CLINICAL PROTOCOL

Transfusion of Biotin-Labeled Red Blood Cells for the Evaluation of Genetic Factors that Contribute to Donor Differences in Red Blood Cell Storage and Post-Transfusion Red Blood Cell Recovery

IND 17609

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PROTOCOL SYNOPSIS

Protocol Title:	Transfusion of Biotin-Labeled Red Blood Cells for the Evaluation of Genetic Factors that Contribute to Donor Differences in Red Blood Cell Storage and Post-Transfusion Red Blood Cell Recovery	
Protocol Number:	PRO19020356	
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Clinical Phase:	Phase II Clinical Trial	
Investigational Product:	Biotin-Labeled Red Blood Cells (BioRBCs)	
Trial Site:	UPMC Montefiore Hospital 3459 Fifth Ave. Pittsburgh, PA 15213	
IND Sponsor:	Mark T. Gladwin, MD Chairman of the Department of Medicine Director, Pittsburgh Heart, Lung, Blood and Vascular Medicine Institute University of Pittsburgh, School of Medicine	
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Study Monitor:	Mark T. Gladwin, MD, IND Sponsor	
Research Facilities:	UPMC Montefiore Hospital Vitalant, Pittsburgh, PA	
Clinical Laboratories:	UPMC Presbyterian Shadyside CP PUH Hematopoietic Stem Cell Laboratory	
Study Rationale:	As stored red cells age in cold storage, complex biochemical and metabolic alterations occur within the erythrocytes that results in hemolysis and reduced red cell post transfusion survival. Intravascular hemolysis following transfusion of aged stored red cells disrupt nitric oxide (NO) bioavailability, via accelerated NO scavenging reaction with cell-free plasma hemoglobin leading to worse outcomes. It remains unknown whether donor genetic background modulates this process. In our recently completed large genome-wide association study (GWAS) study of red blood cell donors, we have identified a	

	number of single nucleotide polymorphisms (SNPs) that are associated with more red blood cell hemolysis in response to storage stress (storage, osmotic, or oxidative hemolysis). Examples of these SNPs may include sickle cell trait (AS), and single nucleotide polymorphisms (SNPs) in ankyrin 1 (ANK1), glucose-6-phosphate dehydrogenase X-linked (G6PD), piezo-type mechanosensitive ion channel component 1 (PIEZ01), spectrin alpha (SPTA), extended synaptotagmin-like protein 2 (ESYT2), Myo9b and SEC14. We hypothesize that these SNPs and others may affect the stability of red blood cells during storage and the recovery of red blood cells in the circulation after transfusion over 60 days. Additional variables that might affect red blood cell post-transfusion recovery include the donor sex and whether a donor is taking testosterone replacement therapy. In general, the quality of a stored unit of red blood cells (RBC) is largely based on in vitro measures of hemolysis but the true quality of an RBC unit is based on the red cell ability to circulate through the vasculature and deliver oxygen to the tissues. Currently, there is limited data on the correlation between in vitro measures of hemolysis and post-transfusion in vivo survival of stored red blood cells. This project explores the gaps in knowledge between in vitro measures of hemolysis and in vivo lifespan of the RBC after transfusion.
Study Objectives:	The primary objective of this study is to define the post transfusion recovery of fresh or stored BioRBCs from blood donors who carry genetic mutations that can compromise RBC function and recovery in response to ex vivo cold storage and after transfusion into subjects. The secondary objective is to define the effects of donors sex and testosterone replacement therapy on post-transfusion recovery and the correlation between in vitro correlates of the RBC storage lesion (e.g., storage and stress-induced hemolysis, membrane microvesicle formation) and post transfusion recovery of BioRBCs.
Study Hypothesis:	We hypothesize that genetic mutations and biologic variables (sex, race/ethnicity) in blood donors modulate the recovery of RBC after cold storage and transfusion.
Study Aims:	Aim 1: To explore the effect of selected SNPs on in-invitro measures of red cell biophysical properties, storage integrity, and post-autologous transfusion RBC recovery. Aim 2: To examine the relationship between sex and sex hormones (testosterone) and in vitro markers of storage integrity and the in vivo 2-4 hours recovery and survival of RBCs stored under standard blood banking conditions.

Study Design:	The proposed clinical investigation will evaluate the *in vivo* lifespan of RBC in healthy volunteers after autologous transfusion over two different periods of storage. We will collect blood samples and label them with a naturally occurring vitamin, biotin. The RBCs will then be re-infused back into the same participant at 2 time points (5-7 days and 35-42 days after storage). To evaluate RBC post transfusion survival, blood samples will be taken to quantify biotin labeled RBCs at 2-4 hours, 1 month and 2 months after each transfusion. On Day 125-150 post blood donation, subjects will be followed to evaluate anti-biotinylated RBC antibody formation. Throughout the study, subjects will be monitored carefully for occurrences of adverse events, laboratory test abnormalities, and changes in vital signs. This protocol may cover up to (9) separate but similarly designed studies that may test 8 groups with candidate SNPs that were associated with hemolysis in REDS III Omics GWAS studies. Candidate SNPs may include, but are not limited to sickle cell trait (AS), and single nucleotide polymorphisms (SNPs) in ankyrin 1 (ANK1), glucose-6-phosphate dehydrogenase X-linked (G6PD), piezo-type mechanosensitive ion channel component 1 (PIEZ01), spectrin alpha (SPTA), and extended synaptotagmin-like protein 2 (ESYT2), Myo9B and SEC14. A subset of recalled donors with these SNPs will be asked to provide a blood sample to be tested for functional effects on red cell biophysical properties, stability in storage, and release of eDAMPs, and compared to donors with no implicated SNPs. We will develop an enriched SNP profile that defines a high risk and a low risk donor for further evaluation in autologous reinfusion studies as a low cost precision transfusion medicine array. A 9th group of subjects on testosterone replacement therapy will also be evaluated.
Planned Sample Size:	Up to potentially 223 healthy volunteers (145 from RBC-OMICS + 78 from Biotin infusion)
Duration of Treatment:	The autologous reinfusion study consists of two blood transfusions of one day each separated by approximately one month.
Major Inclusion Criteria:	• Age 18 years or older • Subjects will be selected based on having at least one SNP hypothesized to affect RBC transfusion storage and recovery. Controls will not have any of these SNPs. The eight genetic mutations may include sickle cell trait (AS), and single nucleotide polymorphisms (SNPs) in ankyrin 1 (ANK1), glucose-6-phosphate dehydrogenase X-linked (G6PD), piezo-type mechanosensitive ion channel

	component 1 (PIEZ01), spectrin alpha (SPTA), extended synaptotagmin-like protein 2 (ESYT2), Myo9b and SEC14. We may identify additional SNPs as our analysis of the REDS-III GWAS study advances that could replace one of these SNP groups and/or we identify additional non-synonymous SNPs that are associated with susceptibility to hemolysis of stored red blood cells. In addition, we will evaluate 15 subjects on testosterone replacement therapy with 20 controls that are sex and race matched that are not on testosterone replacement therapy. • Weight ≥110 lbs • Hemoglobin ≥ 12.5 g/dL or hematocrit ≥ 38% for women and hemoglobin ≥ 13.0 g/dL or hematocrit ≥ 39% for men. • Meet screening criteria for autologous blood donation
Major Exclusion Criteria:	• Subjects with a past medical history or symptoms of blood dyscrasia, diabetes mellitus, hyperlipidemia, obstructive sleep apnea, renal disease, congestive heart failure, significant cardiac disease and / or known peripheral arterial disease. • Moderate to severe systemic hypertension (systolic blood pressure >140 mmHg and/or diastolic blood pressure > 95 mmHg • Systolic blood pressure <100 mmHg and/or diastolic blood pressure < 60 mmHg on the study day. • Positive Direct Antiglobulin Test (DAT) • Consumption of biotin supplements or raw eggs within 30 days • Previous or known allergy to biotin • Treatment with antibiotics in the week before initiating study participation to avoid suppression of erythropoiesis, which may accompany infection. • Blood loss in the previous 8 weeks due to epistaxis, trauma, hemoptysis, gastrointestinal bleeding, diagnostic phlebotomy (>30ml). • Subjects who report tobacco or marijuana smoking within 6 months of study. • Cognitively impaired subjects, or institutionalized persons and subjects unable or unwilling to complete written informed consent • Subjects with a history of blood donation within the previous 56 days. • Use of other investigational drugs/devices within 30 days of screening. • Subjects taking any medication for the treatment of diabetes including insulin • Positive urine pregnancy test. • History of prior transfusion reaction to blood products.

	• Donors with naturally occurring antibodies against BioRBCs will be excluded from the study
Study Endpoints:	The primary endpoint is the labeled red blood cell recovery rate over 60-days post transfusion of 35-42 day stored Bio-RBCs from blood donors with specified genetic mutations, compared with control subjects without the specified mutation. This protocol may evaluate up to eight (8) genetic mutations as independent studies, compared with pooled control groups (without any of the identified SNPs) as well as a group of subjects on testosterone replacement therapy. The eight genetic mutations mayinclude sickle cell trait (AS), and single nucleotide polymorphisms (SNPs) in ankyrin 1 (ANK1), glucose-6-phosphate dehydrogenase X-linked (G6PD), piezo-type mechanosensitive ion channel component 1 (PIEZ01), spectrin alpha (SPTA), extended synaptotagmin-like protein 2 (ESYT2), Myo9b and SEC14. All control subjects will be tested and must not have any of these SNPs. We may identify additional SNPs as our analysis of the REDS-III GWAS study advances that could replace one of these SNP groups and/or we identify additional non-synonymous SNPs that are associated with susceptibility to hemolysis of stored red blood cells. In addition, we will evaluate 15 subjects on testosterone replacement therapy with 20 controls that are gender and race matched that are not on testosterone replacement therapy. The secondary endpoints include effects of gender on post transfusion recovery, the 2-4 hours post transfusion survival of fresh or stored Bio-RBCs, and to determine the association between in vitro correlates of the RBC storage lesion (e.g., storage and stress-induced hemolysis, membrane microvesicle formation) and post transfusion recovery. A subset of recalled donors with these SNPs will be asked to provide a blood sample to be tested for functional effects on red cell biophysical properties, stability in storage, and release of eDAMPs, and compared to donors with no implicated SNPs. We will develop an enriched SNP profile that defines a high risk and low risk donor for further evaluation in autologous reinfusion studies as a low cost precision transfusion medicine array.

1. OBJECTIVE, SPECIFIC AIMS, BACKGROUND, AND SIGNIFICANCE

1.1 OBJECTIVE

The primary objective of this study is to define the post transfusion recovery of fresh or stored Bio-RBCs from blood donors who carry genetic mutations that can compromise RBC function and recovery in response to ex vivo cold storage and after transfusion into subjects.

This protocol may cover up to nine () separate but similarly designed studies that may test eight groups with SNPs and a cohort of patients on testosterone replacement therapy (TRT).

The eight genetic mutations that we will evaluate may include sickle cell trait (AS), and single nucleotide polymorphisms (SNPs) in ankyrin 1 (ANK1), glucose-6-phosphate dehydrogenase X-linked (G6PD), piezo-type mechanosensitive ion channel component 1 (PIEZ01), spectrin alpha (SPTA), extended synaptotagmin-like protein 2 (ESYT2), Myo9b and SEC14. We may identify additional SNPs as our analysis of the REDS-III GWAS study advances that could replace one of these SNP groups and/or we identify additional non-synonymous SNPs that are associated with susceptibility to hemolysis of stored red blood cells. In addition, we will evaluate 15 subjects on testosterone replacement therapy with 20 controls that are gender and race matched that are not on testosterone replacement therapy.

The secondary objective is to define the effects of donors sex and testosterone replacement therapy on post-transfusion recovery and the correlation between in vitro correlates of the RBC storage lesion (e.g., storage and stress-induced hemolysis, membrane microvesicle formation) and post transfusion recovery of Bio-RBCs. This study will verify whether the negative impact of TRT on RBC storage stability observed in mice is clinically relevant in transfusion of blood from TRT donors.

