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Effect of Adenosine 2A Receptor Agonist Regadenoson on Microvascular Blood Flow in Sickle Cell Anemia

Principal Investigators:

Joshua Field, MD, MS (Medical College of Wisconsin)

Jonathan Lindner, MD (Oregon Health & Sciences University)

Co-Investigators:

Joel Linden, PhD (La Jolla Institute of Allergy & Immunology)

Roberto Machado, MD (University of Illinois – Chicago)

Michael Widlansky, MD MPH (Medical College of Wisconsin)

Jason Rubenstein, MD (Medical College of Wisconsin)

Nancy Wandersee, PhD (Medical College of Wisconsin)

Conducted by:

The Medical College of Wisconsin, Milwaukee, Wisconsin

The University of Illinois, Chicago, Illinois

Oregon Health and Sciences University, Portland, Oregon

Dana Farber Cancer Institute, Boston, MA

La Jolla Institute of Allergy and Immunology, La Jolla, CA

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Table of Contents

1. Background and Significance

Sickle cell disease (SCD) is an autosomal recessive disorder that is symptomatic in the homozygous state. One of every 400 African-American newborns in the United States has sickle cell disease [1]. The basis for sickle cell disease is a point mutation in the sixth codon of the β-globin gene. Under hypoxic conditions, the resulting hemoglobin S (ααSS) polymerizes to form an intra-erythrocyte viscous gel that induces rigid, dense and deformed erythrocytes. Within the vasculature, sickle erythrocytes expressing adhesion molecules interact with adhesion molecule receptors on endothelial cells, activating the endothelial cells[2]. The activated endothelial cells call forth further adhesion of inflammatory cells and platelets to vascular endothelium causing recurrent microvascular occlusion and leading to tissue ischemia and ultimately end-organ damage. The activated inflammatory cells (particularly iNKT cells) release cytokines that potentiate inflammation, adhesion and obstruction in the affected microvessels[3,4]. The two most common morbidities in sickle cell disease, pain episodes[5]and acute chest syndrome (ACS)[6], are secondary to this inflammatory vaso-occlusion and there are no specific therapies available to acutely treat these complications.

Acute vaso-occlusive episodes (pain and acute chest syndrome) are the leading cause of morbidity and mortality among individuals with sickle cell disease and few therapeutic options are available[7,8]. Pain episodes result from accelerated vaso-occlusion within bone marrow, joints and soft tissues. Typically characterized by bony pain in the legs, arms, chest and back, patients with HbSS will have, on average, one pain episode per year that requires hospitalization[9]. Acute chest syndrome is defined as a new infiltrate on chest radiograph and is often accompanied by respiratory symptoms, chest pain, fever and cough[10]. Laboratory evidence consistent with acute chest syndrome include increased rate of hemolysis, leukocytosis and hypoxemia[11].

Hydroxyurea is the only FDA-approved drug for the treatment of vaso-occlusion. The primary effect of hydroxyurea is to increase fetal hemoglobin production thereby disrupting hemoglobin polymerization and, ultimately, erythrocyte sickling [12]. Although such anti-sickling therapies have an important role in the *prevention* of pain and acute chest syndrome episodes, this strategy is not effective in the acute setting. Therapeutic options for pain and acute chest syndrome events are limited in the acute setting to supportive care with fluids, antibiotics, opioids and, in the case of severe acute chest syndrome episodes, blood transfusion. Although transfusion therapy is effective for the treatment of acute chest syndrome, the risk of alloimmunization in patients with sickle cell disease is 3% per unit transfused[13],

greatly limiting the availability of compatible blood in the future. Alternative strategies for the treatment of acute exacerbations of vaso-occlusion are needed. Promising approaches to decrease the severity of acute vaso-occlusive episodes include interrupting endothelial adhesion and/or decreasing inflammation[3]. Corticosteroids are the most rigorously studied anti-inflammatory agents in sickle cell disease and have been shown to decrease length of hospital stay[14,15]. However, patients treated with corticosteroids experienced rebound vaso-occlusion and 25% of study participants receiving corticosteroids required re-hospitalization[14]. This study will examine the effects of regadenoson, an adenosine$_{2A}$ receptor agonist that has demonstrated potent anti-inflammatory activity (Objective 1) and hydroxyurea (Objective 2) on microvascular blood flow using contrast-enhanced ultrasound.

Identifying an outcome measure to assess the clinical efficacy of sickle cell disease therapies has been a barrier to drug development. Prior and ongoing studies evaluating drugs aimed to decrease the severity of vaso-occlusion have used length of hospital stay [14,15], pain scores (clinical trials.gov #NCT00252122) and transfusion requirements (clinical trials.gov #NCT01089439) to assess clinical efficacy. Although interventions with a very large effect size may demonstrate benefit using these outcomes, confounding factors such as differing physician practice, inter-individual variability in pain estimates and concomitant medical problems may complicate the assessment of drug effect.

Contrast-enhanced ultrasound has the potential to significantly improve our ability to measure the efficacy of drug candidates. It is notable that in multiple previous studies, it has been possible to detect < 10% changes in skeletal muscle vascular perfusion, indicating that contrast-enhanced ultrasound can detect small changes in vascular perfusion[16-20]. Dr. Lindner's (PI) laboratory has used contrast-enhanced ultrasound to evaluate flow disturbances in the coronary, renal and skeletal muscle microcirculation related to inflammatory diseases and rheologic disorders such as ischemia-reperfusion injury[21], hyperlipidemia[22], insulin resistance[20] and endothelial dysfunction[23]. Pertinent to sickle cell disease, contrast-enhanced ultrasound is able to not only quantify regional perfusion, but is also able to separately assess tissue capillary blood volume, capillary RBC velocity, and spatial heterogeneity of capillary perfusion. Contrast-enhanced ultrasound is a portable technique that can be performed at bedside in about 5 minutes.

Two separate FDA-approved agents (Definity and Optison) are available to assess skeletal muscle in doses that are within the regulatory agency guidelines. There have been over 3 million administered doses of the microbubble contrast agents with an excellent safety profile, including in very unstable conditions such as myocardial

infarction. The reported incidence of pain associated with Definity is 1.2%[40]; although personal communications with practicing cardiologists suggest that the incidence is much lower. In our studies of patients with SCD, the incidence of pain events per CEU exam was 4.1% (4/97). Dr. Lindner (Co-PI) has performed studies in a murine model implicating complement deposition on the microbubbles as the mechanism for the pain events associated with Definity[41]. In the renal vasculature, the complement-laden microbubbles adhere to the endothelium and generate bradykinin, which alters nociception and causes pain[41]. We would like to further evaluate the mechanism of pain in this patient population. Currently, there is no literature to suggest that patients with SCD, or any other pro-inflammatory disorder, are more susceptible to pain reactions due to Definity than other groups. To determine whether complement deposition contributed to the pain events associated with Definity in patients with SCD, we will perform in vivo and flow cytometry analyses on plasma samples.

The ultimate goal in studying regadenoson is to improve vaso-occlusion and dampen the severity of vaso-occlusive episodes. Regadenoson's mechanism in sickle cell disease is to inhibit iNKT cell activation thereby decreasing inflammation that contributes to white blood cell, platelet and endothelial cell activation. Decreasing inflammation and interrupting the multi-cellular interactions of vaso-occlusion should result in increased blood flow. If blood flow is not increased following regadenoson, it is unlikely to provide clinical benefit in reducing the severity of pain or acute chest syndrome episodes. For this proposal, we will quantify regional perfusion in the skeletal muscle and myocardium in sickle cell disease subjects using contrast-enhanced ultrasound.

2. Study Objectives

2.1 Primary Objective: To determine changes in microvascular blood flow using contrast-enhanced ultrasound (CEU) in 27 adults age 18 years or older with sickle cell anemia before, during and after a 24 hour infusion of regadenoson.

2.2 Secondary Objectives:

- **To determine the association between complement deposition on lipid microbubbles and pain events associated with Definity in adults with SCD.**
- To examine differences in microvascular blood flow using contrast-enhanced ultrasound (CEU) in adults with sickle cell anemia age 18 and older at baseline compared to healthy, African American controls.

- To examine differences in microvascular blood flow using contrast-enhanced ultrasound (CEU) in adults with sickle cell anemia age 18 and older at baseline state compared to a pain crisis.
- To evaluate the activation of iNKT cells before, during and after regadenoson

3. Study Population

The study population with consist of four study groups, and one technique optimization control group:

- Regadenoson treatment group
- Sickle Cell CEU measurement group
- Healthy Control subjects group
- Technique Optimization Control subjects group
- Sickle Cell Control group

3.1 Regadenoson Eligibility Criteria

Regadenoson Study Treatment Inclusion Criteria:

1. Diagnosis of sickle cell disease confirmed by hemoglobin analysis
2. Ages 18 to 70 years
3. Laboratory parameters:
   a. Hemoglobin ≥ 6 g/dL
   b. Platelets $\geq$ 150,000/mcL
   c. ALT $\leq$ 2.5 X upper limit of normal
   d. Serum creatinine < 1.5 mg/dL
   e. INR < 1.5
   f. PTT < 40 seconds
4. Reliable IV access as determined by physician

Regadenoson Study Treatment Exclusion Criteria:

1. Subject is not at baseline (see definition of baseline **section 3.7**) at the time of study treatment
2. Current physician diagnosis of active asthma (within last 12 months) or current use of asthma medications (inhaled corticosteroids, leukotriene antagonists). Participants using bronchodilators will be allowed to participate
3. Second or third degree AV block or sinus node dysfunction
4. Known or suspected right to left sided cardiac shunts
5. History of a bleeding diathesis
6. History of clinically overt stroke within the past 3 years
7. History of severe hypertension not adequately controlled with anti-hypertensive medications (SBP ≥ 200 mmHg and/or DBP ≥ 110 mmHg)
8. Receiving chronic anti-coagulation or anti-platelet therapy
9. History of metastatic cancer
10. Receiving other investigational study agents, or have received a study agent in the last 30 days
11. Uncontrolled intercurrent illness including, but not limited to:
    a. Ongoing or active infection
    b. Symptomatic congestive heart failure
    c. Unstable angina pectoris
    d. Cardiac arrhythmia
    e. Psychiatric illness/social situations that would limit compliance with study requirements
12. Pregnancy (must be excluded by a negative urine pregnancy test in all women of childbearing potential) or breastfeeding
13. Know HIV infection
14. Participants who have previously undergone a hematopoietic stem cell transplant
15. Participants who are taking medications that may interact with the investigational agent, including dipyridamole, aminophylline, and theophylline

