Cardiovascular Complications of Sickle Cell Disease

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1. Background:

Sickle-cell disease (SCD) is a group of inherited blood disorders characterized by red blood cells that assume an abnormal, rigid, sickle shape. Sickle cells contain abnormal hemoglobin, an iron-rich protein that carries oxygen for distribution to the body, which causes the sickle shape. Sickling decreases the cells' flexibility, predisposes the cells to lysis, and results in their restricted movement through blood vessels, depriving downstream tissues of oxygen. SCD patients are often perturbed by periodic painful attacks and a risk of systemic complications due to these vaso-occlusive episodes and hemolysis, the clinical hallmarks of SCD [1]. The polymerization of deoxy-hemoglobin, changes in red cell membrane structure and function, disordered cell volume control, and increased adherence to vascular endothelium all play an important role in the pathophysiologic events responsible for vaso-occlusive complications [2]. The most frequently occurring form of SCD is the homozygous form called sickle cell anemia (Hemoglobin SS, HbSS), followed by HbSC disease and sickle hemoglobin-beta thalassemia. In the United States, 6 to 10 percent of African-American newborns have the heterozygous form called sickle cell trait [3]. Life expectancy is substantially shortened, with most studies reporting an average of up to 50 years for age [4]. Important cardiovascular complications of SCD include pulmonary hypertension and left ventricular systolic and diastolic dysfunction.

Pulmonary Hypertension: Acute and chronic pulmonary complications including pulmonary hypertension (PH) occur frequently in patients with SCD and represent the most common cause of death during adulthood in these patients[5]. PH, a complex pulmonary vascular syndrome, is defined as an increase in blood pressure (>25mm Hg at rest and >30mm Hg with activity) in the pulmonary artery, pulmonary vein, or pulmonary capillaries. PH can lead to a significant increase in morbidity and mortality. Severe PH can manifest as either an acute or chronic presentation. Acute PH can induce a sudden increase in right ventricle (RV) afterload, with increased end-diastolic volume, and reduced RV ejection fraction. Chronic PH, on the other hand, leads to progressive RV systolic pressure overload and dilatation, resulting in gradual RV dysfunction, failure, and death. PH develops in most forms of hereditary and chronic hemolytic anemia, including SCD, thalassemia, hereditary spherocytosis, and paroxysmal nocturnal hemoglobinuria suggesting that there is a clinical syndrome of hemolysis-associated pulmonary hypertension [6-8]. Retrospective studies from tertiary care referral centers suggest a prevalence of PH ranging from 30 to 40 percent [2, 9] in patients with SCD. Postmortem studies of patients with SCD often

show evidence of pulmonary vascular bed obliteration, smooth muscle hypertrophy, and parenchymal fibrosis [5].

Survival is decreased among both children and adults whose SCD is complicated by PH [10]. In one study, the median survival was 26 months for patients with PH and SCD, whereas 70 percent of patients without PH were still alive at the end of the nearly 10 year study [11]. Although these studies have demonstrated that patients with SCD have lower pulmonary pressures (but still elevated) and higher cardiac output than patients with primary pulmonary hypertension (PPH), the two-year mortality rates approach 50 percent in both groups. Even modestly elevated pulmonary artery pressures portend a poor prognosis [2, 11].

Etiology of PH- Most studies have looked at PH collectively in SCD and have not determined the prevalence of the different subtypes of PH. Thus, it is unknown whether patients who have SCD and PH have intrinsic pulmonary vascular disease (group 1 pulmonary arterial hypertension, PAH, as defined by the recent World Health Organization (WHO) classification), left heart disease (group 2 PH) from both systolic or diastolic failure, or parenchymal lung disease (group 3 PH), or pulmonary thromboembolic disease (group 4 PH). There is also significant controversy over the contribution of left ventricular dysfunction to increased pulmonary pressures in patients with SCD. Invasive hemodynamic measurements have shown a mixed picture of high pulmonary artery pressures and elevated pulmonary capillary wedge pressures, suggesting that LV diastolic or systolic dysfunction may contribute to the high pulmonary artery systolic pressures and increased risk of death [10, 11]. Diastolic dysfunction in SCD patients may be a consequence of relative systemic hypertension or direct myocardial damage from either microvascular vaso-occlusive disease or iron overload [12]. Pegelow et al. [13] have shown that the normal range of blood pressure is lower in healthy SCD patients compared with the general population. Multiple studies have shown that those SCD patients with blood pressure values higher than expected for their population, “relative systemic hypertension,” had an increased risk of stroke and death. There is also a growing body of results associating relative systemic hypertension with stroke, diastolic dysfunction, and increased mortality in SCD patients. Other potential mechanisms for diastolic dysfunction in patients with SCD include 1) small myocardial infarctions and microvascular dysfunction caused by poor myocardial perfusion related to red blood cell sickling and 2) iron overload which is secondary to the frequent blood transfusions many of these patients require. Dr. Machado, a co-investigator in this proposal, has previously shown that diastolic dysfunction is an independent risk factor for mortality in SCD patients [12]. Therefore, a major goal of our study is to better characterize LV structure and function in the SCD population and to relate these measures with PH and cardiovascular mortality.