1.2 SPECIFIC AIMS

Hypothesis:

We hypothesize that genetic mutations and biologic variables (sex, race/ethnicity) in blood donors modulate the recovery of red blood cells after cold storage and transfusion.

Specific Aims:

Aim 1: To explore the effect of selected SNPs on in vitro meausres of red cell biophysical properties, storage integrity, and post-autologous transfusion RBC recovery.

Aim 2: To examine the relationship between sex and sex hormones (testosterone) and *in vitro* markers of storage integrity and the in vivo 1-hour recovery and survival of RBCs stored under standard blood banking conditions.

1.3. BACKGROUND and RATIONALE

The Red Blood Cell Storage Lesion

Over 12 million blood units are transfused annually in the U.S. and to meet this demand, RBCs are stored in additive solution under hypothermic conditions with a shelf life of 42 days. While cold storage significantly reduces metabolism in red cells, enzymatic reactions proceed during storage and red cells age during this time. The “storage lesion” is the term used to collectively describe the biochemical, structural, physiologic and immunologic changes in red blood cells during storage. The storage lesion has been characterized in detail and includes changes such as

decreased intracellular 2,3-diphosphoglycerate concentrations, hemolysis and formation of red cell microparticles, release of free iron with down-stream effects on nitric oxide bioavailability, and decreased membrane deformability.[1-2] While some of biochemical changes (e.g. intracellular adenosine triphosphate (ATP) and 2,3- diphosphoglycerate (DPG) depletion) are reversible upon transfusion of stored red blood cells, other biochemical changes (e.g. loss of membrane through microparticle formation) are not reversible, and the post-transfusion fate of many other storage lesion alterations remain uncharacterized. As a result, the clinical relevance of the red blood cell storage lesion has been actively studied and generally these changes are not associated with measureable adverse clinical outcomes.[3-7] In this study, we aim to evaluate the intrinsic rate of aging of red cells from individuals with specific SNPs hypothesized to reduce red blood cell survival after storage and transfusion using *in vitro* markers as well as post transfusion recovery and survival.

The eight genetic mutations that we will evaluate may include sickle cell trait (AS), and single nucleotide polymorphisms (SNPs) in ankyrin 1 (ANK1), glucose-6-phosphate dehydrogenase X-linked (G6PD), piezo-type mechanosensitive ion channel component 1 (PIEZ01), spectrin alpha (SPTA), extended synaptotagmin-like protein 2 (ESYT2), Myo9b, and SEC14. We may identify additional SNPs as our analysis of the REDS-III GWAS study advances that could replace one of these SNP groups and/or we identify additional non-synonymous SNPs that are associated with susceptibility to hemolysis of stored red blood cells. In addition, we will evaluate 15 subjects on testosterone replacement therapy with 20 controls that are gender and race matched that are not on testosterone replacement therapy.

Biotin
Biotin (vitamin B7) is a small (244Da) molecule that can be conjugated with sulfo-*N*-hydroxysuccinimide (s-NHS), an ester that reacts with primary amino groups (-NH_2) to form stable amide bonds. The conjugation of s-NHS and biotin forms sulfosuccinimidobiotin (s-NHS-biotin), a compound that readily binds to primary amines on the side chains of lysine residues found on the RBC membranes. S-NHS-biotin does not permeate the RBC membrane due to the charge provided by the sodium sulfoxide group on the succinimidyl ring. Thus, given the RBC remains intact, only primary amines on the RBC membrane surface are biotinylated.

The conventional method to determine RBC survival *in vivo* is *ex vivo* labeling of the RBC with chromium-51 (^{51}Cr). This method has been used for over 60 years to calculate 24-hour recovery of RBCs after reinfusion.[8] The International Committee for Standardization in Hematology (ICSH) developed specific recommendations on how to perform this assay and perform the calculations to determine *in vivo* RBC survival.[9] However, the ^{51}Cr method has significant pitfalls including: 1) Radioactivity, which excludes the study of children, pregnant women and other susceptible populations; 2) Variable ^{51}Cr elution rate from the surface of RBC between study subjects, which may overestimate RBC survival estimates; 3) Lack of an internal standard for comparative RBC survival analysis between populations; 4) A short follow-up period of only 4 weeks due to the brief intrinsic 27.8 day half-life of ^{51}Cr. 5) Multiple transfused RBC populations cannot be studied using this tracking method. Labeling the RBC with s-NHS-biotin overcomes the inherent pitfalls of the ^{51}Cr method and importantly allows the safe and long term tracking of multiple transfused RBCs *in vivo*. BioRBCs provide a superior alternative to radioactive red blood cell labels for *in vivo* lifespan tracking because of the non-radioactive quality of biotin and the durability of the covalent bond of biotin to the red blood cell membrane, which lasts for the lifespan of the cell.

1.4 SIGNIFICANCE

The quality of a unit of stored RBC for transfusion, a procedure performed about 12 million times per year in patients, is currently based on levels of storage hemolysis. However, the storage lesion is a singular manifestation of a multitude of injuries that occur during red cell storage. Hemolysis measures alone failed to capture the progressive accumulation of these changes, some of which may affect the ability of transfused red blood cells to traverse the microcirculation. Furthermore, there is limited data on the correlation between *in vitro* measures of hemolysis and post-transfusion survival of stored red blood cells. This project explores the current gaps in understanding the impact of genetic and biologic factors in blood donors on red blood cell storage stability and post transfusion recovery. We also seek to determine whether *in vitro* markers of storage integrity can predict red cell survival in the patient circulation.

Our studies in human and animal models have demonstrated that genetic variability and biological variables such as sex, age, race or iron availability may significantly modulate predisposition to hemolysis under diverse stress conditions including routine cold storage of RBC units, and in hemolytic disease. For example, we have shown that RBCs from male donors (human or mice) exhibit higher susceptibility to hemolysis during storage, and under osmotic, mechanical or oxidative stress.[10] Furthermore, we have demonstrated the effect of donor sex, testosterone or sickle cell trait on RBC storage and post-transfusion recovery in a murine model of transfusion.[11,12] As members of NHLBI REDS-III and collaborators on the RBC-Omics project, we have further demonstrated in 13,403 informed blood donors that variables such as sex, race, and donation intensity may modulate RBC hemolytic response to cold storage and to osmotic and oxidative stress. Having completed the screening of these donors for hemolytic propensity, we are currently seeking for genes that mediate hemolysis using genome-wide association approach. The biotin method would provide us the unique opportunity to verify whether our in vitro correlates of hemolysis or the newly discovered genes may predict RBC recovery in vivo.

Using data from REDS-III we have identified candidate genetic SNPs that may affect red blood cell biophysical properties, storage integrity, and recovery after storage. The eight genetic mutations that we will evaluate may include sickle cell trait (AS), and single nucleotide polymorphisms (SNPs) in ankyrin 1 (ANK1), glucose-6-phosphate dehydrogenase X-linked (G6PD), piezo-type mechanosensitive ion channel component 1 (PIEZ01), spectrin alpha (SPTA), extended synaptotagmin-like protein 2 (ESYT2), Myo9b and SEC14. We may identify additional SNPs as our analysis of the REDS-III GWAS study advances that could replace one of these SNP groups and/or we identify additional non-synonymous SNPs that are associated with susceptibility to hemolysis of stored red blood cells. In addition, we will evaluate 15 subjects on testosterone replacement therapy with 20 controls that are gender and race matched that are not on testosterone replacement therapy.

2. RESEARCH DESIGN AND METHODS

2.1 CLASSIFICATION AND METHODOLOGICAL DESIGNS

The proposed clinical investigation is a single-center pilot study aimed to evaluate the post transfusion recovery of fresh or stored Bio-RBCs from blood donors who carry genetic mutations that can compromise RBC function and recovery in response to ex vivo cold storage and after transfusion into subjects. In this investigation, biotin will be used as a research tool to track transfused red blood cells in the recipient's circulating blood volume to evaluate the 1-hour post transfusion survival and mean life span.

2.2 DETAILED DESCRIPTION OF STUDY DESIGN

2.2.1. STUDY DESIGN OVERVIEW

Subjects for autologous blood reinfusion studies will be selected in 2 ways. First healthy individuals without any known candidate SNPs will be identified as controls. A subset of REDSIII RBC Omics donors with candidate SNPs will be recalled to provide a blood sample to be tested for functional effects on red cell biophysical properties, stability in storage, and release of eDAMPs, compared to donors with no implicated SNPs. (See 2.2.3) We will develop an enriched SNP profile that defines a high risk and low risk donor for further evaluation in autologous reinfusion studies as a low cost precision transfusion medicine array.

Autolgous reinfusions studies: Potential subjects will undergo a screening visit to determine that eligibility requirements are met. Subjects who meet the inclusion criteria and none of the exclusion criteria will be scheduled within 4 weeks of screening for a baseline assessment.

Eligible subjects will donate a unit (500 mL) of whole blood at one of the Central Blood Bank (Vitalant Pittsburgh) locations licensed by FDA. The blood will be processed and leukoreduced into a packed RBC unit (300-350 mL) according to standard procedures. This unit will then be split into two bags, with equal volume, labeled as "Autologous" RBC unit and stored under standard blood bank conditions in the blood bank under the supervision of Darrell Triulzi, MD. Units will be preserved in adenine, dextrose, sorbitol, sodium chloride and mannitol (ADSOL) solution and stored in a monitored temperature controlled refrigerator at 1-6° until needed, according to standard blood-banking practice. That is, half of a unit (150 mL) will be stored for 5-7 days, and the second half will be stored for 35-42 days. Stored RBCs will be labeled with biotin (i.e. biotinylated autologous red blood cells) at 15 µg/mL on the day of infusion and administered intravenously on 5-7 days, and the second half will be labeled at 3 µg/mL on the day of infusion and administered intravenously into the same subject on 35-42 days.

Final biotin concentrations after transfusion (recipient circulation) are 0.2 µg/mL and 0.04 µg/mL, days 5-7 and 35-42, respectively. Subjects will be closely monitored for evidence of transfusion reactions, and vital signs will be taken at baseline and every 15 minutes during the transfusion and then 15 minutes after the transfusion ends by qualified personnel at the Clinical Translational Research Center (CTRC) of UPMC Montefiore Hospital (MUH-CTRC).

To evaluate RBC post transfusion survival, blood samples will be taken to quantify biotin labeled RBCs pre-transfusion and at 2-4 hours, 1 month and 2 months after each transfusion. Additional blood samples will be obtained at Visit 1 for naturally occurring to anti-BioRBC antibodies and on Day 35-42 and Day 125-150 to evaluate the formation of antibodies to the biotinylated RBCs and any signs of hemolysis. The overall study design is depicted in Figure 1 below.

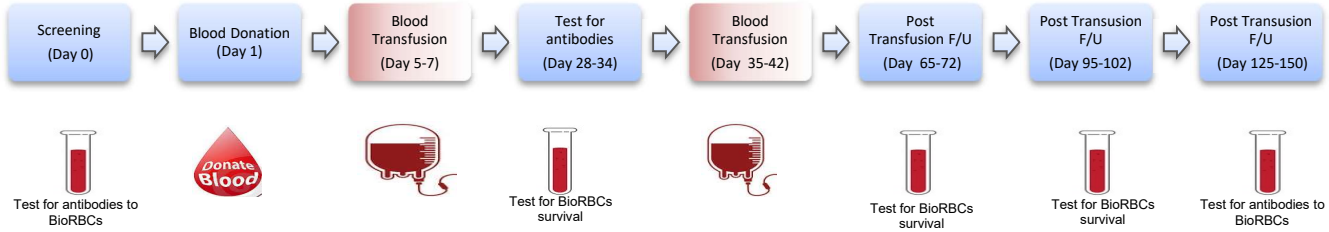

Figure 1. Schematic Diagram of Study Design

Throughout the study, subjects will be monitored carefully for occurrences of adverse events, laboratory test abnormalities, and changes in vital signs. The expected duration of each subject's participation in this proposed clinical investigation is approximately 8 months. For the REDS-III RBC-OMICs subgroup, the subject's participation is approximately 12 months.