16. Caffeine and theophylline may not be taken within 12 hours of starting regadenoson. Dipyridamole and aminophylline may not be taken within 48 hours of starting regadenoson
17. Prior hypersensitivity reactions to either regadenoson or ultrasound contrast agents

3.2 Sickle Cell CEU Subject Eligibility Criteria:

Sickle Cell CEU Subject Inclusion Criteria:

1. Diagnosis of sickle cell disease confirmed by hemoglobin analysis
2. Males and females age 18-70 years

Sickle Cell CEU Subject Exclusion Criteria:

1. Known pregnancy
2. Known history of HIV
3. History of stem cell transplant
4. Current involvement in a therapeutic clinical trial
5. Known or suspected right to left sided cardiac shunts
6. Prior hypersensitivity reactions to ultrasound contrast agents
7. History of severe hypertension not adequately controlled with anti-hypertensive medications (SBP ≥ 200 mmHg and/or DBP ≥ 110 mmHg)
8. Uncontrolled intercurrent illness including, but not limited to:
    a. Ongoing or active infection
    b. Symptomatic congestive heart failure
    c. Unstable angina pectoris
    d. Cardiac arrhythmia

3.3 Healthy Controls Eligibility Criteria

Enrollment Inclusion Criteria for Healthy Control Subjects:

1. African American
2. Ages 18 to 70 years

Enrollment Exclusion Criteria for Healthy Controls:

1. Sickle cell disease or sickle cell trait confirmed by Sickledex
2. Known or suspected right to left sided cardiac shunts
3. Diagnosis of type 1 or type 2 diabetes mellitus
4. Hypertension defined as (SBP ≥ 200 mmHg and/or DBP ≥ 110 mmHg) or 160/90 on anti-hypertension medication
5. History or current diagnosis of dyslipidemia or taking lipid lowering drugs
6. Diagnosis of coronary artery disease or peripheral vascular disease
7. Body weight greater than 10% of ideal weight which would hinder ability to obtain ultrasound images, as assessed by study ultrasound tech
8. Uncontrolled intercurrent illness including, but not limited to:
    a. Ongoing or active infection
    b. Symptomatic congestive heart failure
    c. Unstable angina pectoris
    d. Cardiac arrhythmia
9. Pregnancy (must be excluded by a negative urine pregnancy test in all women of childbearing potential) or breastfeeding
10. Known HIV infection
11. Prior hypersensitivity reactions to ultrasound contrast agents

3.4 Technique Optimization Controls Eligibility Criteria

Enrollment Inclusion Criteria for Technique Optimization Controls:

1. Ages 18 to 70 years

Enrollment Exclusion Criteria for Technique Optimization Controls:

1. Known sickle cell disease or sickle cell trait
2. Known or suspected right to left sided cardiac shunts
3. Uncontrolled intercurrent illness including, but not limited to:
    a. Ongoing or active infection
    b. Symptomatic congestive heart failure
    c. Unstable angina pectoris

d. Cardiac arrhythmia

4. Known pregnancy or breastfeeding

Prior hypersensitivity reactions to ultrasound contrast agents

3.5 Sickle Cell Control Subject Eligibility Criteria:

Sickle Cell Control Subject Inclusion Criteria:

1. Diagnosis of sickle cell disease confirmed by hemoglobin analysis.
2. Ages 18 to 70 years

Sickle Cell Control Subject Exclusion Criteria:

3. Subject is not at baseline (see definition of baseline section 3.8) at the time of study treatment.
4. Known pregnancy
5. Known history of HIV
6. History of stem cell transplant
7. Receiving other investigational study agents, or have received a study agent in last 30 days.
8. Known or suspected right to left sided cardiac shunts
9. Prior hypersensitivity reactions to ultrasound contrast agents
10. History of severe hypertension not adequately controlled with anti-hypertensive medications (SBP ≥ 200 mmHg and/or DBP ≥ 110 mmHg)
11. Uncontrolled intercurrent illness including, but not limited to:
    a. Ongoing or active infection
    b. Symptomatic congestive heart failure
    c. Unstable angina pectoris
    d. Cardiac arrhythmia

3.6 Selection of Subjects

Subjects will be recruited in Milwaukee and Chicago. Subjects in all study treatment arms will be recruited from the Froedtert Adult Sickle Cell Disease Clinic in

Milwaukee, which currently serves more than 200 adults with sickle cell disease. Regadenoson is currently being administered to patients in Milwaukee and therefore the infrastructure is in place to study this drug. Subjects for the Healthy control group and technique optimization control group will be recruited in Milwaukee using an advertisement in the Adult Sickle Cell Disease Clinic at Froedtert Hospital, the Adult Translational Research Unit at the Medical College of Wisconsin, within the BloodCenter of Wisconsin Headquarters building, the Blood Research Institute building, and BloodCenter of Wisconsin Donor Centers, including the Milwaukee, Brown Deer, and Wauwatosa locations.

Subjects will also be recruited in Milwaukee and Chicago for the Definity complement deposition blood sample. The University of Illinois in Chicago, follows over 500 adults with sickle cell disease and may also identify healthy controls (according to criteria in **section 3.3**) to give blood samples, using a method approved by their local IRB.

3.7 Definitions

Acute Chest Syndrome (ACS): ACS is an acute illness characterized by fever and/or respiratory symptoms, accompanied by a new pulmonary infiltrate on a chest X-ray. Diagnostic criteria include, but are not limited to: a new segmental radiographic pulmonary infiltrate, in addition to <u>at least one</u> of the following: temperature ≥38.5ºC, >2% decrease in SpO_2 from a documented steady-state value on room air (FiO_2 = 0.21), PaO_2 <60 mmHg, tachypnea (per age-adjusted normal), intercostal retractions, nasal flaring, or use of accessory muscles of respiration, chest pain, cough, wheezing, or rales

Baseline: The subject is well, reporting no more than baseline pain and is at least 2 weeks from a hospitalization or emergency department visit for any reason, or 4 weeks from a hospitalization if the subject was diagnosed with Acute Chest Syndrome (ACS)

Pain Crisis (pain): An episode characterized as pain related to SCD in the extremities, back, abdomen, chest or head lasting at least 2 hours and leading to a hospitalization or emergency department visit [9]

Red Blood Cell Exchange (RBCX): An exchange blood transfusion via manual exchange (phlebotomy plus transfusion of blood products) or erythrocytapheresis

Study Physician: In this protocol, “study physician” refers to the physician/investigator who oversees protocol therapy. The study physician may or may not be the same person as the treating physician, who provides the patient’s standard care.

Treating Physician: In this protocol, "treating physician" refers to the hospital or outpatient supervising physician who provides the patient's standard care. The treating physician may or may not be the same person as the study physician, who oversees protocol therapy.

4. Study Design

4.1 Regadenoson Study Treatment (Milwaukee): To examine the effects of regadenoson on microvascular blood flow, we will administer regadenoson at 1.44 mcg/kg/hr, to 27 evaluable subjects with sickle cell disease ages 18 to 70 years, for 24 hours. Study coordinators will identify participants from rosters of the sickle cell disease clinic populations in Milwaukee. Following informed consent and after confirming eligibility, Subjects will receive infusional regadenoson when they are at baseline (see definition of baseline **section 3.7**). Prior to starting the infusion, a limited functional 2D echocardiogram and signal-average electrocardiogram will be performed. Contrast-enhanced ultrasound, correlative study lab measurements and optional "red and white blood cell" exploratory lab measurements will be obtained at baseline, approximately 6 hours after the infusion starts, and prior to stopping the infusion. A limited functional 2D echocardiogram may also be repeated prior to stopping the regadenoson infusion. Subjects will be monitored for 6 hours after the infusion is complete and will repeat the contrast-enhanced ultrasound measurement at the conclusion of the observation period. Subjects will also be asked to complete a cardiac questionnaire. The primary outcome measure will be a 40% increase in skeletal muscle microvascular blood flow when the 24 hour measurements are compared to the baseline measurements.

4.2 Sickle Cell CEU subjects (Milwaukee): To determine variability in contrast-enhanced ultrasound measurements among adults with sickle cell disease we will also obtain skeletal muscle and myocardial contrast-enhanced ultrasound values, limited functional 2D echocardiograms, a signal-average electrocardiogram, correlative laboratory study blood samples and optional "red and white blood cell" exploratory blood samples, from 30 evaluable subjects with sickle cell anemia at baseline.

Due to variability in an individual's blood flow, an additional baseline contrast-enhanced ultrasound may be performed at least 7 days later, but within 30 days, when the subject is at baseline, if feasible. The same study procedures, with exception of the signal-averaged electrocardiogram, will also be performed when the subjects are admitted to the hospital for a pain crisis (see definition in **Section 3.7**).

Of note, the initial measurement may be taken when the subject is in pain, followed by a baseline measurement, which should be obtained when the subject is experiencing their baseline pain (see definition in **Section 3.7**). Subjects will also complete a cardiac questionnaire at baseline. Of these 30 subjects, up to 2 of the individuals may have additional 2D Echo and contrast-enhanced ultrasound measurements taken prior to, and following a red blood cell exchange transfusion (see definition in **Section 3.7**), if this therapy has been recommended clinically. The contrast-enhanced ultrasound measurement should be taken within 72 hours of completing the transfusion, or as soon as reasonably possible. The subjects receiving red blood cell exchange transfusions will have additional correlative laboratory blood samples and optional "red and white blood cell" exploratory blood samples taken at the time of contrast-enhanced ultrasounds. Pain scores will be collected at the time of each study blood draw.

