	2.6	
Stroke	0	0	0.04	0.21	0.51	0.77	0.92	0.98	2.23

We anticipate the ability to perform multiple genotype –phenotype association studies after whole genome SNP typing is complete.

23. Overall Timeline

Study phase	Phase 1				Phase 2												Phase 3				
	2013				2014				2015				2016				2017				2018
Calendar Year	2013	2013	2013	2013	2014	2014	2014	2014	2015	2015	2015	2015	2016	2016	2016	2016	2017	2017	2017	2017	2018
Quarter	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1
Sickle Cell Project																					
Aim A: Esstablishing a SCD Cohort/Biorepository																					
Design of CRFs and central data warehouse																					
Sickle cell protocol development																					
Sickle cell IRB approvals [US and Brazil Aims A-C)	█																				
Pilot test electronic database each Hemocenter																					
OMB clinical exemption (all aims)																					
Aim A manual of operations	█	█																			
Training all study personnel	█	█																			
Enrollment of cohort/sample collection for biorepository			█	█	█	█															
Annual follow-up visits for clinical information update								█	█	█	█	█	█	█	█	█	█	█	█	█	
Analysis and reporting																			█	█	█
Aim B: Post-transfusion immune modulation																					
Protocol Development																					
Manual of operations	█	█																			
Training all study personnel			█	█																	
Identifying/recruiting subjects at each center					█	█	█	█	█	█	█	█	█								
Clinical data capture & sample collection					█	█	█	█	█	█	█	█	█								
Testing of samples (BSRI)													█	█	█						
Analysis and reporting																	█	█	█	█	█
Aim C: Red blood cell allo-immunization																					
Protocol Development																					
Manual of operations			█	█																	
Training all study personnel					█	█															
Selecting antibody positive patients from cohort							█	█													
Recruiting additional antibody positive									█	█											
Selecting antibody negative controls from cohort											█	█									
Testing samples													█	█	█						
Analysis and reporting																	█	█	█	█	█

24. Survey Considerations and OMB Requirements all Aims

All ethical committee approvals in Brazil and Institutional Review Board approvals in the United States will be in place prior to enrollment. We anticipate receiving a Clinical Exemption for Aims A, B and C. The approval process is expected to take up to one year.

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