Samples of the various subgroups will be analyzed for storage and stress-induced hemolysis, membrane microvesicle formation and for sickle cell trait (AS), and SNPs in ANK1, G6PD, PIEZ01, SPTA, ESYT2, Myo9b, and SEC14. And/or we identify additional non-synonymous SNPs that are associated with susceptibility to hemolysis of stored red blood cells. We may identify additional SNPs as our analysis of the REDS-III GWAS study advances that could replace one of these SNP groups**.** These findings will be compared with SNPs from the sample analyses of the REDS-III GWAS study. The analysis will occur in the labs of Dr. Gladwin in the Vascular Medicine Institute by qualified study personnel.

2.2.2. VISIT SCHEDULES FOR AUTOLOGOUS REINFUSION STUDIES

Outlined below are study procedures that will be performed at screening, during the study treatments, and at follow-up. See Appendix A for the complete study assessment and procedures.

REDS-III RBC-OMICs Subgroup Blood Draw Visit

Pre-Screening

The screening procedure involves a telephone interview for the purpose of identifying potential subjects and determining their eligibility for the study. In the REDS-III RBC-OMICS study, the subject consented to allow only de-identified data to be shared in the future. With IRB approval and the subject's consent, the biotin study team will access the REDS-III RBC-OMICs subject identifiable genetic data for use in determining which study group the subject is eligible for in the biotin study. The subject's genetic data (SNPs) does not have any application to the subject's clinical care, no actionable information, and will not be shared with the subject.

Blood draw visit (Day 0)

This visit will be at MUH-CTRC or on the 8th floor of the Translational Research Center. This visit is approximately 60-90 minutes. This subgroup will have a small blood draw for in-vitro testing only. The results of the testing may identify this subject as a candidate for autologous reinfusion study. The biotin study team will schedule an RBC-OMICs control subject (without the targeted SNPs) at different times on the same day as an RBC-OMICs biotin subject with one of the targeted SNPs. An RBC-OMICs control subject will perform the same blood draw study visit procedures on the same day as an RBC-OMICs biotin subject with one of the targeted SNPs. The biotin study team will compare red blood cells biophysical properties in an RBC-OMICs control subject with an RBC-OMICs biotin subject with one of the targeted SNPs. If an RBC-OMICs control subject and an RBC-OMICs biotin subject with one of the targeted SNPs cannot be scheduled on the same day blood draw visit, the biotin study team will coordinate both subjects schedules to have them come into MUH-CTRC another day for a same day blood draw visit. The biotin study team may ask an RBC-OMICs control subject to return to MUH-CTRC up to a maximum of 6 times in one year to do additional optional control blood draw visits. RBC-OMICs control subjects will do one screening visit to test red blood cells and as many as 6 additional optional blood draw visits to provide as control samples. The biotin study team may ask the RBC-OMICs control subjects during the subject's blood draw visit or may contact the subject by phone and/or email in the future to do additional optional blood draw visits up to a

maximum of 6 times within one year. If the RBC-OMICs control subject does not want to perform an additional optional blood draw visit, the biotin study team will ask the other RBC-OMICs control subjects to participate in the additional optional blood draw visit. If an RBC-OMICs control subject will have an additional optional blood draw visit scheduled on the same day as an RBC-OMICs biotin subject with one of the targeted SNPs, the biotin study team will coordinate both subjects schedules to have them come into MUH-CTRC another day for the same day blood draw visit. Each RBC-OMICs control subject will not exceed the blood donation volumes (approximately 500 mls) within a 8 week period with the timing of each additional optional blood draw visit and/or any unrelated blood donations. The biotin study team will verbally ask the RBC-OMICs control subjects if they donated whole blood or red blood cells within 56 days prior to scheduling each blood draw visit. An RBC-OMICs control subject will not come more often than once a week for an additional optional blood draw visit at MUH-CTRC. The RBC-Omics subjects will not be asked to donate with the same week per expedited review guideline for blood draw. The biotin study team under the direction of the PI will monitor each RBC-OMICs control subject's blood draw visits and each RBC-OMICs control subject's total number of blood draws to be in compliance with the protocol; not to exceed 6 times in one year. If the RBC-OMICs biotin subject with one of the targeted SNPs has abnormal red blood cell biophysiological properties, then the length of time from the subject's blood draw visit to when and if the subject can enroll in the biotin study (if qualifying) could vary by several months.

- Obtain written consent.
- Blood draw samples for Dr. Gladwin's lab to test for in vitro studies of RBC membrane structure and function, RBC cellular response to oxidative stress, response to mechanical and storage stress, and RBC cellular metabolism. Other in vitro tests may be developed and performed.
- Blood draw sample for Wake Forest University to test for RBC hydration and water/ion transport.
- Blood draw sample to Vitalant to test for ektacytometry and RBC aggregation assays. Dr. Yingze Zhang's lab to help facilitate the processing of samples for Dr. Gladwin's lab, Wake Forest University, and Vitalant.
- Samples may be sent to Yale University for other vitro tests.
- AE assessment

Total blood volume drawn from an RBC-OMICs control subject or an RBC-OMICs biotin subject with one of the targeted SNPs at each visit will be about 3 ¼ tablespoons (approximately 48 mLs).

The RBC-OMICs control subjects may return to MUH-CTRC up to maximum of 6 times in one year to perform blood additional optional blood draw visits. RBC-OMICs control subjects will do one screening visit to test red blood cells and as many as 6 additional optional blood draw visits to provide as control samples.The total blood volume drawn from up to a maximum of six blood blood draw visits will be about 19.5 tablespoons of blood (approximately 288 mls).

Biotin Infusion Study Visits

Pre-Screening

The screening procedure involves a telephone interview for the purpose of identifying potential subjects and determining their eligibility for the study.

Visit 1 (Day 0)
Subjects will be asked to fast a minimum of 8 hours prior to coming into MUH-CTRC.

- Obtain written informed consent.
- Review Inclusion and Exclusion criteria
- Medical history review and demographics
- Physical examination to include body weight, height, and vital signs including temperature (Temp), heart rate (HR), respiratory rate (RR), blood pressure (BP), oxygen saturation measured by pulse oximetry (pulse ox)
- Urine pregnancy test in women of childbearing potential (i.e. women who are not at least 1 year post-menopausal or who have not undergone a surgical sterilization procedure)
- Clinical lab testing (CBC, creatinine, AST, ALT, hemoglobin A1c and lipid profile). A blood test may be repeated if an abnormal test results from Visit 1. The blood test will be repeated by recollecting the blood sample from the donor.
- Draw blood for genetic testing
- Hemoglobin electrophoresis for sub-groups of healthy African-American volunteers
- Direct Antiglobulin Test (DAT)
- Blood draw for Type and Cross (ABO/Rh type and antibody screen) prior to transfusion. A blood draw for a check-type sample may be required by Vitalant
- Blood draw to test naturally occurring antibodies to BioRBCs.

Total blood volume drawn will be about 3 tablespoons (approximately 32.0 mls).

Visit 2 (Day 1)
If the subject is eligible from visit 1, then Vitalant's form "Physician Order for Autologous Blood" will be sent to Vitalant staff via the UPMC secure email system. This form will have the subject's private health information and the study ID number that is reviewed and signed/dated by the PI or Co-I in this study prior to visit 2. In order for the blood to be released from Vitalant to the HSCLab, Vitalant will need this form. The blood donation visit will be scheduled within approximately 4 weeks of Visit 1 at Vitalant Pittsburgh. All procedures will be performed by professionally trained Vitalant Pittsburgh staff in accordance with blood bank's standard blood donation policy and procedures. By signing the study consent, patients are authorizing the blood bank to release the donated blood to use in the study (if there are no infections) or to inform us that we cannot use the sample. Study team will not be informed of what test is positive (if applicable). But, by Vitalent confirming whether we can use it or not, it reveals that a patient will not have any of the infections or that a patient may have one of them. Vitalent will call coordinator, leave confidential voice message, or send a secure email for the coordinator who will record in the research record whether the blood donation sample is usable in the study.

- Confirm eligibility following Visit 2.

Total blood volume donated will be approximately 500 mls. Subjects will get back 300 mls back over 2 transfusions.

Visit 3 (Day 5-7)
This visit will be scheduled between approximately 5-7 days following blood donation in MUH-CTRC. A "Physician's Processing Request form" will be sent to the HSCLab staff via the UPMC secure email system. This form will have the subject's private health information and the study ID number that is reviewed and signed/dated by the PI or Co-I in this study. In order for the blood to be released from HSCLab to CTRC, HSCLab will need this form. The Vitalant form,"Patient

Identification Form" that is sent to HSCLab by the study team via the UPMC secure email system will contain the subject's private heath information and study ID number. In order for the blood to be released from Vitalant to HSCLab on the day of biotinylation, Vitalant will need this form.

- Brief physical examination to include vital signs (Temp, HR, RR, BP, pulse ox)
- Urine pregnancy test in women of childbearing potential
- Blood draw for whole blood sample will be sent to Wake Forest University
- Blood draw for clinical lab testing (CBC)
- Blood draw prior to transfusion to detect presence of BioRBCs. Results not needed to proceed with the study.
- Transfuse 150 mL of 5-7 days old BioRBCs
- Obtain the subject's leftover BioRBCs in IV line/unit of blood for storage and stress hemolysis
- Vital signs (Temp, HR, RR, BP, pulse ox) approximately every 15 minutes during the transfusion and approximately 15 minutes post transfusion
- Blood draw approximately 2-4 hours post transfusion to determine baseline survival for 5-7 days old BioRBCs.
- AE assessment
- Given meal

Total blood volume drawn will be about 2 tablespoons (approximately 14 mls).

Visit 4 (Day 6-8)
Subject will return approximately 24 hours (± 4 hours) after the first blood transfusion visit to MUH-CTRC.

- Blood draw approximately 24 hours following the first transfusion visit to test survival of BioRBCs
- Blood draw for clinical lab testing (CBC)
- AE assessment

Total blood volume drawn will be about 1 tablespoon (approximately 8 mls).

Visit 5 (Day 32-41)
This visit will be scheduled approximately alittle over one month (ranges between 32-41 days) after completion of 1st transfusion in MUH-CTRC.

- Blood draw to test antibodies to BioRBCs. If anti-BioRBC antibodies are detected following 1st transfusion, the subject will be withdrawn from the study. A negative test result must be confirmed prior to proceeding with 2nd transfusion.
- Blood draw for Type and Cross (ABO/Rh type and antibody screen) prior to transfusion
- AE assessment

Total blood volume drawn will be about 1 tablespoon (approximately 10 mls).

Visit 6 (Day 35-42) This visit will be scheduled between approximately 35-42 days following blood donation in MUH-CTRC. A "Physician's Processing Request form" will be sent to the HSCLab staff via the UPMC secure email system. This form will have the subject's private health information and the study ID number that is reviewed and signed/dated by the PI or Co-I in this study. In order for the blood to be released from HSCLab to CTRC, HSCLab will need this form. The Vitalant form,"Patient Identification Form" that is sent to HSCLab by the study team via the UPMC secure email system will contain the subject's private heath information and study ID

number. In order for the blood to be released from Vitalant to HSCLab on the day of biotinylation, Vitalant will need this form.

- Brief physical examination to include vital signs (Temp, HR, RR, BP, pulse ox)
- Review medical history and demographics
- Urine pregnancy test in women of childbearing potential
- Blood draw for whole blood sample will be sent to Wake Forest University
- Blood draw for clinical lab testing (CBC)
- Blood draw prior to transfusion to detect presence of BioRBCs. Results not needed to proceed with the study.
- Transfuse 150 mL of 35-42 days old biotin-labeled RBCs
- Obtain the subject's leftover BioRBCs in IV line/unit of blood for storage and stress hemolysis
- Vital signs (Temp, HR, RR, BP, pulse ox) approximately every 15 minutes during the transfusion and approximately 15 minutes post transfusion
- Blood draw approximately 2-4 hours post transfusion to determine baseline for 35-42 days old BioRBCs.
- AE assessment
- Given meal

Total blood volume drawn will be about 2 tablespoons (approximately 14 mls).

Visit 7 (Day 36-43)
Subject will return approximately 24 hours (± 4 hours) after the second blood transfusion visit to MUH-CTRC.