4.3 Healthy Control subjects (Milwaukee): To determine baseline variability in contrast-enhanced ultrasound measurements, we will also obtain skeletal muscle and myocardial contrast-enhanced ultrasound values, correlative study lab measurements and optional "red and white blood cell" exploratory lab measurements from 20 evaluable age and gender-matched African American controls without sickle cell disease or sickle cell trait at baseline, 1 day and 30 days (plus or minus seven days). Controls will be given an option to obtain additional 6 hour skeletal muscle and myocardial contrast-enhanced ultrasound on each of their visits(Day0, Day 1 and Day 30). Subjects may choose to do the optional 6 hr time point CEU at one, all or none of their visits. At baseline, a limited functional 2D echocardiogram and signal-average electrocardiogram will be performed.

4.4 Technique Optimization Control subjects (Milwaukee): To refine the contrast-enhanced ultrasound measurement technique, we will obtain skeletal muscle and myocardial contrast-enhanced ultrasound values from up to 20 healthy volunteers, study-wide. Volunteers will not have sickle cell disease or sickle cell trait. Participants may also have an additional contrast-enhanced ultrasound performed at least one day after the initial measurement.

4.5 Sickle Cell Control subjects (Milwaukee): To learn about the impact of CEU technique and definity microbubbles on the rise in microvascular blood flow rate, we will obtain skeletal muscle and myocardial contrast-enhanced ultrasound values from up to 20 sickle cell subjects. These subjects will follow the study procedure similar to regadenoson arm, however they will not receive regadenoson, hence acting as sickle cell control subjects. The 24 hr and 30 hr CEU studies will be optional for these subjects.

The subjects who have already completed Regadenoson arm or Sickle Cell CEU Arm of the study may also be enrolled in this arm. Likewise, subject may participate in the Regadenoson or Sickle Cell CEU Arm after completed participation in the 'Sickle Cell Control' Arm, if they meet study entry criteria for the respective Arm.

Blood Samples for Definity complement deposition (Milwaukee and Chicago):
To gather more valuable information about the mechanism for pain and other adverse events to contrast agents, such as Definity, we would like to collect blood samples from up to 14 subjects with sickle cell and up to 10 healthy controls, study-wide. Subjects with sickle cell and healthy controls who provide their consent will have a one-time blood sample while at their baseline state (see definition in **Section 3.7**), which will be used to evaluate complement deposition on study contrast (Definity) microbubbles. Labs for clinical complement values (C3, C4, CH50) will also be obtained. This may be a standalone blood draw or performed at another scheduled study visit. If this sample is drawn, the total volume of blood drawn for the study from any subject will not exceed 50ml per 8 week period.

Individuals who meet basic study entry criteria for any of the study arms (excluding the Technique Optimization Controls Arm), or subjects who previously participated in the study, may be approached for providing this sample. Of note, self-reported health status, (including sickle cell, pregnancy state, or other) may be allowed in evaluating eligibility for obtaining this blood sample, however should be confirmed via applicable screening procedures (Section 5), if the individual should go on to participate in other study arms.

5. Screening Procedures

*Screening evaluations are to be conducted within 30 days prior to start of protocol therapy (regadenoson, sickle cell CEU subjects, healthy controls, sickle cell controls, or technique optimization control subjects).

Historical Hemoglobin analysis and sickle dex test are acceptable.

5.1 Regadenoson Screening Procedures

- ✓ Vital signs, height and weight
- ✓ Documentation of pain score prior to lab draw
- ✓ Blood Tests:
  - Complete blood count with or without differential & platelet count
  - Comprehensive metabolic panel
  - Coagulation tests
  - Study lab draw for Definity complement deposition and values (optional)
- ✓ Urine pregnancy test (in women of childbearing potential)

- ✓ Collect demographics, race, ethnicity, medical diagnostic history
- ✓ Concomitant medications reviewed
- ✓ Determine caffeine and theophylline will not be taken 12 hours prior to receiving regadenoson (infusion can be delayed for 12 hours if the subject ingests caffeine or theophylline)
- ✓ Determine dipyridamole and aminophylline will not be taken in the 48 hours prior to regadenoson infusion
- ✓ A study physician or designee may read and assess a cardiac EKG tracing to rule out 2° or 3° heart block prior to the administration of regadenoson. A recent (≤30 days) EKG may also be used for the same purpose, if it is available.

5.2 Sickle Cell CEU Subject Screening Procedures

- ✓ Vital signs, height and weight
- ✓ Urine pregnancy test in all women of childbearing potential
- ✓ Collection of demographics, race, ethnicity, medical diagnostic history and medication history

5.3 Healthy Control Subjects Screening Procedures

- ✓ Sickledex blood test
- ✓ Collection of demographics, race, ethnicity, medical diagnostic history and medication history
- ✓ Urine pregnancy test in all women of childbearing potential

5.4 Technique Optimization Control Subjects Screening Procedures

- ✓ Collection of medical diagnostic history and medication history
- ✓ Physical exam, if subject's medical history suggests possible cardiac disorders requiring further assessment, or per study physician's discretion

5.5 Sickle Cell Control Subjects Screening Procedures

- ✓ Collection of demographics, race, ethnicity, medical diagnostic history and medication history
- ✓ Vital signs, height and weight
- ✓ Urine pregnancy test in all women of childbearing potential
- ✓ Collection of demographics, race, ethnicity, medical diagnostic history and medication history

6. On-Study Procedures

6.1 Regadenoson (Within 4 hours prior to infusion)

- Signal Averaged ECG (+/- 30 days from infusion visit date / Baseline)

- Reconfirm eligibility and that subject is at baseline (if necessary)
- Concomitant medication use reviewed prior to infusion:
  - Assess caffeine and theophylline intake for previous 12 hours (delay the onset of the infusion for at least 12 hours if the subject recently ingested caffeine or theophylline)
  - Assess dipyridamole and aminophylline intake in previous 48 hours.
- Limited functional 2D echocardiogram prior to infusion
- Contrast-enhanced ultrasound is performed prior to infusion
- Documentation of pain score prior to lab draw
- Correlative laboratory studies and optional "red and white blood cell" exploratory study labs (up to 6ml) are drawn prior to infusion
  - *If subject agrees, and gives the Definity complement sample, "red and white blood cell" samples may only be collected if at least +/-8 weeks from Definity complement sample being drawn (as not to exceed 50ml blood collected in 8 week period)
- Complete cardiac questionnaire (can be completed any time prior to the end of observation period)

Regadenoson Administration Procedure:

Prior to starting the study drug infusion, a baseline heart rate and blood pressure will be established with at least 2 measurements, 5-10 minutes apart. If the last two consecutive measurements have a difference greater than 5 beats per minute (heart rate) or 10 mm Hg (systolic blood pressure), they should be repeated every 5 minutes until they stabilize. The baseline measurement is the last heart rate and blood pressure measurement prior to study drug infusion. Oxygen saturation and heart rate will be measured during drug infusion and blood pressure will be taken every 30 minutes for 2 hours (+/-10 minutes), hourly for the next 2 hours, then every two hours (+/-15 minutes) for the duration of the infusion, and during the observation period.

Blood draws will be performed prior to infusion and at the times contrast-enhanced ultrasound is performed, for laboratory measurements. Pain will be assessed at the same time as blood draws using a standardized pain scale. Blood pressure monitoring and blood draws will be performed on a limb or site other than the regadenoson infusion.

The infusion will be stopped for any for any toxicity that is designated a serious adverse event (SAE). For this study, an SAE will be defined according to the CTEP Active Version of the NCI Common Terminology Criteria for Adverse Events (CTCAE) with the exception of heart rate and blood pressure. SAEs are CTCAE grade 3 events as well as significant changes in heart rate or blood pressure (defined in **section 11.3**). Before initiation of the regadenoson infusion, resuscitation equipment and trained staff will be available.

After the 24 hour infusion (or if the infusion is stopped early for an SAE), there will be a 6 hour observation period during which the subject will be monitored. Oxygen saturation and heart rate will be measured, blood pressure will be recorded every 2 hours (+/-15 minutes) and blood samples will be collected at the end of the observation period at 30 hours. Caffeine may not be ingested during the infusion or observation period.

Regadenoson (6 hours after infusion start +/- two hours, or earlier if necessary, per discretion of study physician)

- Contrast-enhanced ultrasound
- Documentation of pain score prior to lab draw
- Correlative laboratory studies drawn

Regadenoson (24 hours from infusion start within two hours before infusion stop, or earlier if necessary, per discretion of study physician)

- Limited functional 2D echocardiogram
- Contrast-enhanced ultrasound
- Documentation of pain score prior to lab draw
- Correlative laboratory studies and optional exploratory study labs (up to 6ml) ardrawn
- Infusion may over-run up to 1 hour, with PI permission (if difficulty completing study procedures before infusion stopped)
    - *If subject agrees, and gives the Definity complement sample, “red and white blood cell” samples may only be collected if at least +/-8 weeks from Definity complement sample being drawn (as not to exceed 50ml blood collected in 8 week period)

Regadenoson (30 hours from infusion start +/- two hours, or earlier if necessary, per discretion of study physician)

- Contrast-enhanced ultrasound
- Documentation of pain score and adverse events, if applicable
- Correlative laboratory studies drawn

Post-regadenoson follow-up period (30 days post infusion)

- Subjects will be contacted by phone weekly (plus or minus 3 days) for 4 weeks following the conclusion of regadenoson therapy to assess for any potential toxicities related to study drug

6.2 Sickle Cell CEU Subject

Sickle Cell CEU Subject (Baseline)

- Review medical diagnostic history and medication history
- Vital signs, height/weight
- Documentation of pain score prior to lab draw
- Correlative laboratory studies and optional “red and white blood cell” exploratory study labs are drawn
- Study lab draw for Definity complement deposition and values (optional)
- Signal-average ECG (plus or minus 30 days from baseline)
- Limited functional 2D echocardiogram
- Contrast-enhanced ultrasound
- Complete cardiac questionnaire

Sickle Cell CEU Subject (Second Baseline, ≤30 days if feasible)

- Review medical diagnostic history and medication history (if different)
- Vital signs,
- Documentation of pain score prior to lab draw
- Correlative laboratory studies drawn
- Contrast-enhanced ultrasound
- Limited functional 2D echocardiogram (optional)

Sickle Cell CEU Subject (Pain Crisis)

- Review medical diagnostic history and medication history
- Vital signs, height/weight
- Documentation of pain score prior to lab draw
- Correlative laboratory studies and optional “red and white blood cell” exploratory study labs are drawn
- Contrast-enhanced ultrasound
- Limited functional 2D echocardiogram (optional)

Sickle Cell CEU Subject (before red blood cell exchange) – up to 2 subjects

****Note – the ‘Before’ red blood cell exchange event may qualify as either a Baseline or Pain Crisis event, depending on patient circumstances**

- Review medical diagnostic history and medication history
- Vital signs, height/weightDocumentation of pain score prior to lab draw
- Correlative laboratory studies drawn
- Contrast-enhanced ultrasound
- Limited functional 2D echocardiogram (optional)

Sickle Cell CEU Subject (after red blood cell exchange) – up to 2 subjects

- Review medical diagnostic history and medication history
- Vital signs,
- Documentation of pain score prior to lab draw
- Correlative laboratory studies and optional “red and white blood cell” exploratory study labs are drawn

- Contrast-enhanced ultrasound within 72 hours following transfusion
- Limited functional 2D echocardiogram (optional)

6.3 Healthy Control Subject Procedures

Healthy Control Subject (Day 0)

- Review medical diagnostic history and medication history, if applicable
- Vital signs, height and weight collected
- Correlative laboratory studies and optional "red and white blood cell" exploratory study labs are drawn
- Study lab draw for Definity complement deposition and values (optional)
- Signal-average ECG (plus or minus 30 days from baseline)
- Limited functional 2D echocardiogram
- Contrast-enhanced ultrasound
- Optional Contrast-enhanced ultrasound at 6 hours

Healthy Control Subject (Day 1)

- Vital signs
- Correlative laboratory studies drawn
- Contrast-enhanced ultrasound
- Optional Contrast-enhanced ultrasound at 6 hours

Healthy **Control Subject (Day 30 plus or minus 7 days)**

- Review medical diagnostic history and medication history, if applicable
- Vital signs
- Correlative laboratory studies drawn
- Contrast-enhanced ultrasound
- Optional Contrast-enhanced ultrasound at 6 hours

For all the study arms, CBC with diff, platelet count, and comprehensive metabolic panel are standard of care if done within 30 days, and need not be repeated.

6.4 Technique Optimization Control Subject Procedures

Technique Optimization Control Subject

- Review medical diagnostic history and medication history, if applicable
- Vital signs collected
- Contrast-enhanced ultrasound

Technique Optimization Control Subject (2nd measurement, if applicable)

- Vital signs collected
- Contrast-enhanced ultrasound

6.5 Sickle Cell Control Subject

Sickle Cell Control Subject (0 hour):

- Signal Averaged ECG (+/- 30 days from infusion visit date)
- Reconfirm eligibility and that subject is at baseline (if necessary)
- Concomitant medication use reviewed
- Vital signs
- Limited functional 2D echocardiogram
- Contrast-enhanced ultrasound is performed
- Documentation of pain score prior to lab draw
- Correlative laboratory studies and optional “red and white blood cell” exploratory study labs are drawn
- Study lab draw for Definity complement deposition and values (optional)
- Complete cardiac questionnaire (can be completed any time prior to the end of the 24 hr CEU study)

Sickle Cell Control Subject (6 hours +/- 2hr from baseline):

- Vital signs
- Contrast-enhanced ultrasound
- Documentation of pain score prior to lab draw
- Correlative laboratory studies are drawn

Sickle Cell Control Subject (24 hours +/- 2hr from baseline –optional):

- Vital signs
- Contrast-enhanced ultrasound
- Documentation of pain score prior to lab draw
- Correlative laboratory studies and optional “red and white blood cell” exploratory study labs are drawn

Sickle Cell Control Subject (30 hours +/-2hr from baseline - optional):

- Vital signs
- Contrast-enhanced ultrasound
- Documentation of pain score prior to lab draw
- Correlative laboratory studies are drawn

Table 1. Study Schedule of Procedures for Regadenoson Subjects

Procedure	Screening	Baseline	6 hours (+/-2hr)	24 hours	30 hours (+/-2hr)	30 Day Follow up
Documentation- MedHx, medications, Tx & clinical data	X					
Signal-average ECG	X[f]					
PT/INR, PTT	X					
β-HCG[c]	X					
CBC	X					
Comprehensive metabolic panel[b]	X[b]					
VS (including SBP, HR, O_2 sat)	X	X[a]				
Regadenoson infusion		X[J]				
Adverse event evaluation	X					
Limited functional 2D Echo		X		X		
Contrast-Enhanced Ultrasound		X	X	X	X	
iNKT cell measurement		X	X	X[d]	X	
Inflammatory Markers		X		X		
Exploratory "red and white blood cell" study labs[g]		X[h,i]		X[h,i]		
Definity complement deposition blood sample (optional)	X[i, k]					
Complement (C3, C4, CH50) (drawn if optional Definity complement sample drawn)	X[i, k]					
Pain Scores[e]	X	X	X	X	X	
Cardiac Questionnaire		X				
Coordinator calls subject <u>weekly</u> to assess for toxicities after regadenoson infusion						

<u>a:</u> BP, O_2 saturation, and HR will be taken prior to infusion and at the following times after the start of the infusion: every 30 min (+/- 10 min) for 2 hrs,. Then hourly for 2 hours, then every 2 hours for the rest of the infusion (+/-15 min). After the infusion ends, there will be a 6 hr observation period, during which BP, HR and O_2 saturation will be taken every 2 hrs (+/- 15 min). <u>b:</u> Albumin, alkaline phosphatase, total bilirubin, serum creatinine, BUN, calcium, chloride, glucose, potassium, total protein, SGOT [AST], SGPT [ALT], sodium

c: Urine pregnancy test (women of childbearing potential)
d: The 24 hour blood draw should occur prior to stopping the drug.
e: Pain scores will be taken just prior to each blood draw using a visual analog scale
f: Signal-averaged ECG may be performed within 30 days of the infusion visit
g: Optional lab testing for blood flow adhesion and leukocyte activation
h: up to 6ml blood may be collected at each timepoint (optionally)
i: if subject agrees to Definity complement sample, optional "Red and white blood cell" samples may only be collected if it is at least +/-8 weeks of Definity complement sample (as not to exceed 50ml in 8 week period)
J; Regadenoson infusion may over-run for up to 1 hour, with PI permission
k: during study visit or standalone blood draw

Table 2. Study Schedule of Procedures for Sickle Cell CEU Subjects

Procedure	Screening	Baseline	Baseline #2	Pain Crisis	Pre-RBCX	RBCX Transfusion[a]
Documentation- medical hx, medications, transfusions & clinical data	X	X	X	X	X	X
Vital Signs ,Height and Weight	X	X	X	X[c]	X [c]	X [c]
β-HCG[e]	X					
CBC w/diff, plt. Count, reticulocyte [c]		X[g]		X[g]	X	X[g]
Comprehensive metabolic panel [b, c]		X[g]		X[g]	X	X[g]
Adverse event evaluation	X					
Signal-average ECG		X				
Limited functional 2D Echo		X	X[i]	X[i]	X[i]	X[i]
Contrast-enhanced ultrasound		X	X	X	X	X[h]
Cardiac questionnaire		X				
iNKT cell measurement		X	X	X	X	X
Inflammatory Markers		X	X	X	X	X
Exploratory "red and white blood cell" study labs[j]	X			X		
Definity complement deposition blood sample (optional)	X[k]					
Complement (C3, C4, CH50) (drawn if optional Definity complement sample drawn)	X[k]					
Pain Scores[f]		X	X	X	X	X

a: Red blood cell exchange transfusion
b: Albumin, alkaline phosphatase, total bilirubin, serum creatinine, BUN, calcium, chloride, glucose, potassium, total protein, SGOT [AST], SGPT [ALT], sodium
c: Standard of care measurement or intervention
e: Urine pregnancy test (women of childbearing potential)
f: Pain scores will be taken just prior to each blood draw using a visual analog scale
g: Labs (CBC, metabolic panel) are considered standard of care if they have not been measured within 30 days
h: Contrast-enhanced ultrasound to be performed within 72 hours after red blood cell exchange transfusion
i: Limited Functional 2D Echocardiogram is optional
j: Optional lab testing for blood flow adhesion and leukocyte activation
k: during study visit or standalone blood draw

Table 3. Study Schedule of Procedures for Healthy Control Subjects

Procedure	Screening	Day 0	Day 1	Day 30
Documentation- Med Hx, demographics, medications & data	X	X		X
Sickledex blood test	X			
Vital signs, height, and weight		X	X	X
β-HCG [a]	X			
CBC w/diff, plt. Count, reticulocyte		X		
Comprehensive metabolic panel [b,]		X		
Reticulocyte count		X		
Signal-average ECG		X		
Limited functional 2D Echo		X		
Contrast-enhanced ultrasound (CEU)		X	X	X
Optional 6 hr time point CEU		X	X	X
iNKT cell measurement		X	X	X
Inflammatory Markers		X	X	
Exploratory "red and white blood cell" study labs[c]		X		
Definity complement deposition blood sample (optional)	X[d]			
Complement (C3, C4, CH50) (drawn if optional Definity complement sample drawn)	X[d]			
Adverse event evaluation		X		

a: Urine pregnancy test (women of childbearing potential)
b: Albumin, alkaline phosphatase, total bilirubin, serum creatinine, BUN, calcium, chloride, glucose, potassium, total protein, SGOT [AST], SGPT [ALT], sodium
c: Optional lab testing for blood flow adhesion and leukocyte activation
d: during study visit or standalone blood draw