- Blood draw approximately 24 hours following the second transfusion visit to test survival of BioRBCs
- Blood draw for clinical lab testing (CBC)
- AE assessment

Total blood volume drawn will be about 1 tablespoon (approximately 8 mls).

Visit 8 (Day 65-72)
This visit will be scheduled between approximately 65-72 days, approximately 2 months post 1st transfusion or 1 month post 2nd transfusion in MUH-CTRC.

- Vital signs (Temp, HR, RR, BP, pulse ox)
- Blood draw to determine BioRBC survival.
- Blood draw to test antibodies to BioRBCs.
- Blood draw for clinical lab testing (CBC)
- AE assessment

Total blood volume drawn will be about 1 tablespoon (approximately 12 mls).

Visit 9 (Day 95-102)
This visit will be scheduled between approximately 95-102 days, approximately 2 months post 2nd transfusion in MUH-CTRC.

- Vital signs (Temp, HR, RR, BP, pulse ox)
- Blood draw to determine BioRBC survival.
- Blood draw for clinical lab testing (CBC)

- AE assessment

Total blood volume drawn will be about 1 tablespoon (approximately 12mls).

Visit 10 (Day 125-150)
This visit will be scheduled between approximately 125-150 days, approximately 3-4 months post 2nd transfusion to test biotin antibodies in MUH-CTRC.

- Vital signs (Temp, HR, RR, BP, pulse ox)
- Blood draw to determine BioRBC survival.
- Blood draw to test antibodies to BioRBCs.
- Blood draw for clinical lab testing (CBC)
- AE assessment

Total blood volume drawn will be about 1 tablespoon (approximately 12mls).

Additional blood draws

Following the transfusion visits, subjects may be asked to return periodically over the next 3 months for further blood draws important for the biotinylation analysis of the their blood cells. The total volume will not exceed 20ml. Subjects will be compensated each time needed to return to MUH for a blood draw.

2.3 INVESTIGATIONAL PRODUCT

The investigational product is Biotinylated Red Blood Cells (BioRBCs). We will use sulfo-NHS-biotin to label human RBCs with biotin. Sulfo-NHS-Biotin is supplied in pre-weighed 1mg microtubes (EZ-Link Sulfo-NHS-Biotin, Thermo Scientific, Rockford, IL, USA) as a lyophilized white powder and is stored at -20°C.

Dosages: The dose of biotinylated red blood cells is designated by the concentration of biotin (µg/mL) used to label them. The actual total dose of biotin delivered per product is a function of the labeling dose (3 or 15 µg/mL) and labeling efficiency (approximately 1%), as all free biotin is washed from the product. The labeling efficiency will be determined for each product by quantitative flow cytometry. In our scale-up studies, RBC product labeled at 30 µg/mL contained a total biotin dose of 54.8 ± 34.0 µg biotin/product (mean, SD). Biotinylated autologous RBCs labeled at 15 µg/mL will be administered intravenously on day 5-7 in a volume of approximately 150 mL (0.5 - 1% of the subject's total intravascular volume). The subject will again receive BioRBCs labeled at 3 µg/mL on day 35-42 intravenously. As most of the biotin is washed away during labeling, the total biotin dose subjects will receive is approximately 27.5 µg during the first transfusion and 5.5 µg biotin during the second transfusion. The BioRBCs will circulate in the subject's bloodstream for the lifespan of the cells (approximately 120 days). Two separate infusions of BioRBCs over a period of 5-6 weeks will result in exposure to BioRBCs for 5-6 months (approximately 165 days).

Quantitative Estimation of Total Biotin Dose: For each batch of biotin labeled RBC, we perform quantitative flow cytometry to determine the average number of molecules of biotin per conjugated RBC and estimate the total dose of biotin administered to the patient. Biotin is quantified by labeling the biotin-conjugated cells with titrated Phycoerythrin (PE)-conjugated streptavidin and

measuring the PE mean fluorescence intensity of the labeled RBC by flow cytometry. The Mean Fluorescence Intensity (MFI) of the labeled RBC is interpolated on a calibration curve derived from PE-conjugated beads (blank and 4 levels of PE) with known numbers of PE molecules per bead (Quantum R-PE MESF beads, Bangs Laboratories). The results are reported as molecules of Mean Equivalent Soluble Fluorescence (MESF) per labeled RBC. Under carefully controlled labeling conditions, MESF/RBC can be interpreted as molecules of biotin/RBC. The total dose of biotin delivered to the patient may then be estimated as follows:

1) $Total\ Biotin\ Molecules = \ MESF/RBC\ x\ total\ RBC$

2) $Total\ Biotin\ (mol) = \frac{Total\ Biotin\ Molecules}{6.02\ x\ 10^{23}}$

3) $Total\ Biotin\ Dose\ (g) = Total\ Biotin\ (mol) x\ 244.31$ (formula weight of biotin).

Determination of molecules of equivalent soluble fluorochrome (MESF)

We determined MESF for 16 independent samples (labeled at 2 biotin concentrations). Sulfo-NHS-biotin labeling concentration (μg/mL total reaction mixture) correlated with MESF but there was more variability at the lowest dose (3 μg/mL).

There are 3 sequential independent products labeled at 3 and 30 μg biotin/mL total reaction mixture. Calibration of the results in MESF shows that RBC-bound biotin of RBC labeled at the same sulfo-NHS-biotin concentration, using the same cGMP-compliant procedure could vary by more than 2-fold. Despite the variability, excellent separation was obtained between unlabeled RBC and RBC labeled at the two biotin concentrations.

2.3.1. Investigational Product Preparation and Dispensing

The packed RBCs will be biotin-labeled under sterile conditions by trained personnel at the UPMC Hematopoietic Stem Cell Laboratory (HSCLab), an International Organization for Standardization Class 7 (ISO7) Current Good Manufacturing Practice (cGMP) compliant facility. The HSCLab has a Master file on file with the FDA that is cross referenced by this protocol. Preparation of the biotin solution and filter sterilization occurs immediately before addition to the autologous red blood cell sample, which is handled under sterile conditions. The sterile biotin reagent and autologous RBCs are combined at a constant rate and are incubated at room temperature for 30 minutes to allow biotin to bind to the surface of the RBC membrane. After the incubation period, the product is washed and re-suspended in saline with 5% human serum albumin. A BioRBC sample is removed by sterile technique for release testing. Release criteria include a final RBC packed cell volume ≥ 25 mL and total endotoxin content of < 5EU/kg recipient actual body weight. The product will be withheld if either of these criteria is not met. Additional product quality testing will include quantitative flow cytometry to determine the actual dose of biotin in each product, and pre- and post labeling 14 day aerobic, anaerobic and fungal sterility cultures.

The biotinylated autologous RBCs will be provided in 300 mL transfer bags for infusion, at a volume of ~150 mL and a hematocrit of approximately 35% to 40%. The HSCLab is (Foundation for the Accreditation of Cellular Therapy (FACT) accredited and uses International Society of Blood Transfusion (ISBT) 128 Standard Terminology for Blood, Cellular Therapy, and Tissue

Product Descriptions labeling and packaging, according to Good Clinical Practice and regulatory requirements. HSCLab will dispense the BioRBC product upon receiving a signed and dated physician's processing request form. The HSCLab will maintain accurate records of investigational products including subject dispensing, product destruction and documentation of adequate storage of investigational products.

Further details on investigational product preparation and dispensing are provided in a separate laboratory standard operating procedure.

2.3.2. Administration of Investigational Product

BioRBCs will be administered intravenously on two separate occasions (5-7 and 35-42 days following donation). BioRBCs will be transfused at a rate of 100-150 mL/hr. This range will allow us to reduce infusion rate if recipient complains of any discomfort or pain. Recipient vitals will be taken at baseline and approximately every 15 minutes during the infusion and then approximately 15 minutes after the infusion ends by qualified personnel at the Clinical Translational Research Center (CTRC) of UPMC Montefiore Hospital.

2.3.3. Dose Selection

Experience with biotin labeling method to study RBC survival in humans is expanding. Biotinylated RBCs (BioRBCs) were first used to study RBC survival in rabbits in 1987.[13] Since then, the use of BioRBCs has successfully been expanded to human studies evaluating RBC 24-hour recovery and lifespan. Investigators have used BioRBCs to study RBC volume and survival in healthy adults.[14-15] BioRBCs have also been used in patients with sickle cell disease to study autologous RBC survival and post-infusion membrane changes,[16] and it is currently being used to study both autologous and allogeneic RBC survival in neonates in the intensive care unit.[17] The primary safety concern in using the BioRBC method in humans is the potential formation of antibodies against the BioRBCs. The formation of anti-BioRBC antibodies was observed in 3 subjects out of 20 adults studied (15%).[18] The anti-BioRBC antibodies appeared 4-5 months after the infusion of BioRBCs, reacted only against BioRBCs (i.e. antibodies did not behave as autoantibodies), did not affect BioRBC 24-hour recovery or survival, and were transient in nature (i.e. anti-BioRBC antibody activity disappeared within 1-4 months after initial detection).

Biotin has also been directly administered to healthy adults both orally and intravenously with daily oral doses being as high as 2000 μg and IV doses as high as 4500 μg without any reported adverse effects.[19,20] In this study, subjects will receive a total biotin dose of approximately 0.2 μg/ml (27.5 ug biotin) during the first transfusion visit and 0.04 μg/ml biotin (5.5 ug of biotin) during the second transfusion visit.

2.3.4. Testing for Antibodies to BioRBCs

On Day 0, plasma samples will be obtained to test subject's naturally occurring anti-BioRBC antibodies. On Day 32-41 and Day 125-150, plasma samples will be obtained from the subjects. These plasmas will be tested for anti-BioRBC antibodies using a commercial sensitive and specific IgG gel card agglutination assay.

The gel card assay is based on RBC agglutination. The gel cards will be used according to the manufacturer's recommendations (MTS Anti-IgG Card, MTS084024, Ortho Clinical Diagnostics, Rochester, NY). Briefly, 50 μL of the target BioRBC-N or unlabeled RBC (at 0.8% hematocrit in

Diluent 2) are prepared from a group O+ convenience donor and layered atop the buffer in gel card microtubes. Test plasma (25 µL) is added to the target BioRBC or RBC in the gel column and incubated at 37˚C for 30 min (ID-MTS Incubator MTS9680 (Ortho Clinical Diagnostics, Rochester, NY). Gel cards are subsequently centrifuged at 895 RPM (80-90 RCF) for 10 min (ID-MTS Centrifuge MT515060, Ortho Clinical Diagnostics, Rochester, NY). The presence of BioRBC agglutination is quantified using the manufacturer's gradation-specific definitions of reactivity. Reactivity scores range from 0 (no agglutination) to 4+ (agglutinates are trapped on the surface of the gel bed).[21]

Detailed testing procedures are provided in a separate laboratory standard operating procedure.

2.3.5. Treatment Period

The study consists of two blood transfusions of one day each separated by approximately one month.

2.3.6. Investigational Product Storage and Accountability

The packed RBCs will be stored under standard blood banking conditions (1-6°C) and transferred to HSCLab one day before the transfusion procedure. All aliquots will be sterile and made using a closed system. The HSCLab will label the RBC within 48 hours of receipt. Preparation of the biotin solution and filter sterilization occur immediately before it is added to the autologous red blood cell sample, which will be prepared immediately prior to infusion to research subjects. In no case will the time from preparation of the biotin solution and biotin-labeled autologous red blood cells to research subject infusion exceed an assigned period of 8 hours.

2.3.7 Concomitant Medications

Subjects will be advised to avoid any nutritional supplement containing biotin (Vitamin B7) and to refrain from eating raw eggs 30 days prior to blood donation and throughout the study period. Potential subjects will be requested to bring a list of any over the counter drugs and/or any nutritional or herbal supplements that they may be taking to the initial screening visit. This list will be documented in the research chart and carefully evaluated by the Physician Investigator for potential interactions with biotin. The Physician Investigator will have final decision regarding the acceptability of concomitant medications. Subjects will be requested to contact the research team prior to taking any new medications (including over-the-counter drugs and vitamin or herbal supplement) during their participation in the clinical Investigation.