Table 4. Study Schedule of Procedures for Technique Optimization Control Subjects

Procedure	Screening	Initial Measurement	Second Measurement (if applicable)
Documentation- medical history & concomitant medications	X		
Physical Exam (if necessary)	X		
Vital signs		X	X
Contrast-enhanced ultrasound		X	X
Adverse event evaluation		X	X

Table 5. Study Schedule of Procedures for Sickle Cell Control Subjects

Procedure	Screening	0 hours	6 hours (+/- 2hr)	24 hours (+/- 2hr, optional)	30 hours (+/- 2hr, optional)
Documentation- medical history, medications, transfusions & clinical data	X				
Vital signs, height and weight	X				
Signal-average ECG	X				
β-HCG[a]	X				
CBC w/diff, plt. Count, reticulocyte[b]		X			
Comprehensive metabolic panel[b]		X			
VS (including SBP, HR, O_2 sat)		X			
Adverse event evaluation	X				
Limited functional 2D Echo		X			
Contrast-Enhanced Ultrasound		X	X	X	X
iNKT cell measurement		X	X	X	X
Inflammatory Markers		X		X	
Exploratory "red and white blood cell" study labs[c]		X[e]		X[e]	

Definity complement deposition sample (optional)	**X[f]**				
Complement (C3, C4, CH50) (drawn if optional Definity complement sample drawn)	**X[f]**				
Pain Scores[d]		**X**	**X**	**X**	**X**
Cardiac Questionnaire		**X**			

a: Urine pregnancy test (women of childbearing potential)
b: Standard of care measurement or intervention if done with 30 days, or per physician discretion
c: Optional lab testing for blood flow adhesion and leukocyte activation
d: pain score taken just prior to blood draw
e: If optional Definity blood sample is also collected, up to 5ml blood may be collected at each timepoint (optionally)
f: during study visit or standalone blood draw

7. Investigational agent

7.1 Regadenoson

Regadenoson is a selective adenosine$_{2A}$ receptor agonist. Activation of adenosine$_{2A}$ receptors inhibits inflammation by directly targeting the iNKT cell. We suspect that iNKT cells have an important role in sickle cell disease vaso-occlusion and that inhibiting iNKT cells through activation of A_{2A}Rs may decrease inflammation and improve vaso-occlusion.

Regadenoson has an FDA indication for radionuclide myocardial perfusion imaging in patients unable to undergo adequate exercise stress. Regadenoson has the same FDA-approved indication as the injectable formulations of adenosine and dipyridamole[24].

Adenosine and dipyridamole are nonselective adenosine receptor agonists, targeting adenosine A_1, A_{2B}, and A_3 receptors, as well as the A_{2A}R[25,26]. Regadenoson has low affinity for the A_{2A} receptor, resulting in increased “off rates” from that receptor and a short duration of effect.

Maximal plasma concentrations are achieved within 1 to 4 minutes after regadenoson injection. In a 3-compartment model, the initial half-life was 2 to 4 minutes, the intermediate half-life was 30 minutes, and the terminal half-life was 2 hours[24,26]. Body mass index, body weight, age, and height had no influence on regadenoson pharmacokinetics[24,27,28]. Clearance is not dose-dependent; therefore, weight-adjusted dosing is not necessary. Renal excretion accounts for approximately 58% of the total elimination[29]. Average plasma renal clearance exceeds glomerular

filtration rate, suggesting renal tubular secretion plays a role in regadenoson elimination[24].

Dosage adjustments are not necessary in patients with renal function impairment[24]. In healthy volunteers, the regadenoson plasma concentration-time profile is multi-exponential in nature and best characterized by 3-compartment model. The maximal plasma concentration of regadenoson is achieved within 1 to 4 minutes after injection of regadenoson and parallels the onset of the pharmacodynamic response. The half-life of this initial phase is approximately 2 to 4 minutes. An intermediate phase follows, with a half-life on average of 30 minutes coinciding with loss of the pharmacodynamic effect. The terminal phase consists of a decline in plasma concentration with a half-life of approximately 2 hours[24,26].

A population pharmacokinetic analysis including data from subjects and patients demonstrated that regadenoson clearance decreases in parallel with a reduction in creatinine clearance and clearance increases with increased body weight. Age, gender, and race have minimal effects on the pharmacokinetics of regadenoson[26,27,29].

Metabolism

The metabolism of regadenoson is unknown in humans. Incubation with rat, dog, and human liver microsomes as well as human hepatocytes produced no detectable metabolites of regadenoson[24].

Excretion

In healthy volunteers, 57% of the regadenoson dose is excreted unchanged in the urine (range 19–77%), with an average plasma renal clearance around 450 mL/min, i.e., in excess of the glomerular filtration rate. This indicates that renal tubular secretion plays a role in regadenoson elimination[24].

For a list of toxicities associated with regadenoson, please refer to **section 8.1**.

Our group is currently conducting a Phase I Clinical Trial of regadenoson in Children and Adults with sickle cell disease. The results from this safety and dose-seeking trial show that an infusional dose of 1.44 mcg/kg/hr is safe in adults with sickle cell disease (greater than 18 years of age) at baseline state (i.e., not in pain crisis) when given for 24 hours. For this study, we will use the same dose and similar monitoring parameters that were used in the phase I trial to ensure patient safety.

7.2 Administration

Doses will be calculated based on the subject's weight at screening.

Based on the ongoing phase I dosing and safety study (*Safety of Adenosine 2A agonist Lexiscan in Children and Adults with Sickle Cell Disease*, MCW IRB# PRO13470, we will deliver a dose of 1.44 mcg/kg/hr of regadenoson.

Full details of administration activities can be found in **section 6.1**.

7.3 Packaging and Preparation

Regadenoson is available in a prefilled syringe. Each syringe contains 0.4 mg in 5 ml solution. For continuous infusions, a syringe pump or portable pump will be used. Regadenoson should be administered in a peripheral vein with a catheter or needle that is 22 gauge or larger. Regadenoson (Lexiscan) is manufactured by Astellas Pharma, US. Astellas Pharma US, Inc. Deerfield, IL 60015.

7.4 Storage and Stability

Store at controlled room temperature, 25°C (77°F); excursions permitted to 15° to 30°C (59°–86°F).

7.5 Compatibility

No formal pharmacokinetic drug interaction studies have been conducted with Lexiscan.

Effects of Other Drugs on Regadenoson

- Methylxanthines (e.g., caffeine and theophylline) are non-specific adenosine receptor antagonists and may interfere with the vasodilation activity of regadenoson. Patients should avoid consumption of any products containing methylxanthines as well as any drugs containing theophylline for at least 12 hours before regadenoson administration. Aminophylline may be used to attenuate severe or persistent adverse reactions to regadenoson.

- In clinical studies, regadenoson was administered to patients taking other cardioactive drugs (i.e., β-blockers, calcium channel blockers, ACE inhibitors,

nitrates, cardiac glycosides, and angiotensin receptor blockers) without reported adverse reactions or apparent effects on efficacy.

- Dipyridamole may change the effects of regadenoson. When possible, withhold dipyridamole for at least two days prior to regadenoson administration.

Effect of Regadenoson on Other Drugs

Regadenoson is not known to inhibit the metabolism of substrates for CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, or CYP3A4 in human liver microsomes, indicating that it is unlikely to alter the pharmacokinetics of drugs metabolized by these cytochrome P450 enzymes.

7.7 Handling

No special precautions for handling are required.

7.8 Availability

The regadenoson used in this study must be considered an investigational new drug and the IND is pending. Regadenoson will be purchased with study funds from the manufacturer by the study at no charge to the study subject.

7.10 Ordering

Regadenoson will be purchased with study funds from the manufacturer at no charge to the study subject. The supplier of regadenoson is manufactured by Astellas Pharma, US. Astellas Pharma US, Inc. Deerfield, IL 60015.

7.11 Drug Accountability

Drug accountability logs will be maintained for all study drugs. At a minimum, these logs must contain quantity of drug received and dispensed, date received and dispensed, subject number, expiration date, dose, quantity returned, and initials of individual dispensing the study drug. Used medication vials can be discarded according to institutional standards.

7.12 Destruction and Return

At the end of the study, unused supplies of regadenoson should be destroyed according to institutional policies. Destruction will be documented in the Drug Accountability Record Form.

7.13 Discontinuation Criteria

Subjects may be removed from the study, or treatment stopped by the Investigator for any of the following reasons:

1. Subject experiences an unacceptable adverse event that warrants withdrawal from the study, as judged by the investigator.

2. Occurrence of any CTCAE grade 3 toxicity with the exception of heart rate and systolic blood pressure.

3. The below criteria will be used to determine whether a subject is experiencing a drug-related toxicity and treatment should be stopped:

   A baseline heart rate and blood pressure will be established with at least 2 measurements, 5-10 minutes apart. If the last two consecutive measurements have a difference greater than 5 beats per minute (heart rate) or 10 mm Hg (systolic blood pressure), they should be repeated every 5 minutes until they stabilize. The baseline measurement is the last heart rate and blood pressure measurement prior to study drug infusion.

   An increase or decrease of > 30 beats per minute from baseline will be immediately rechecked along with blood pressure. Participants who are either sleeping or relaxing in bed will be awakened or encouraged to move their limbs prior to the repeat measurement. If the heart rate is confirmed > 30 beats per minute above or below baseline, it will be rechecked again 15 minutes later along with blood pressure. Heart rate differences that remain > 30 beats per minute from baseline after 15 minutes and are accompanied by 1 of the following will be considered a toxicity and will be criteria for stopping:

   1) A sustained decrease in blood pressure of > 30 mmHg from baseline,
   2) A sustained systolic blood pressure < 75 mmHg, or
   3) Signs of hypoperfusion (confusion, cool extremities, mottled skin).

   Additionally, a heart rate < 40 beats per minute will be rechecked immediately and participants who are either sleeping or relaxing in bed will be awakened or encouraged to move their limbs prior to the recheck. If the rechecked heart rate is confirmed < 40 beats per minute, it will be checked 15 minutes later. Sustained values < 40 beats per minute will be considered criteria for stopping. Physicians will be notified when heart rate values are: greater than 30 beats per minute above or below baseline or < 40 beats per minute (prior to the 15 minute recheck).

   A decrease in systolic blood pressure of > 30 mmHg from baseline will be immediately rechecked. Participants who are either sleeping or relaxing in bed

will be awakened or encouraged to move their limbs prior to the repeat measurement. If systolic blood pressure is confirmed > 30 mmHg below baseline, it will be rechecked again 15 minutes later. Systolic blood pressure decreases that remain > 30 mmHg after 15 minutes will be considered a toxicity and criteria for stopping. Additionally, a systolic blood pressure < 75 mmHg will be rechecked immediately and participants who are either sleeping or relaxing in bed will be awakened or encouraged to move their limbs prior the recheck. If the rechecked systolic blood pressure is confirmed < 75 mmHg, it will be checked 15 minutes later. Sustained values < 75 mmHg will be considered a toxicity and will be criteria for stopping. Physicians will be notified when the systolic blood pressure is verified as: a decrease of > 30mmHg from baseline or < 75 mmHg. The study drug may be stopped at any time per the discretion of the physician and these events would be criteria for stopping.

If the infusion is stopped for a toxicity, participants will be observed off therapy for an additional 6 hours.

Management of the above adverse events is outlined in **section 8.1**.

4. Participant decides to withdraw from the study
5. General or specific changes in the participant's condition render the participant unacceptable for further treatment in the opinion of the treating investigator

8. Toxicities

8.1 Regadenoson

Common
Dyspnea, headache, flushing, chest discomfort, dizziness, angina pectoris, chest pain, and nausea

Less Common
Abdominal discomfort, dysguesia, feeling hot, bronchoconstriction (wheezing), and hypotension and tachycardia (as defined by criteria for toxicity in Section 7.13.6)

Rare
Regadenoson also has the potential to cause sinus bradycardia or first, second or third degree heart block, heart abnormalities (including atrial fibrillation/atrial flutter), seizure or stroke

Rarely, hypersensitivity episodes have also been reported.

Mild episodes are defined as events that include hives or urticaria, but are not characterized by respiratory compromise, angioedema or reduced blood pressure. See below for hypersensitivity management.

Severe episodes are defined as events that include either respiratory compromise (brochospasm, hypoxemia, stridor, dyspnea), anaphylaxis, angioedema, or reduced blood pressure. See below for hypersensitivity management.

Regadenoson Toxicity Management:
Most toxicities associated with regadenoson are self-limiting and resolve within 15 minutes of discontinuing the drug. If an increase or decrease in heart rate or decrease in systolic blood pressure is recorded, according to protocol guidelines, the drug will be discontinued. Within minutes these parameters should return to normal. If a more significant decrease in blood pressure occurs, aminophylline is an antidote to regadenoson. The dose of aminophylline is 50 to 250 mg given as a slow IV injection (e.g., 50-100 mg given over 30-60 seconds).

For **mild hypersensitivity episodes**, consider the administration of:

- Methylprednisolone 1 mg/kg IV
- Diphenhydramine 50 mg IV (1 mg/kg in children)

For **severe episodes**, consider the administration of:

- Epinephrine 1 mg per ml (1:1000) IM 0.3 to 0.5 mg every 5-15 minutes (children 0.01 mg/kg up to 0.5 mg dose every 5-15 minutes)
- Methylprednisolone 1 mg/kg IV
- Diphenhydramine 50 mg IV (children 1 mg/kg)
- Ranitidine 50 mg IV or PO (children 1 mg/kg) for mild episodes

8.2 Optison (perflutren lipid microsphere) CEU contrast agent

Rare
headache, nausea and/or vomiting, flushing, dizziness

Very Rare
Pain (which may be severe)**Hypersensitivity episodes have been reported.**

Mild episodes are defined as events that include hives or urticaria, but are not characterized by respiratory compromise, angioedema or reduced blood pressure.

Severe episodes are defined as events that include either respiratory compromise (brochospasm, hypoxemia, stridor, dyspnea), anaphylaxis, angioedema, or reduced blood pressure.

Optison Toxicity Management:

The subject should be advised to report any sudden onset of symptoms and report it to the ultrasound tech. The contrast agent should be stopped immediately if hypersensitivity or other serious AE develops.
For possible hypersensitivity reactions, please stop administration of the contrast agent and use the suggestions provided previously in **section 8.1**.

9. Correlative studies

9.1. CEU methodology

Low-mechanical index power modulation imaging (IE33, Philips Ultrasound) will be performed at a transmission frequency of 1.7 MHz with a phased-array transducer at a mechanical index of 0.15.[20,30]. We will image the deep forearm flexor muscles (short-axis), the myocardium (3-apical views) and kidney (optional) after establishing a continuous infusion of lipid-shelled octafluoropropane microbubbles (Optison, GE Healthcare), which will be prepared in the following way: 1 vial (0.5ml) diluted into 15-30ml with sterile saline, and infused at a rate of approximately 90-120ml/hr. Adjustment may be necessary to achieve desired imaging effect, but will not exceed manufacturer's dosing recommendation. This dose may be repeated during the CEU if necessary, not to exceed manufacturer's packaging recommendations. Images of the forearm may also be acquired 2 minutes after initiating hand-grip exercise (12 Hz at 50% maximal tension) to assess flow reserve. Images will be acquired for 15 seconds after a destructive (MI 1.0) 5 frame pulse sequence. Time-intensity data fit to the function: $y=A(1-e^{\square t})$ where *y* is intensity at time *t*, *A* is the plateau intensity, and the rate constant is the microvascular flux rate reflecting RBC flux rate [31,32]. There may be a 30 minute break during the course of the contrast-enhanced ultrasound procedure. Data analysis will take place in the laboratory of Jonathan Lindner, MD (Co-PI) in Portland, OR. Microvascular blood volume (MBV) will be quantified by $(A)/(1.06\times I_{LV}\times F)$ where 1.06 is tissue density (g/cm^3), and *F* is the scaling factor for a different infusion rate for I_{LV} (0.3 ml/min) which will be reduced to avoid dynamic range saturation Quantitative perfusion (ml/min/g) will be calculated by the product of MBV and. Spatial distribution of microvascular flow in skeletal muscle will be determined by using fractal analysis in the spatial domain on parametric images as previously described.

9.2 iNKT cell measurements

iNKT cell measurements will be performed in the lab of J. Linden (Co-I) in La Jolla, CA. 4 ml whole blood will be collected per time point into vacutainer EDTA tubes and stored at 4ºC until shipping in cold containers. A rapid express system that we have

developed will permit all samples to reach Dr. Linden's laboratory in La Jolla, CA, in 24 hours.

Two methods will be used to identify iNKT cells:

1) CD1d tetramers, loaded with the α-GalCer-like glycolipid PBS57 (defined as tetramer^{+}CD3^{+})

2) 6B11 antibodies (defined as CD45^{+}6B11 high)

Concordance between cells identified by CD1d tetramers and 6B11 antibodies is >90%. Further, only cells staining CD45^{+}6B11 high will be called iNKT cells, recognizing that our sensitivity for detecting iNKT cells will be lower, but we will have fewer false positives. FACS analysis and counting beads are then used to determine the absolute numbers of iNKT cells and the percent of iNKT cells among all lymphocytes (live, CD45^{+} low side scatter cells).

9.3 Limited Functional 2D Echocardiogram

All patients will undergo limited functional rest 2D echocardiography to assess for patent foramen ovale (PFO), atrial septal defect (ASD), right-to-left cardiac shunts, and aortic arch diameter. Right ventricular (RV) and left ventricular (LV) and valvular function will also be assessed. The following views will be used for assessment: parasternal long axis (PLAX), parasternal short axis (PSAX), subcostal, and suprasternal. Color flow Doppler will be used across all valves to assess regurgitation. The LV ejection fraction will be calculated according to the "method of discs". Normal LV function will be defined as an LVEF > 55%. Left atrial area and volume and right atrial volume will be measured at end systole using the apical 4-chamber view, as well as apical 2-chamber, and apical 3-chamber views. RV areas at end-diastole and end-systole will be determined in the apical 4-chamber view, as well as apical 2-chamber, and apical 3-chamber views. The tricuspid regurgitant jet velocity will be recorded as the highest value obtained with a good quality Doppler envelope from either modified apical 4-chamber or parasternal views. Tissue Doppler imaging (TDI) will also be performed to obtain the MV annulus in the medial/lateral view, and the TV annulus in the lateral view. Data analysis will take place in the laboratory of Jonathan Lindner, MD (Co-PI) in Portland, OR.

9.4 Signal-average Electrocardiogram

A signal-averaged electrocardiogram (SAECG) is a more detailed type of 12-lead ECG, during which multiple tracings are obtained over a period of approximately 10-

20 minutes. The subject will be asked to lie as still as possible for the duration of the procedure, while the ECG leads are placed, and the cardiac rhythms are recorded. Three techniques will be used to analyze the signals, including temporal signal averaging, spatial signal averaging, and spectral analysis. Temporal signal averaging, averages a number of QRS complexes over time [34]. Beats are detected by a computer algorithm, based on voltage thresholds or other criteria, then aligned by a constant feature of the signal, the "fiducial" [35]. Once beats have been aligned, their arithmetic mean is taken. This process diminishes random noise that is not synchronized to the QRS complex. ECG spatial averaging involves the summation and averaging of electrical potentials simultaneously recorded from multiple pairs of closely spaced electrodes [36]. This process allows a real-time, beat-by-beat analysis of individual QRS complexes, but requires electrical shielding of the patient and equipment. The advantage of this technique over temporal signal averaging is that it allows assessment of irregular rhythms with changing conduction. Spectral analysis considers the QRS complex (or P-wave) to be composed of multiple simple waveforms, typically sinusoids. Spectral analysis thus decomposes the QRS complex or P-wave into these constituent signals, represented by component frequencies and corresponding phase and amplitudes [37] [38]. Spectral decomposition will be performed using Fourier analysis [39].