2.4 STUDY ENDPOINTS

Primary Endpoint:
The primary endpoint is the labeled red blood cell recovery rate over 60-days post transfusion (using data from 2-4 hours, 1 and 2 month) of 35-42 day stored Bio-RBCs from blood donors with specified genetic mutations, compared with control subjects without the specified mutation.

This protocol may evaluate up to eight (8) genetic mutations as independent studies, compared with pooled control groups, as well as a group of subjects on testosterone replacement therapy. The eightgenetic mutations that we will evaluate may include sickle cell trait (AS), and single nucleotide polymorphisms (SNPs) in ankyrin 1 (ANK1), glucose-6-phosphate dehydrogenase X-linked (G6PD), piezo-type mechanosensitive ion channel component 1 (PIEZ01), spectrin alpha

(SPTA), extended synaptotagmin-like protein 2 (ESYT2), Myo9b, and SEC14. We may identify additional SNPs as our analysis of the REDS-III GWAS study advances that could replace one of these SNP groups and/or we identify additional non-synonymous SNPs that are associated with susceptibility to hemolysis of stored red blood cells. In addition we will evaluate 15 subjects on testosterone replacement therapy with 20 controls that are gender and race matched that are not on testosterone replacement therapy. Each group will be considered a separate study for statistical purposes. Controls will be selected based on not having any of the SNPs that will be evaluated in these studies.

Secondary Endpoints:

- Define the 1-hour post transfusion survival of fresh or stored Bio-RBCs
- Determine the association between gender and in vitro correlates of the RBC storage lesion and post transfusion recovery of BioRBCs at selected time points (i.e. 2-4 hours, 60 days). The in vitro measurements of stored RBCs test include spontaneous cold storage hemolysis, osmotic hemolysis, mechanical fragility, AAPH-induced oxidative hemolysis, and membrane microvesicle formation. RBC predisposition to hemolysis is quantified as percent hemolysis, which accounts for the levels of RBC-derived free hemoglobin in response to cold storage (5-7 and 35-42 days following donation) or exposure of washed and stored RBCs to osmotic or oxidative stress.

Storage hemolysis: quantification of storage hemolysis is based on the equation:

Storage hemolysis (%) $= \frac{(100-\mathrm{HCT})\times \mathrm{Hb_{supernatant}}}{\mathrm{Hb_{total}}}$, for which HCT refers to the sample hematocrit (HCT), $Hb_{supernatant}$ refers to the levels of free hemoglobin obtained after centrifugation (1500x g, 10min, 18°C) measured in the supernatant, and Hb_{total} refers to the total amount of sample hemoglobin before centrifugation.

Stress hemolysis assays: For the evaluation of stress-induced hemolysis, stored RBCs are washed (1500x g, 10min, 18°C) three times with phosphate-buffered saline (PBS) to remove plasma and additive solution, and immediately subjected to osmotic or oxidative stress assays.

RBC osmotic hemolysis: Osmotic hemolysis is determined by incubating (4h at 22°C) stored and washed RBCs in pink test buffer (a hypotonic Bis-Tris buffer containing 25mmol/L sodium chloride, 70mmol/L Bis-Tris buffer, and 135mmol/L glycerol; pH 6.6) at a final concentration of 1.6%±0.2% after which samples were centrifuged (1500x g, 10min, 18°C), and percent osmotic hemolysis is determined by: Osmotic hemolysis (%) = $\frac{Hb_{osmotic}}{Hb_{total}} \times 100$, for which $Hb_{osmotic}$ corresponds to supernatant cell-free hemoglobin of pink test-treated RBCs and Hb_{total} refers to the total amount of hemoglobin of each sample.

RBC oxidative hemolysis: Testing for the impact of genetic mutations on RBC susceptibility to oxidative hemolysis is performed by incubating (1.5-h, 37°C) stored and washed RBCs in the presence of 2,2'-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH, 150mmoL). After incubation, samples are centrifuged (1500x g, 10min, 18°C), and AAPH-induced oxidative hemolysis is determined by: Oxidative hemolysis (%) = $\frac{Hb_{AAPH}-Hb_{control}}{Hb_{total}} \times 100$, where Hb_{AAPH} corresponds to supernatant cell-free hemoglobin of AAPH-treated RBCs, $Hb_{control}$ corresponds to supernatant cell-free hemoglobin of untreated RBCs, and Hb_{total} refers to the total amount of hemoglobin of each sample.

Membrane microvesicle formation: RBC-derived microvesicles will be quantified by centrifugation and collection of supernatants from stored RBC units. Microvesicles will be quantified and characterized (size) by flow cytometric analysis as described by Donadee and Gladwin.[22]

Correlation between primary and secondary endpoints: We will define the associations between the primary endpoint (60-day in vivo survival of transfused Bio-RBCs) and each of those secondary endpoints by regression analysis using Pearson's r.

2.5 STATISTICAL ANALYSIS

2.5.1 Sample Size Determination

In healthy volunteers, the mean (SD) of 30 and 60-days RBC biotin recovery rate is 75% (12.5%) and 50% (10%) (calculated from 24-hour 100% recovery value), respectively[23, 24]. For our first study, evaluating patients with the sickle cell trait SNP, we expect SCD trait to reduce 30 and 60-day recovery to 62% and 25%. A sample size of 15 subjects with sickle cell trait (HbAS) and 20 control African American subjects (HbAA; 10 males and 10 females) would provide 84% power to detect a difference of 13% (SD =12.5%) for 30 days and a power of >95% to detect a difference of 25% (SD = 12.5%) for 60 days RBC recovery rate between AS and AA. This sample size has at least 80% power to detect the interaction between time and genetic group as measure of different recovery rate. We will also recruit an additional 20 Caucasian control (HbAA, 10 males and 10 females) to gain power for testing the effect of other RBC mutations identified in our genome-wide association studies of blood donors (NHLBI RBC-Omics). We will use explanatory analysis for the evaluation of post transfusion recovery of RBCs from subjects with rare variants (<10% of the population) due to a projected small number of subjects carrying such mutations. All 40 control subjects will be tested and selected to not have any of the SNPs that we are studying (listed below).

This power calculations informs our other planned studies that will compare up to 7 additional single nucleotide polymorphisms (SNPs) patient groups of 15 each, compared with our control groups without the SNPs (n=20 race and gender matched). Successful enrollment of the sickle trait and 7 SNP groups would be 120 total subjects. The eight genetic mutations that we will evaluate may include sickle cell trait (AS), and single nucleotide polymorphisms (SNPs) in ankyrin 1 (ANK1), glucose-6-phosphate dehydrogenase X-linked (G6PD), piezo-type mechanosensitive ion channel component 1 (PIEZ01), spectrin alpha (SPTA), extended synaptotagmin-like protein 2 (ESYT2), Myo9b and SEC14. We may identify additional SNPs as our analysis of the REDS-III GWAS study advances that could replace one of these SNP groups and/or we identify additional non-synonymous SNPs that are associated with susceptibility to hemolysis of stored red blood cells. In addition, we will evaluate 15 subjects on testosterone replacement therapy with 20 controls that are gender and race matched that are not on testosterone replacement therapy.

Each study will be conducted as an independent analysis of 15 subjects with the SNP and 20 race and gender matched subjects without the SNP using power calculations estimated from the SCD trait example above.

Biotin infusion study: The study team will target 70 evaluable subjects. The study team will approach 86 subjects with 90% expected to agree to yield 78 subjects who will sign the biotin infusion consent. The study team expects 90% of these subjects to complete the biotin infusion study yielding the target 70 evaluable subjects. The biotin infusion subjects include 20 Whites, 20 African American without sickle cell trait, 15 African Americans with sickle cell trait, and 15 males on testosterone. In addition, 20 subjects with SNPs from the REDS-III RBC-OMICS study will be included for a total of 98 subjects to sign the biotin infusion consent and yield approximately 90 subjects who complete the study.

REDS-III RBC-OMICs study: The study team will target 105 subjects (with SNPs) who will sign the REDS-III RBC-OMICs consent. Included in the105 are the 20 subjects who will also sign the biotin infusion consent. In addition, approximately 40 RBC-OMICs control subjects will be tested and selected to not have any of the SNPs that we are studying. An RBC-OMICs control subject will perform the same blood draw study visit procedures at different times on the same day as an RBC-OMICs subject with one of the targeted SNPs. The study team will compare the red blood cells biophysical properties in an RBC-OMICs control subject to an RBC-OMICs subject with one of the targeted SNPs. The RBC-OMICs control subjects may be asked to return up to a maximum of 6 times in one year to perform additional optional RBC-OMICs control blood draw visits at MUH-CTRC. There will be a maximum of 6 number of blood draw visits for each of the 40 RBC-OMICs control subjects.

2.5.2 Statistical Analysis

The primary assessment of RBC transfusion efficacy is post transfusion survival. Data from RBC recovery at 2-4 hours, 1 month and 2 months after each transfusion will be evaluated using mixed effect model (with restricted maximum likelihood variance estimator and bootstrapping) to calculate the effect of time, genetic group and their interaction on RBC survival. RBC recovery at each time point will be performed using the equation below.

$$Percent\ survival\ (\%) = \frac{BioRBCs\ in\ cirulation\ at\ T_y\ (1 \le y \le 120\ days)}{Baseline\ BioRBCs\ \ in\ circulation\ at\ T_0\ (20\ \ min\ following\ RBC\ infusion)} * 100$$

Secondary endpoints: The equation above will be used to define the 2-4hours post transfusion survival of BioRBCs. The other endpoints test for changes in RBC predisposition to hemolysis in cold storage by quantifying the rates of storage, osmotic, mechanical and oxidative hemolysis and the formation of RBC membrane-derives microvesicles. A sample size of 8 samples would detect a 10% difference in our assays' outputs with a power of 85%. We will use commercial software for statistical analyses (STATA version 15.0, StataCorp., College Station, Texas, USA). We will use mixed effect model (with restricted maximum likelihood variance estimator and bootstrapping) to calculate the effect of time, genetic group and their interaction on RBC survival. Normality tests will help to determine the appropriate transformation of outcome data. We will also use nonlinear term of time in these models (if applicable). We will use marginal test (from the primary models) to assess the effect of genetic group on 60-days and 2-4hours RBC survival separately. As a confirmatory analysis, Wilcoxon signed rank test will determine the differences between two groups for 60-days and 2-4 hours survival rate as well.[10] The hypotheses will be tested primarily in old blood. A separate analysis will be performed in fresh blood transfusion as well. All P value <0.05 will be reported as statistically significant.

2.5.3 Handling Missing Data

Every effort will be made to minimize the missing data in the study. We will record reasons for drop-out and compare baseline characteristics between participants who do and do not complete the study. However, since there are several scheduled visits following each transfusion, the investigators in this study can use regression analysis to predict and estimate 1-hour red cell post transfusion survival or mean lifespan based on the known red cell life span estimate of 120 days. Critical values for this estimation will be obtained on the day of blood donation and on day of RBC infusions. For this purpose, we will fit mixed effect models using 1-hour, 60 days and 120 days survival for non-missing observations including the subjects' characteristics. This model will be used to impute the missing value for other subjects of study. Alternatively, in at each time point a

prediction model will be created from patients' characteristics of non-missing observations to impute the missing data point per time of observation. These two approaches with complete set analysis will be compared.

2.5.4 Data Management

The study forms will be paper-based in that the data will be first recorded on paper forms at the time of the study visits. A relational database will be constructed on a local server with daily backups where only select research team members will have access to the database. The database will include data entry forms with the same appearance as the paper forms to facilitate accurate data entry and routine data edit checks for consistency both within and between forms. After data entry, the paper forms will be archived in secure file cabinets. All study subjects will be assigned unique study identifiers that will appear on all data collection instruments, documents, and files used in the statistical analysis and manuscript preparation. The case report forms with study IDs will be housed in the same chart as personal identifier documents are while the subject is active in the protocol. Only limited team members will have access to charts and database. No personal information concerning study participants will be released without their written consent. Other data quality assurance measures will include verifying the data, out of range data checks, and repeated evaluation of the data process.
The REDS-III RBC-OMICs subjects research records may contain the subject's SNP for the study team to know which SNP subgroup the subject is in.