9.5 Exploratory study of inflammatory markers

Since inflammation is known to promote vascular occlusion of sickle erythrocytes, the hallmark of a sickle cell pain event, measuring markers of inflammation at baseline and during a vaso-occlusive crisis may provide insight into the mechanism of inflammation, as it relates to sickle cell vaso-occlusion. Additionally, measuring inflammatory markers before and after administration of regadenoson may provide further evidence about the effects of regadenoson on sickle cell vaso-occlusion. Based on mouse studies we expect that an effective concentration of regadenoson will decrease plasma levels of IFN-gamma, certain chemokines and various other proinflammatory mediators.

Samples will be collected from participants in Milwaukee, in the Regadenoson Arm, Sickle Cell CEU Arm, Healthy Control Arm, and Sickle Cell Control Arm at baseline, and 24 hours after baseline, and/or the start of regadenoson infusion, for measurement of markers of inflammation. Samples will be collected in 4 mL vacutainer tubes with EDTA. They will be spun for plasma, transferred to anonymized plastic cryovials and stored at -80ºC or below until ready for analysis. The samples will be destroyed after the analysis is completed.

9.6 **Exploratory studies of "red and white blood cell" blood flow adhesion and leukocyte activation (optional)**

Samples will be collected optionally from participants in Milwaukee in all study arms (with exception of the Technique Optimization Controls Arm). Up to 10ml whole blood will be collected into anticoagulated vacutainer tubes, spun for serum and plasma separation, and the supernatant transferred to anonymized cryovials and stored at -80ºC or below until ready for analysis. The pellets (non-plasma or serum fraction) of the vacutainer tubes may be used to isolate erythrocyte and leukocytes (see description below), for further examination in this study. Any remaining samples will be destroyed after the study analysis is completed.

A flow adhesion assay of isolated blood cells (erythrocytes or leukocytes) will be performed under conditions of controlled flow, to determine red cell or leukocyte rolling and adhesion. For this assay, we will count how many blood cells become adherent or roll along the surface of the flow chamber. Furthermore, we may examine red cell or leukocyte physiology or structural characteristics, such as red cell stiffness of membrane or flow cytometry studies. Sample and data analyses will take place at the lab of Cheryl Hillery, MD, at the Blood Research Institute in Milwaukee, WI.

9.7 **Exploratory Study of complement deposition on Definity microbubbles in vivo (optional)**

Complement deposition on the surface of lipid microbubbles will be compared between samples collected in subjects with SCD who had pain events associated with Definity (n=4), patients SCD who did not have a pain event (n=10) and healthy African American controls (n=10). We will collect up to 4ml whole blood into anticoagulated tubes, and spin for plasma, transfer to anonymized cryovials, and store at -80C until ready for analysis. Samples will be analyzed at the laboratory of Jonathan Lindner, MD in Portland, OR.

In Dr. Lindner's laboratory in Portland, plasma will be incubated with lipid microbubbles and then flow cytometry will be performed to measure surface complement deposition on the microbubbles. Approximately 4ml blood will also be collected for isolation of serum and analyzed for levels of circulating complement (C3, C4, CH50) at a clinical laboratory.

10. Subject Remuneration

10.1 Regadenoson Study Subjects

For their time and efforts in study activities, regadenoson study subjects will receive $200.00 for the completion of the:

- Infusion Visit and related study activities
- Complete 4 weekly telephone follow-up calls for assessment of AE's

When on-study procedures are partially completed, participants will receive $25 remuneration.

10.2 Sickle Cell CEU Subjects

For their time and efforts in study activities, sickle cell CEU subjects will receive $35.00 for the completion of each study visit (baseline, baseline #2, admission for pain crisis, and/or pre- and post- RBCX).

10.3 Healthy Control Study Subjects

For their time and efforts in study activities, healthy control study subjects will receive $100.00 for the completion of the study activities and $35 for any additional 6 hr time point CEU (optional).
When testing is partially completed, participants will receive $25 remuneration.

10.4 Technique Optimization Control Study Subjects

For their time and efforts in study activities, technique optimization controls will receive $25 for each contrast-enhanced ultrasound measurement visit.

10.5 Sickle Cell Control Study Subjects

For their time and efforts in study activities, sickle cell control subjects will receive $35.00 for the completion of each CEU (0hr, 6 hr, 24hr and 30 hr).

Definity Complement Blood Sample

Subjects who provide consent and agree to give the optional Definity Complement blood sample will receive $25 for their time and this additional blood sample.

Participants in Chicago may be offered a larger compensation amount, per the discretion of the local Principal Investigator. The amount will be based on the locally established rate of compensation for similar studies and/or procedures and must be approved by the local (UIC) IRB.

11. Adverse events

Adverse event collection and reporting is a routine part of every clinical study. All adverse events will use the descriptions and grading scales found in the CTEP Active Version of the NCI Common Terminology Criteria for Adverse Events (CTCAE).

11.1 Adverse Event Monitoring

11.1.1 Study Staff

Information on all adverse events, whether reported by the subject, directly observed, or detected by physical examination, laboratory test or other means, will be collected, recorded, followed, and reported as described in the following sections.

The PI will monitor and review adverse events monthly or more often as needed.

11.2 Definitions

Adverse Event (AE) **–** Any untoward or unfavorable medical occurrence in a human subject, including any abnormal sign (for example, abnormal physical exam or laboratory finding), symptom, or disease, temporally associated with the subject's participation in research, whether or not considered related to the subject's participation in research.

Serious Adverse Event (SAE) – Any adverse event temporally associated with the subject's participation in research that meets any of the following criteria:

- Results in death;
- Is life-threatening (places subject at immediate risk of death from the event as it occurs);
- Requires inpatient hospitalization or prolongation of existing hospitalization, within 48 hours of a CEU or other research-related activity (*see below);
- Results in a persistent significant/incapacity;
- Results in a congenital anomaly/birth defect; or
- Any other adverse event that, based upon appropriate medical judgment, may jeopardize the subject's health and may require medical or surgical intervention to prevent on the other outcomes listed in this definition

Note that seriousness and severity are separate concepts. The term "severe" refers to the intensity of a specific event; a severe event may be of minor medical significance (e.g., a severe leg cramp). The term "serious" is based on an outcome or action criteria that are usually associated with events that pose a threat to the patient's life or functioning. An event that is mild in severity is serious if it leads to one of the outcomes defined above.

Grade 4 and 5 events will always be considered Serious Adverse Events. Many Grade 3 and some Grade 1 and 2 events may meet the definition of a Serious Adverse Event.

* Individuals with sickle cell disease are not uncommonly hospitalized for sickle cell-related ailments, such as pain exacerbations or others, which are part of the disease process. It is important to note that any adverse event should be assessed with regard to activities that are related to research.

- **SAE's which originate from a study-related intervention, such as CEU, are most likely to occur immediately and the PI should acknowledge all hospitalizations, perceived prolongation of hospitalizations or ED visits, which occur within 48 hours of a study intervention being performed.**

Unexpected Adverse Event- Any adverse event occurring in one or more subjects in a research protocol, the nature, the severity, or frequency of which is not consistent with either:

- The known of foreseeable risk of adverse events associated with the procedures involved in the research that are described in;
  - the protocol related documents or the current IRB-approved informed consent document and;
  - other relevant sources of information, such as a product labeling or package insert or;
- The expected natural progression of any underlying disease, disorder, or condition of the subject(s) experiencing the adverse event and the subject's predisposing risk factor profile for the adverse event.

Unanticipated problem involving risks to subjects or others (UP) - Any incident, experience or outcome that meets all the following criteria:

- Unexpected (in terms of nature, severity, or frequency) given:
  - The research procedures that are described in the protocol related-documents, such the IRB-approved research protocol and the informed consent form document and,
  - The characteristics of the subject population being studied;
- Related or possibly related to the subject participation in research; and
- Suggests that the research places subjects or others at a greater risk of harm (including physical, psychological, economic, or social harm) related to the research than was previously known or recognized.

Grading of Adverse Events – Events will be graded by using the Common Terminology Criteria for Adverse Events (CTCAE) Criteria version 4.03.

Attribution – the determination and documentation of whether an adverse event is related to a medical procedure.

Attribution Categories:

1. Not Related –Event clearly related to other factors (e.g., clinical state, other therapies; concomitant drugs)
2. Possibly Related – Sequence of events is compatible with study drug, device, or procedure, but could have been produced by other factors
3. Probably Related - Sequence of event is compatible with study drug, or procedure and cannot be explained by other factors without much doubt
4. Definitely Related - Sequence of event is compatible with study drug, or procedure and beyond doubt cannot be explained by other factors

A list of the adverse events and potential risks associated with the agents administered in this study can be found in **sections 8.1-8.3**, as well as the Informed Consent Form.

11.3 Timeline for Reporting of Adverse events, Serious Adverse Events, Unexpected Adverse Events and Unanticipated Problems

All serious adverse events, all unexpected adverse events, and all unanticipated problems that occur after the initial dose of study treatment, during treatment, or during the defined follow-up period will be reported to the overall PI within 24 hours of learning of the event's occurrence by phone, email, or SAE form regardless of attribution to the study drug. A follow-up SAE report should be submitted within 24-48 hours following initial notification.

11.4 Recording Adverse Events

Participating investigators will assess the occurrence of AEs and SAEs at all participant evaluation time points during the study. All AEs and SAEs whether reported by the participant, discovered during questioning, directly observed, or detected by physical examination, laboratory test or other means, will be recorded in the participant's study and/or medical record and on the appropriate study-specific case report forms (CRFs).

11.5 Reporting Adverse Events to Institutional Review Boards

For multi-site studies where a MCW/FH investigator is serving as the principal investigator, each participating investigator is required to abide by the reporting requirements set by the MCW/FH Institutional Review Board (IRB). The study must be conducted in compliance with FDA regulations, local safety reporting requirements, and reporting requirements of the principal investigator. Each investigative site will be responsible to report SAEs that occur at that institution to their respective IRB, according to local policy. It is the responsibility of each

participating investigator to report serious adverse events to the Sponsor/ Investigator and/or others as described in Section 11.6 below.

A copy of the submitted institutional SAE form should be forwarded to:
Dr. Joshua Field: Joshua.field@bcw.edu
Study Coordinator: zachary.williams@bcw.edu

The MCW/FH Principal Investigator will submit SAE reports from outside institutions to the MCW/FH IRB according to policies and procedures in reporting adverse events.

11.6 Reporting Events to the Sponsor/Investigator

11.6.1 Serious Adverse Event Reporting Requirements

All serious adverse events that occur after the initial dose of study treatment, during treatment, or during the defined follow-up period must be reported to the MCW/FH Overall Principal Investigator on the local institutional SAE form. This includes SAEs described by the criteria outlined in **section 11.2**, as well as the following:

- Grade 2 (moderate) and Grade 3 (severe) events that are unexpected and at least possibly related/associated with the intervention.
- All Grade 4 (life-threatening or disabling) events that are unexpected or not specifically listed in the protocol as not requiring reporting.
- All Grade 5 (fatal) events while the participant is enrolled and actively participating in the trial **OR** when the event occurs during the defined follow-up period of the last study intervention.

Participating investigators must report each serious adverse event to the Overall Principal Investigator within 24 hours of learning of the occurrence. In the event that the participating investigator does not become aware of the serious adverse event immediately (e.g., participant sought treatment elsewhere), the participating investigator is to report the event within 24 hours after learning of it and document the time of his or her first awareness of the adverse event. Report serious adverse events by email or telephone to:

Dr. Joshua Field
Joshua.field@bcw.edu
414-937-3848

Email is preferred; however, in urgent situations after normal business hours, the Principal Investigator can be reached at 414-318-8597

Within 24-48 hours, the participating investigator must provide follow-up information on the serious adverse event, utilizing the appropriate SAE Reporting Form. Follow-up information should describe whether the event has resolved or continues, if and

how the event was treated, and whether the participant will continue or discontinue study participation.

Follow-up reports should be emailed to Dr. Field at the email address above and to the study coordinator at zachary.williams@bcw.edu

11.6.2 Non-Serious Adverse Event Reporting Requirements
Non-serious adverse events will be reported to the Principal Investigator on the toxicity Case Report Forms.

11.6 Food and Drug Administration (FDA) Notification by Sponsor/ Investigator for Investigational Agent

The Sponsor-Investigator will report to the FDA any adverse event that is serious, unexpected and reasonably related (i.e., possible, probable, definite) to the investigational study treatment.

Unexpected fatal or life-threatening experiences associated with the use of the investigational study treatment will be reported to FDA as soon as possible but in no event later than 7 calendar days after initial receipt of the information.

All other serious unexpected experiences associated with the use of the investigational study treatment will be reported to FDA as soon as possible but in no event later than 15 calendar days after initial receipt of the information.

Events will be reported to the FDA by telephone (1-800-FDA-1088) or by fax (1-800-FDA-0178) using Form FDA 3500A (Mandatory Reporting Form for investigational agents). Forms are available at http://www.fda.gov/medwatch/getforms.htm.

12. Data and Safety Monitoring

12.1 Data Management and Collection

Research information, including consent forms and will be maintained secure as described in the Confidentiality **section 13.3**.

Data will be collected onto study-specific case report forms. The data gathered will be entered into an electronic data set maintained by the investigator. Each participant will have a unique identifier, or study ID, assigned. This study ID will be used to label all shipped biological specimens. All data and samples will be coded with their respective unique identifier. Only the study coordinator and investigator will have access to the study ID key. The study coordinator will also retain all source documentation for each subject, which will be stored in locked cabinets, inside a locked secured building.

12.2 Data Safety Monitoring Board (DSMB)

The overall study principal investigator will appoint at least three physicians and/or scientists with research experience as members of the DSMB group. They will monitor accruing data in order to confirm that the patients in the trial are being cared for safely, and make recommendations as appropriate. The DSMB will be responsible for:

1) Reviewing and analyzing the progress of the study;
2) Reviewing recruitment and event rates.
3) Approving amendments to the trial protocol, if warranted;
4) Monitoring the risk/benefit ratio, safety and response of study treatments and diagnostic procedures;
5) Ensuring complete and responsible reporting and data quality;
6) Approving amendments to the trial protocol, if warranted;
7) Consult with the site monitor physician as needed
8) Provide a summary of their findings in a written report

The DSMB will meet at least annually by phone, or in person, or more often if warranted to review serious adverse events.

Local Data Safety Monitoring Plan -The local principal investigator and/or members of the research team will monitor the study for regulatory compliance, inclusion/exclusion criteria, accrual/withdrawal rates, and breech of confidentially annually prior to submission to the IRB. Any adverse events will be reviewed immediately with the Principal Investigator and will be reported to the local respective IRB per site IRB regulations. All study events will be summarized once a year for the IRB, during annual continuing review progress report.

The local IRB will determine when it is necessary to inform participants of any new findings that reveal additional risk or information that may alter their willingness to participate in the trial.

13. Human Subjects Protection

13.1 Informed Consent

Subjects are required to sign an informed consent prior to screening, and before undergoing any study procedures or assessments, in accordance with ICH E6; 4.8, "Informed Consent of Trial Subjects." When substantial modifications are made to the informed consent, the IRB may require that all subjects currently enrolled in the study will be re-consented; ICH E6; 4.8 guidelines would still apply.

Subjects will be provided with a copy of the signed informed consent form used in the study, procedures, and assessments. Subjects will also be provided with the contact telephone numbers of the investigator and qualified personnel who can assist with their questions and concerns.

13.2 Protected Health Information

Protected health information that will be collected as part of this study includes name, date of birth, dates for hospitalization, and medical, diagnostic history and medications (as mentioned in **Sections 5 and 6**). This information will be stored for 3 years beyond the completion of the study. After blood is used for study testing it will be discarded.

13.3 Confidentiality

Loss of confidentiality is always a potential risk when collecting protected health information. Research Records will remain confidential. Volunteer subject's records will be available to the study staff and to each site's IRB and for audit purposes. Only authorized personnel will have access to the protected health information and research records. In order that confidentiality can be maintained, the principal investigators will keep records in locked cabinets/room and results of tests will be coded to prevent association with volunteers' names. All electronic data will be entered into a secured data set that is password protected. Identifying information will not be included on laboratory samples, faxes, or correspondence. Laboratory samples will be shipped with only unique study numbers that will be linked to a key kept by the site Principal Investigator.

All study team members are trained in HIPAA privacy regulations and other applicable site privacy policies. No information will be released, nor will participation

in the research be acknowledged, to any party except where compulsory according to law or intuitional policy. The results of the research study may be published, but volunteers' names or identities will not be revealed.

13.4 Follow-up and Record Retention

The current proposal involves on-going data collection for the duration of the study at each site. The records will be maintained per site policy but at a minimum of two years per FDA requirements.

14. Statistical Considerations

14.1 Description of Endpoints:

14.1.1 Primary Endpoint: Primary outcome measure will be a 40% increase in skeletal muscle microvascular blood flow when 24 hour measurements are compared to baseline in subjects receiving regadenoson.

14.1.2 Secondary Endpoints:

- Secondary outcome measure will be that there will be more complement deposited on lipid microbubbles from the plasma from the subjects with SCD who suffered pain events compared to the other groups.
- Secondary outcome measure will be a 40% increase in skeletal muscle microvascular blood flow when 12 week measurements are compared to baseline in subjects receiving hydroxyurea.
- Decrease in iNKT cell activation as measured by NFkB when 24 hour measurements are compared to baseline in subjects receiving regadenoson.

14.2 Statistical Analysis

Primary outcome measure: change in skeletal muscle microvascular blood flow during regadenoson infusion, obtained at baseline and at end of infusion (24 hours); Secondary outcome measures: myocardial microvascular blood flow, functional microvascular density, reduction in iNKT cell activity (as measured by phospho-NF-kB expression).

We anticipate that at baseline sickle cell disease subjects will have skeletal muscle blood flow similar to that of subjects with diabetes who are hyperemic, for whom data suggests a mean blood flow of 0.7 (standard deviation 0.3) [30]. We seek to detect a 30% increase from baseline in skeletal muscle microvascular blood flow, to a mean of 1.0, following the administration of regadenoson (Aim 1). With 27 patients enrolled on the 24 hour regadenoson study of Aim 1, we will have 90% power to

detect this difference, testing at the one-sided 0.05 significance level. For these calculations we assume that the unknown correlation of measurements within patient is positive, and that therefore the maximum value for the variance of the difference will be two times the variance of a single observation, or 0.18 (standard deviation 0.42). Secondary outcome measures will be summarized and differences assessed for regadenoson therapy, and tested with the one-sample t-test if symmetry holds and the Wilcoxon signed rank test if it does not. Serial measures of iNKT cell number and activation state and markers of inflammation will be obtained during therapy. We will construct longitudinal mixed models of these outcomes, controlling for demographic factors.

Since we do not know the magnitude of complement deposition on the lipid microbubbles in this setting, power calculations will use the Wilcoxon rank sum test, assessing number of standard deviations of difference. We will have 87% power to detect a 1.5 standard deviation difference and 80% power to detect a 1 standard deviation difference in complement deposition between the subjects who did and did not have reactions, testing at the 0.10 one-sided significance level.

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