3. HUMAN SUBJECTS

The anticipated age range for the study population will be age 18 years or older based upon the target population. Healthy subjects of any ethnic background and with no significant past medical history will be eligible for enrollment.

The proposed study population will consist of potentially 223 (145 from the RBC-OMICs + 78 from Biotin infusion) healthy subjects. In order to achieve this number, it is anticipated that approximately 231 (145 from RBC-OMICS + 86 from Biotin infusion) participants may need to be enrolled into this study to account for subjects who may not qualify the eligibility criteria or not be able to donate blood or are withdrawn for any reasons.

3.1 SUBJECT POPULATION

3.1.1 Inclusion of Women and Minorities

Women and individuals from minority groups especially African Americans or individuals originally from malaria endemic regions who meet the inclusion criteria, and have none of the exclusion criteria, will be enrolled without restriction as dictated by the study protocols. Every effort will be made to enroll participants in this research in a distribution, which mirrors the study population of the Pittsburgh area.

3.1.2 Inclusion of Children

This investigation will not enroll children based upon lack of safety data in children with regards to the investigational product.

3.1.3 Inclusion of Prisoners

This investigation will not enroll prisoners.

3.2 INCLUSION CRITERIA

The following criteria will be required on ALL subjects:

- Male or female age 18 years or older at screening.
- Weight ≥110 lbs.
- Hemoglobin at or above 12.5 g/dL or hematocrit ≥ 38% for women and hemoglobin ≥ 13.0 g/dL or hematocrit ≥ 39% for men.
- Meet screening criteria for autologous blood donation

Additional Inclusion Criteria for Other Sub-Groups:

Healthy volunteers (subjects who complete the study=20)
- 10 males and 10 females
- Caucasian

Healthy African American volunteers with sickle cell trait (subjects who complete the study=15)
- Self-identification of African American race
- Sickle cell trait (HbAS) status confirmed by hemoglobin electrophoresis

Healthy African American volunteers without sickle cell trait (subjects who complete the study=20)
- 10 males and 10 females
- Self-identification of African American race
- Normal Hb (HbAA) and sex and age match to HbAS donors

Healthy volunteers enrolled in REDS-III Red Blood Cell Omics (RBC-Omics) study (105=subjects who complete the study)
- Recalled blood donors from the RED-III RBC-Omics study with non-synonymous SNPs identified in ANK1, G6PD, PIEZ01, SPTA, ESYT2, Myo9b, SEC14 and/or we identify additional non-synonymous SNPs that are associated with susceptibility to hemolysis of stored red blood cells. We may identify additional SNPs as our analysis of the REDS-III GWAS study advances that could replace one of these SNP groups. The SNPs will be tested for functional effects on red cell biophysical properties, stability in storage, and release of eDAMPs, compared to donors with no implicated SNPs. We will develop an enriched SNP profile that defines a high risk and low risk donor for further evaluation as a low cost precision transfusion medicine array. As determined in the aforementioned primary and secondary endpoints.

Healthy Volunteers Receiving Testosterone Replacement Therapy (subjects who complete the study=15)
- Male only

We aim to determine the impact of testosterone replacement therapy (TRT) on transfusion safety by defining the post transfusion recovery of stored biotinylated-RBCs from male donors who receive TRT. TRT has been recently identified as of one of the most overused medical practices in the US, with a 4-fold increase in the number of young patients (ages 18-45) since 2003. The lack of clear guidelines and risk assessment of RBC transfusion from TRT patients deem such donors eligible for donation, except for cases of suspected polycythemia. We have shown that TRT in mice enhances predisposition to osmotic and oxidative stress, whereas orchiectomy is associated with improved post transfusion recovery of stored RBCs as compared with intact males.[10,11] This study will verify whether the negative impact of TRT on RBC storage stability observed in mice is clinically relevant in transfusion of blood from TRT donors.

3.3 EXCLUSION CRITERIA

Subjects meeting any of the following exclusion criteria at baseline will be excluded from participating in study:

- Subjects with a past medical history or symptoms of blood dyscrasia, diabetes mellitus, hyperlipidemia, obstructive sleep apnea, renal disease, congestive heart failure, significant cardiac disease and / or known peripheral arterial disease.
- Moderate to severe systemic hypertension, (systolic blood pressure >140 mmHg and/or diastolic blood pressure > 95 mmHg)
- Systolic blood pressure <100 mmHg and/or diastolic blood pressure < 60 mmHg on the study day.
- Positive Direct Antiglobulin Test (DAT)
- Consumption of biotin supplements or raw eggs within 30 days
- Treatment with antibiotics in the week before initiating study participation to avoid suppression of erythropoiesis, which may accompany infection.
- Blood loss in the previous 8 weeks due to epistaxis, trauma, hemoptysis, gastrointestinal bleeding, diagnostic phlebotomy (> 30ml)
- Subjects who report tobacco or marijuana smoking within 6 months of study.
- Cognitively impaired subjects, or institutionalized persons and subjects unable or unwilling to complete written informed consent
- Subjects with a history of blood donation within the last 56 days.
- Use of other investigational drugs/devices within 30 days of screening.
- Subjects taking any medication for the treatment of diabetes including insulin
- Females of childbearing potential who are pregnant or unwilling to undergo pregnancy testing; females with positive pregnancy testing on screening day will be excluded.
- History of prior transfusion reaction to blood products.
- Allergic reaction to biotin
- Donors with naturally occurring antibodies against BioRBCs will be excluded from the study

4. IRB APPROVAL AND FDA AMENDMENTS

The Investigator will obtain, from the University of Pittsburgh Institutional Review Board (IRB), prospective approval of the clinical protocol and corresponding informed consent form(s);

modifications to the clinical protocol and corresponding informed consent forms, and advertisements (i.e., directed at potential research subjects) for study recruitment.

The only circumstance in which a deviation from the current IRB-approved clinical protocol/consent form(s) may be initiated in the absence of prospective IRB approval is to eliminate an apparent immediate hazard to the research subject(s). In such circumstances, the Investigator will promptly notify the University of Pittsburgh IRB of the deviation. The Investigator should also notify the sponsor of this event.

The University of Pittsburgh IRB operates in compliance with FDA regulations at 21 CFR Parts 50 and 21 CFR 56, and in conformance with applicable International Conference on Harmonization (ICH) Guidelines on Good Clinical Practice (CGP).

In the event that the University of Pittsburgh IRB requires, as a condition of approval, substantial changes to a clinical protocol submitted under an FDA-accepted IND application, or in the event of the Investigator's decision to modify the previously accepted clinical protocol, the Sponsor will submit (i.e., in advance of implementing the change) a Protocol Amendment to the IND describing any change to a Phase 2 or Phase 3 protocol that significantly affects the safety of subjects, the scope of the investigation, or the scientific quality of the study.

5. RECRUITMENT AND INFORMED CONSENT PROCEDURES

5.1 RECRUITMENT METHODS

The following recruitment methods will be used to identify potential subjects. All recruitment procedures and materials must be reviewed and approved by the IRB prior to their use.

(1) Potential subjects may be recruited from university and the University of Pittsburgh Research Participant Registry (Pitt+Me), and community advertisements including, but not limited to newspapersinternet ads, bulletin boards, posters, and flyers.

(2) Potential subjects for this study may also be recruited from the REDS-III RBC-Omics Study (IRB# 13115; PI: Dr. Triulzi). Subjects who were enrolled in the studies have provided previously their written informed consent to be contacted for future studies. For the REDS-III RBC-Omics Study, the study investigators/coordinators of the REDS-III RBC-Omics study will identify potential subjects from the original REDS-III RBC-Omics Study cohort. They will review eligibility criteria based on previously gathered research data on the subjects and if eligible, reach out via phone*and/or email to inform the subject. They will provide the subject with the biotin study coordinator phone number. The REDS-III RBC-OMICs subjects who choose to call will be asked to give verbal permission (waiver to document consent) to allow the biotin study team to review their identifiable genetic information. This will be used to determine if subjects are eligible for the biotin study or to serve as the biotin control.

(3) Potential subjects may also be recruited via our existing contacts with normal healthy volunteers, support groups, and clinics throughout the University of Pittsburgh areas.

(4) For subjects who are not patients in the physician investigators' UPMC Presbyterian/Montefiore-based clinics, we will obtain a waiver of written HIPAA permission to review their medical records for eligibility.

(5) Subjects referred from facilities outside the UPMC Epic medical record system will be provided a medical record release for their physician to release needed documents to confirm eligibility.

(6.) The study team may be using a secure UPMC email system to do pre-screening questions using the biotin screening script. The study team may email the biotin screening script pre-screening questions using a secure UPMC email system to the subject, as soon as the subject fills out the questions, then the subject will email it back to study team. Then the study team will print out a hard copy of subject's responses to the pre-screening questions and then the study team will delete the subject's email.The study team will keep the hard copy of the pre-screening questions in a locked cabinet in a locked office on a unit that has restricted access until the subject comes in for a screening visit. The subject's pre-screening form will be stored for approximately several months and will be destroyed if the subjects do not go to a screening visit and sign consent.

5.2 INFORMED CONSENT PROCEDURES

Prior to performing any of the study procedures the subjects must provide informed consent. The information about this study will be given to potentially eligible subjects in language understandable to them. The physician investigator or the study coordinator will verbally present a general outline of the research study, including potential risks and benefits of participation, to the prospective participant. The consent form, outlining the design of the study and including the risks and benefits of participating, will be reviewed with the prospective participant by the physician investigator who will answer any questions at that time. Prospective participants will have ample time and opportunity to ask questions and will be informed about the right to withdraw from the study at any time without any disadvantage and without having to provide any reasons for this decision. Participants will receive a copy of the informed consent form.

The RBC-OMICs control subjects may be asked to return up to a maximum of 6 times in one year to MUH-CTRC for additional optional blood draw visits. The biotin study team will ask the subjects questions related to the consent to confirm subjects comprehension of the study.

The physician investigator or study coordinator will verbally present from the consent that the individual genetic results or incidental findings will not be shared with subjects as these tests are research-related and these initial finding will need additional research before their clinical significance is understood and appropriate actions are determined.

During the course of the study, participants will be provided with any new information that arises (i.e., new study procedure, change in risk/benefit profile) that may affect a participant's decision whether or not to continue participation in the study. Subjects who already signed consent will be presented with a revised consent form with the new study procedures and/or what has changed since they last provided consent. A copy of the revised consent will be given to the subjects for their records.

6. POTENTIAL RISKS AND BENEFITS

6.1 POTENTIAL RISKS

As with any experimental procedure, there may be adverse events or side effects that are currently unknown, and certain of these unknown risks could be permanent, severe or life threatening. Every attempt will be taken to minimize these risks.

Risks of BioRBC Infusion: The risks of BioRBC infusion include the possibility that the biotinylated cells may be destroyed and cleared from the circulation. The amount of transfused BioRBCs represents about 2% of the total blood volume in the subject and removal of the BioRBCs are not likely to cause any health problems. During the biotinylation process, the autologous RBCs will be removed from the blood bank and processed under a sterile tissue culture hood. The biotinylation process will be performed in a GMP cellular therapy facility; however there is a minimal risk of bacterial contamination. This risk is reduced by requiring that each BioRBC sample be tested for and passes endotoxin contamination testing prior to infusion.

Risk of Antibody Formation: In a previous study in which adults were transfused with BioRBCs, 3 of 20 (15%) subjects developed antibodies to BioRBCs, but none developed anemia or any other health problems.[15] In study subjects that developed antibodies, the antibodies disappeared from the blood over a period of months. To reduce this risk of antibody formation, a lower dose of biotin will be used throughout this study compared to the previous study,[15] and subjects will be screened for antibody formation throughout the study and during follow-up visits. In summary, we do not anticipate side effects in the proposed biotin doses.

Rare risks include immune-mediated hemolytic reaction to transfused biotinylated RBCs. Given the small volume of BioRBC infused, no significant clinical effects of hemolysis of biotinylated RBC is expected.

Risks of Transfusion Reactions: In this study, healthy subjects receive only small volume of autologous packed BioRBCs. The risk of experiencing a transfusion reaction is considered minimal; no excess risk of transfusion reactions is expected from the small autologous infusions used in this study. However, adverse reactions that are known to occur following transfusion. Specifically, transfusions of conventional blood products are known to be associated with adverse transfusion reactions such as fever, chills, nausea/vomiting, itching, rash, hives, hemolysis, bronchospasm, hypertension and hypotension, transfusion-related acute lung injury (TRALI), transfusion-associated circulatory overload (TACO), and anaphylaxis.

Risk of Blood Donation: The risks include nausea, fainting or dizziness, low blood pressure, bruise at needle puncture site, possible nerve damage, blood loss and infection. Other less common risks include phlebotomy complications, fainting (vasovagal) reactions, chance of identifying communicable disease and stress, emotional distress on subject. We will follow the AABB's blood drawing guidelines along with the hospital's standard operating procedures for adult venipunctures.

Risks of Venipuncture /Intravenous Needle Insertion**:** Common Risks: bruising, soreness, phlebitis. Infrequent Risks: infiltration (a leakage of anything that has been given through the vein (such as the saline) into the arm tissue that surrounds the vein and holds the IV), mild pain and discomfort at the injection or needle insertion site as well as possible infection and bleeding. Rare: severe pain; swelling; fainting. In order to minimize the potential for fall injuries, subjects will be closely monitored for dizziness or light-headedness before they are allowed to stand.

Risks of Collection of Private Health Information, Biospecimens and Genetic Analysis: Participation in this research study does involve the potential risks of a breach of confidentiality of the medical record information and associated privacy of the participants. This research study will result in identifiable information that will be placed into the subject's medical records held at the University of Pittsburgh Medical Center. The nature of the identifiable information resulting from participation in this research study that will be recorded in the medical record includes laboratory test results. This potential for breach of confidentiality, including with regard to genetic analysis, could impact future insurability, employability, or reproduction plans, or have a negative impact on family relationships, and/or result in paternity suits or stigmatization. If the subject is eligible from visit 1, then Vitalant's form "Physician Order for Autologous Blood" will be sent to Vitalant staff via the UPMC secure email system. This form will have the subject's private health information and the study ID number that is reviewed and signed/dated by the PI or Co-I in this study prior to visit 2. In order for the blood to be released from Vitalant to the HSCLab, Vitalant will need this form. A "Physician's Processing Request form" will be sent to the HSCLab staff via the UPMC secure email system. This form will have the subject's private health information and the study ID number that is reviewed and signed/dated by the PI or Co-I in this study. In order for the blood to be released from HSCLab to CTRC, HSCLab will need this form. The Vitalant form,"Patient Identification Form" that is sent to HSCLab by the study team via the UPMC secure email system will contain the subject's private heath information and study ID number. In order for the blood to be released from Vitalant to HSCLab on the day of biotinylation, Vitalant will need this form. The study team will make every effort to keep the subject's medical information and study ID confidential.

6.2 ALTERNATIVE TREATMENTS

If subjects choose not to participate in this study, they are to continue their medical care under the direction of their treating physicians.

6.3 POTENTIAL BENEFITS

There will be no direct benefit to the subjects participating in this study, but the society at large may benefit from the increased knowledge gained from this study. This study will advance the general knowledge regarding the effect of the storage lesion of red cell transfusion on endothelial function, and evaluate the metabolism and interconversion of nitrite to nitric oxide *in vivo* in healthy adults as a means to ameliorate the endothelial effects of the storage lesion, and study the lifespan of transfused red cells. This may have a major impact on how we transfuse red cells in patients.

Based on the preceding assessment of risks and potential benefits, the risks to subjects are reasonable in relation to anticipated benefits. The research presents a balance of risks and expected benefits similar to that available in the clinical setting.

6.4 DATA SAFETY MONITORING

6.4.1 Data Safety Monitoring Board

An Independent Data and Safety Monitoring Board (iDSMB) will be established; to be comprised of individuals who are not involved with this study protocol. The iDSMB will comprise of members including senior experts in transfusion medicine, clinical research and clinical trial design. None

of the DSMB members will have direct involvement in the conduct of the study. All members of the DSMB are required to be independent of the studies being reviewed and need to certify this by signing a DSMB Conflict of Interest and Confidentiality statement.

The iDSMB will conduct interim monitoring of accumulating data from research activities to assure the continue safety of human subjects, relevance and appropriateness of the study, and the integrity of research data.

6.4.2 Data Safety Monitoring Plan

Before the start of the study, the DSMB will review the protocol for any major concern and the plans for data and safety monitoring to ensure that the frequency of monitoring is appropriate for the intervention. In addition, certification of IRB approval of the protocol with the data and safety monitoring plan will be sent to the NHLBI Grant Program Official (PO) prior to implementation.

Once the study is implemented, the DSMB will convene as often as necessary, but not less than every 6 months, to examine the accumulated safety and enrollment data, review study progress, and discuss other factors (internal or external to the study) that might impact continuation of the study as designed. A DSMB meeting may be requested by DSMB members, the PO, IRB, or study PI at any time to discuss safety concerns. Meetings may be held by conference calls or videoconferences or as face-to-face meetings.

During the study, the DSMB will review cumulative study data to evaluate safety, study conduct, and scientific validity and integrity of the trial as well as to assess the performance of overall study operations and any other relevant issues, as necessary. The DSMB will conclude each review with their recommendations to NHLBI, IRB and other applicable oversight bodies as to whether the study should continue without change, be modified, or terminated.

The study PI will ensure that the DSMB is apprised of all new safety information relevant to the study product and the study, including all protocol revisions, summary safety and enrollment data and all safety reports issued by the IND sponsor.

The IND Sponsor will also submit annually an Annual Report to the FDA. The detailed written summary will be provided to the DSMB and the IRB. In addition, the DSMB Report will be submitted to the IRB at the time of continuing review annually or more often as required.

Monitoring of safety and data quality in the proposed study will be the responsibility of all personnel on the project, with primary responsibility and supervision by the Investigator. The Institutional Review Board will approve the Statement of Informed Consent for the study and provide institutional oversight of data and safety issues. The study protocol will be approved prior to recruiting or obtaining consent from any participants. Moreover, the study will be reviewed at a minimum of annual basis (or more frequently as deemed necessary) by the IRB committee. Each participant will sign the Informed Consent Form described above prior to participating in the study. To ensure participant safety, once participants are enrolled in the study, study staff will immediately report all adverse and serious adverse events to one of the Investigators. The Investigator will, per standardized procedures, report them to the IRB for their review. These events should also be communicated to the sponsor of the IND. With regard to monitoring of data quality and protected health information, all required personnel proposed for this project will have the required human subjects and confidentiality training, which includes information about maintaining data integrity and security. Confidentiality will be guarded using established procedures such as storing data in locked cabinets within locked offices or locked data rooms,

coding by study identification numbers rather than any personally identifying information to avoid revealing the identity of subjects, and aggregating data across participants. The key linking names and study identification numbers will be kept separately from the data sets with limited access by study personnel. However, Vitalant staff members and the HSCLab staff members will need to have the study ID number on documents with the subjects name to add to the level of protection when identifying the subject's blood unit and in order for the blood to be released. Only study personnel will have access to the data sets on protected servers. Oversight of all aspects of data management will occur with the Investigator. Subject's de-identified biological samples and data will be stored for future research analyses and may be shared with other researchers', their laboratories or federal repositories.
Only the REDS-III RBC-OMICS subject's de-identified biological samples and data may contain the subject's SNP and will be stored for future research analyses and may be shared with other researchers', their laboratories or federal repositories. The subject's de-identified biological samples and data may contain the subject's SNP for the biotin study team to know which SNP subgroup the subject is in. Three of the analyses that may be done at Wake Forest University, Yale University, and Vitalant.
The future analysis could include genetic analyses, including whole genome sequencing.

Data Monitoring Plan. The proposed study will use the FDA definition of adverse events (AE) and serious adverse events (SAE). Any SAE, which is unexpected and related to study intervention, will be reported immediately to the IRB and will be followed by an additional letter detailing the nature of the SAE. In the event that a participant either withdraws from the study or the investigators decide to discontinue a participant due to a SAE, the participant will be monitored by the co-PIs until (a) a resolution is reached (e.g., the problem has resolved or stabilized with no further change expected), (b) the SAE is determined to be clearly unrelated to the study intervention, or (c) the SAE results in death. Outcomes of SAEs will be regularly reported to the IRB and the sponsor. A summary of the SAEs that occurred during the previous year will be included in the annual progress report as well as in the annual IRB renewal.

6.4.3 Parameters to be monitored

The following progress will be monitored throughout the course of the research to ensure the safety of subjects as well as the integrity and confidentiality of their data.

- An evaluation of the progress of the research study, including subject recruitment and retention, and an assessment of the timeliness and quality of the data.

- A review of collected data (including adverse events, unanticipated problems, and subject withdrawals) to determine whether there is a change to the anticipated benefit-to-risk assessment of study participation and whether the study should continue as originally designed, should be changed, or should be terminated.

- An assessment of external factors or relevant information (eg. pertinent scientific literature reports or therapeutic development, results of related studies) that may have an impact on the safety and study participants or the ethics of the research study.

- A review of study procedures designed to protect the privacy of the research subjects and the confidentiality of their research data.

The severity of adverse changes in physical signs or symptoms will be classified as follows:

- Grade 1 (Mild): asymptomatic or mild symptoms; clinical or diagnostic observation only; intervention not indicated.

- Grade 2 (Moderate): minimal, local or noninvasive intervention indicated; limiting age-appropriate ADL (Activities of Daily Living).

- Grade 3 (Severe): medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self care ADL.

- Grade 4 (Life-threatening): consequences; urgent intervention indicated.

- Grade 5 (Death): event is a direct cause of death.

6.4.4 Frequency of Monitoring

The Investigator will review subject safety data as it is generated. The Investigator, sub-investigators, and the research staff will meet on an approximately monthly interval to re-evaluate study goals, subject recruitment, data coding and retention, documentation and identification of adverse events, complaints and confidentiality of subjects. There will be an evaluation of the progress of the research study, including assessments of data quality, time lines, participant recruitment, accrual, and retention. The Investigator will also review the outcome and adverse event data to determine whether there is any change to the anticipated benefit-to-risk ratio of study participation and whether the study should continue as originally designed or should it be re-evaluated and changed.

The DSMB will also be expected to meet as needed, but not less than every six months to provide an overall summary status report to the regulatory agencies.

6.4.5 Reportable Adverse Events

For this study, a serious adverse event is any untoward clinical event that is thought by either the investigator or the sponsor to be related to the study and results in any of the following outcomes: that is also:

1) Death
2) A life threatening adverse event
3) Inpatient hospitalization or prolongation of an existing hospitalization
4) A persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions
5) A congenital anomaly or birth defect
6) Important medical events that may not result in death, be life threatening, or require
7) hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the patient, or subject, and may require medical, or surgical intervention to prevent one of the serious outcomes listed above.

If clinically important and unexpected adverse experiences or clinically important study-related adverse experiences occur, they will be recorded on the adverse event case report form.

6.4.6 Adverse Events Reporting Timeline

Life-threatening or fatal unexpected adverse events associated with the use of the study drug or procedures must be reported to the IRB within 24 hours of discovery of the incident with subsequent follow-up submission of a detailed written report.

The FDA will be notified by telephone or facsimile transmission of a human adverse event that is fatal or life-threatening no later than 7 calendar days after receiving the respective human adverse event information, followed by the subsequent submission of a written IND Safety Report.

Serious and unexpected adverse events associated with the use of the study drug or procedures will be reported to the IRB with subsequent follow-up submission of a detailed written report in accordance with the respective policies and procedures of the IRB. Written IND Safety Reports will be submitted to the FDA as soon as possible and, in no event, later than 15 calendar days following the investigator-sponsor's receipt of the respective adverse event information.

A summary report of the findings will be prepared and submitted to the regulatory agencies.

The unexpected, life-threatening or fatal, suspected adverse reaction will be reported to the NHLBI within 7 calendar days. Unanticipated problems suggesting greater risk of harm to study participants than was previously known or recognized will be reported to NHLBI within 30 calendar days. Expedited SAE/UP reports to NHLBI will include the following elements:

- Study title, grant/contract number, PI name
- Description and date of the event or problem, including why it merits expedited reporting
- When available, date(s) when the event was reported to applicable governing bodies (e.g., IRB, FDA)
- Any corrective action planned or taken in response to the event or problem (e.g., study suspension, consent or protocol changes, additional training or security measures)

Communications from other applicable oversight bodies (eg: IRB, FDA, iDSMB) regarding any applicable SAE/UP will also be reported to NHLBI.

6.4.7 Reporting of Changes to Protocol and Consent

Any change in research procedures that would result in an increased risk to human subjects will be submitted to the IRB for review and approval prior to implementation of the changes in study practice. In the event that the study is suspended or terminated by the IRB or by the DSMB, or for other reasons, the NHLBI will be notified in writing including details as to the reasons for suspension or termination and any recommendations made by the IRB or DSMB. The FDA will also be notified of any changes to the protocol, suspensions or termination.

6.4.8. Withdrawal of Subjects and Stopping Criteria:

STOPPING RULES:

Withdrawal of Subjects: Subjects must be withdrawn from the study for the following reasons:

- An SAE or a serious post-infusion hemolytic event that is determined to be possibly, probably, or definitely related to the infusion of autologous BioRBC.
- Detection of anti-BioRBC antibodies following first infusion. In the unlikely event that a study subject tests positive for bioRBCs following first infusion, the subject will be withdrawn from

the study. The subject will not receive the subsequent RBC infusion. Since anti-BioRBC antibodies attack BioRBCs, the 2-4 hours post transfusion survival and mean lifespan measurements will be affected. Based on the time of discovery, the 2-4 hours time point measurement may be retained or discarded. The participant will be followed for signs and symptoms of hemolysis as well as the disappearance of the anti-biotinylated RBC antibodies.

Subjects may be withdrawn from the study for the following reasons:

- In the unlikely event that a stored unit does not pass the endotoxinemia test, red blood cells will not be transfused and will have to be withdrawn from the study.
- In the event that a donated RBC unit is misplaced, the subject's participation in the study will be terminated since an autologous transfusion will no longer be possible.
- At their own request
- At the specific request of the Investigator (eg detection of a new significant safety concern related to the study drug).
- Occurrence of intercurrent diseases which in investigator's opinion, continuation of the study drug would be harmful to the subject's well-being.
- Pertinent non-compliance with the conditions for the trial or instructions by the investigator.
- Lost to follow up

Any subject removed from the study will remain under medical supervision until the condition is medically acceptable. Study subjects removed from the study will replaced by enrolling another volunteer with similar demographic

Discontinuation of the Clinical Trial: RBC transfusion is a common clinical procedure. As such, this is a minimal risk clinical study that poses little to no risk to the study subjects, especially since the RBCs to be transfused are autologous. Other than the adverse events described above and those listed in the exclusion criteria, we do not currently anticipate any sources of issues that will discontinue the clinical trial. However, for safety reasons, we will discontinue the study if any of the subjects enrolled experience any unexpected fatal or life-threatening events that can be attributed to the biotinylated autologous RBCs; the study will be halted until data review by investigators and the iDSMB has rendered a final recommendation about study continuation.

6.5 RISK MANAGEMENT PROCEDURES

6.5.1 Protection Against Risks

General Risks of Study Protocol and Procedures

- All research interventions/activities will be conducted in private patient care areas. The collection of sensitive information about subjects is limited to the amount necessary to achieve the aims of the research, so that no unneeded sensitive information is being collected.

- All demographic and clinical information about the subject will be stored on a Case Report Form which will be completed for each subject enrolled into the clinical study. A relational database will be constructed on a local server with daily backups where only select research team members will have access to the database. The electronic data capture system being used for this clinical research study has not been fully certified as being compliant with the FDA regulations at 21 CFR Part 11 due to the limited scope of this clinical research study. Data will be entered on the server over an encrypted network connection, and stored behind

a secure firewall. The case report forms with study IDs will be housed in the same chart as personal identifier documents are while the subject is active in the protocol. Personal information documents will be stored separately once each subject's participation is complete. Only limited team members will have access to charts and database. Other data quality assurance measures will include verifying the data, out of range data checks, and repeated evaluation of the data process. All members of the research team should receive appropriate training about securing and safeguarding research data.

- Specimens will be stripped of subject identifiers and stored according to a similar coding protocol as described above. These specimens will be stored safely in the custody of the Investigator responsible for the individual assays. The Investigators will limit future access to any remaining sample to only those investigators with prior IRB approval for their studies.

- All staff involved in this study are properly credentialed and instructed in the areas of testing, confidentiality, and safety.

- The Investigator will retain the data for the entire period of this study and will retain the specified records and reports until two years after investigations under the IND have been discontinued and the FDA is notified. The Investigator may continue to use and disclose subjects' de-identified information for the purpose of this study for a minimum of seven years after final reporting or publication of the study. If the subject and/or legal representative decide to withdraw or be withdrawn from study participation, they may request that the study data and samples be destroyed. Subject names or other directly identifiable information will not appear on any reports, publications, or other disclosures of clinical study outcomes.

6.5.2 Protection Against Potential Risks of Experimental Intervention

- Involvement of trained staff / investigators with experience in the administration of the investigational product. Subjects will be closely monitored by qualified medical staff during the transfusion procedure. Hospital blood transfusion policies will be followed to assess for the unlikely possibility of transfusion reactions. To ensure that the correct blood unit is reinfused the following process will be followed: Autologous units will be requested by trained staff providing the subject's identifying information. The BioRBCs will be issued to the CTRC in a cooler with the subjects identifying information. The CTRC nursing staff will compare the identifying information on the unit blood identification tag with verbal information from the study subject and document verification.

- Blood that is drawn and sent to research lab for biotinylation will be labeled with a subject ID. This label will be verified with the subject's ID wristband before transfusion. Further, only one subject's blood will be biotinylated at a time to assure that a subject receives only his/her own autologous blood.

- Continuous monitoring by the iDSMB.

- Required Education in the Protection of Human Research Participants: The PI, sub-investigators and all involved research staff are trained in human research protection issues and have completed the Collaborative Institutional Training Initiative (CITI) online courses including education in Human Subject Research, Responsible Conduct of Research, and Good Clinical Practice. Any new research staff will be required to complete the CITI online courses before engaging in human subjects research and being added to a research protocol.

In addition, all members of the research team must continue to supplement this training by completing a refresher course.

7. COSTS AND PAYMENTS

7.1 COSTS

Research subjects or their insurance providers will not be charged for any of the procedures performed for the purpose of this research study.

7.2 PAYMENTS

The subjects will receive the remuneration upon completion of the study. In the event that a subject drops out prior to completion of the study, the subject will be reimbursed for the individual study visits that they completed per the reimbursement schedule.

8. QUALIFICATIONS AND SOURCE OF SUPPORT

8.1 QUALIFICATIONS OF THE INVESTIGATORS

IND SPONSOR AND SUB-INVESTIGATOR:

Mark Gladwin, M.D., is a Distinguished Professor of Medicine, University of Pittsburgh. Dr Gladwin is the Chair of the Department of Medicine and Director of the Vascular Medicine Institute at the University of Pittsburgh. He is an internationally recognized authority in the field of sodium nitrite including both the basic science and a broad range of clinical applications in cardiovascular disease. He is a current IND holder for the investigation of sodium nitrite in lung transplant. For approximately ten years, while at the intramural Research Division of the NHLBI, NIH and later at the University of Pittsburgh, he was PI on similar human subject's studies. As the grant PI and IND Sponsor for this clinical investigation, Dr. Gladwin will work to ensure that effective IND application is maintained with respect to this clinical investigation and will ensure proper monitoring of the investigation. He will be responsible for taking all reasonable steps to ensure the proper conduct of the clinical trial as regards ethics, clinical trial compliance, integrity, and validity of the data collection.

INVESTIGATOR**:**

Darrell Triulzi, M.D: Dr. Triulzi is a Professor of Pathology and Director, Division of Transfusion Medicine, Department of Pathology at the University of Pittsburgh. Dr. Triulzi has extensive experience in performance of clinical trials in transfusion medicine including donor and recipient safety. He served as a PI for the REDS-III network and will oversee collection, storage and distribution of blood received from study subjects participating in this clinical trial. As the Investigator for this study, Dr. Triulzi will provide daily leadership and supervision to all aspects of the clinical trial implementation

SUB-INVESTIGATORS**:**

Albert Donnenberg, Ph.D.: Dr. Donnenberg is a Professor of Infectious Disease and Microbiology in the Graduate School of Public Health, a Professor of Medicine in the School of

Medicine at the University of Pittsburgh. Dr. Donnenberg runs a GMP lab that will biotin-label the RBCs to be transfused as well as run the flow cytometric analysis of blood samples.

Ling Wang, MD, PhD.: Dr. Wang is a Research Assistant Professor in the Department of Medicine, Division of Pulmonary, Allergy, and Critical Care Medicine. Dr. Wang's research interest focuses on investigating the downstream signaling pathways regulated by nitrite and nitric oxide in cellular and animal models in order to identify new therapeutic targets and develop nitrite-based therapy in vascular and cardiopulmonary diseases. In this project, Dr. Wang will assist with donor selection and execute the in vitro assays associated with this protocol.

Janet Lee, MD: Dr. Janet Lee is Professor of Medicine in the School of Medicine and Professor of Environmental Occupational Health in the Graduate School of Public Health at the University of Pittsburgh. Dr. Lee is a board-certified pulmonary and critical care medicine specialist with over 20 years of clinical experience managing patients requiring in-hospital management. Dr. Lee's clinical focus is host defense in the ICU, transfusion consequences in the critically ill, and critical illness syndromes such as acute respiratory distress syndrome. Her research expertise is pulmonary host defense and molecular pathogenesis of acute lung injury from microbial infection. Dr. Lee is Director of the Pulmonary Translational Research Core, and Director of the Acute Lung Injury Center of Excellence in the Department of Medicine at the University of Pittsburgh. Dr. Lee will serve as co-Investigator for this study, assisting Dr. Triulzi with all aspects of clinical trial implementation.

8.2 SOURCES OF SUPPORT

National Institutes of Health

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APPENDIX A: Schedule of Procedures

	REDS-III RBC-OMICs Subjects	Biotin Infusion Subjects									
Visit Number / Study Procedures	V1^	V1	V2§	V3	V4	V5	V6	V7	V8	V9	V10
	Day 0	Day 0	Day 1	Day 5-7	Day 6-8	Day 32-41	Day 35-42	Day 36-43	Day 65-72	Day 95-102	Day 125-150
Informed Consent		x	x								
Consent for REDS-III RBC OMICs subgroup	x	x									
Inclusion/Exclusion Criteria		x	x								
Medical history & demographics	x	x	x				x				
Physical Exam		x		x			x				
Vital Signs		x	x	x			x		x	x	x
Urine Pregnancy Test*		x		x			x				
Laboratory Profiles		x	x								
Genetic Testing		x									
Hemoglobin electrophoresis#		x									
Direct Antiglobulin Test		x									
Blood Donation			x								
Blood Draw for Type & Screen		x				x					
Collect BioRBCs for Storage and Stress Hemolysis				x			x				
Pre-Transfusion Blood Draw to Detect Presence of BioRBCs				x			x				
BioRBCs Transfusion				x			x				

Post-Transfusion Blood Draw to Detect Presence of BioRBCs				x			x		x	x	x
24 hour (± 4 hours) Blood draw					x			x			
Testing for Antibodies to BioRBCs		x				x			x		x
Blood draws for red cell biophysical properties, stability in storage, and release of eDAMPs	x										
AE Assessment	x			x	x	x	x	x	x	x	x

*** Women of childbearing potential only**

 # Hemoglobin electrophoresis will be performed only on healthy African-American volunteers to confirm the sickle cell trait.

^RBC-OMICs control subjects may return to MUH-CTRC up to a maximum of 6 times in one year for additional optional blood draw visits.

§ **All procedures will be performed by professionally trained Vitalant Pittsburgh staff in accordance with bloodbank's standard blood donation policy and procedures.**