As PH is a complex, multifactorial, polygenic disorder, the genetic basis for PH associated with SCD. Recently, mutations in the bone morphogenetic protein receptor type 2 gene (BMPR2) have been identified in ~50% of cases of familial Primary PH [14]. We, therefore, hypothesize that both genetic polymorphisms and environmental factors play a pivotal role in the pathological sequence that leads to PH in SCD patients. The complexity of the disease also lends itself to the use of microarray technology, which allows the efficient and accurate simultaneous expression measurement of thousands of genes. This technology has been most successfully employed in the investigation of cancer, including the classification of histologically-indistinct tumor types with different natural histories [15]. In this proposal we will utilize mid- and high throughput array technologies to detect genome–wide gene expression and targeted genetic polymorphism detection to enhance our understanding of the genetic basis of the cardiopulmonary and vascular disease which afflicts SCD patients.

Diagnosis — Because of the nonspecific clinical manifestations (or absence of manifestations many times) of PH in SCD, noninvasive screening for PH employing two-dimensional (2-D) and Doppler trans-thoracic echocardiography (TTE) is still the first-line for detection. Many TTE-based studies have attempted to better define the subphenotypes of the left and right ventricle including diastolic dysfunction, systolic dysfunction with wall motion abnormalities, as well as the pulmonary artery characteristics of SCD patients. Most of these studies have revealed a possible multi-factorial contribution to the phenotype in SCD patients. Other noninvasive measures that may help identify PH patients include plasma levels of brain natriuretic peptide (BNP). The Multicenter Study of Hydroxyurea in Sickle Cell Anemia study [9] found that BNP >160 pg/mL had a sensitivity, specificity, and positive predictive value of 57, 91, and 78

percent, respectively, in detecting PH, as defined by TTE criteria. BNP levels correlated directly with mortality (RR 2.87, 95% CI 1.2-6.6) and indirectly with hemoglobin levels. There are also a number of other plasma markers that are under investigation including arginine levels and glomerular filtration rate [16]. This proposal aims to screen the blood for an array of established and novel plasma markers from SCD patients to advantage future studies designed to characterize and detect association with the PH phenotype.

Cardiac magnetic resonance (CMR) has also gained increasing clinical application in cardiopulmonary diseases. Due to its 3-dimensional nature, CMR is considered the gold-standard for quantifying left and right ventricular systolic function and size. Additionally, its high tissue contrast allows for a detailed characterization of myocardial tissue. Specifically, the use of techniques such as late gadolinium enhancement can be used to detect the presence of tiny amounts of myocardial scar [17]. Other techniques, such as T2*, have been shown to correlate strongly with myocardial iron content [18]. Just as importantly, CMR perfusion imaging can accurately quantify myocardial blood flow and can provide tremendous insight into the function of the microcirculation [19, 20]. CMR's high spatial and temporal resolution, its 3-dimensional approach, its ability to characterize the tissue, and its ability to evaluate the micro- and macro-circulation make it a comprehensive technique for the evaluation of heart disease. Recently, one CMR study has already shown the presence of cardiac microvascular disease in a subset of adult SCD patients in the absence of infarcted myocardium, myocardial iron overload, or coronary artery disease, increasing the evidence for the contribution of left heart disease to PH development in these patients [21]; unfortunately, strong conclusions could not be made because the study was underpowered. Thus, this proposal will leverage the advantages offered by CMR to better characterize and detect the PH and cardiopulmonary subphenotypes in the SCD patient population.

2. Purpose of the Study:

Aim 1: Using CMR, TTE, and a plasma screen (BNP, hemoglobin, complete blood count, complete metabolic profile, bilirubin, lactate dehydrogenase [22], ferritin, von Willebrand factor (vWF), plasma vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), P- and E-selectin, nitric oxide (NO), erythropoietin, vascular endothelial growth factor (VEGF), pre-B-cell colony enhancing factor (PBEF), macrophage migration inhibitory factor (MIF), sphingosine-1 phosphate (S1P) metabolites levels, and arginine), we plan to comprehensively and quantitatively characterize the cardiopulmonary complications of SCD and gain an improved understanding of the pathophysiology of PH and diastolic dysfunction in patients with SCD.

Aim 2: To utilize mid- and high throughput array technologies to detect genome–wide gene expression (microarray and microRNAs) and targeted genetic polymorphisms (selected candidate genes) in SCD patients linked to a quantitative noninvasive-based PH phenotype as defined by Aim 1 in SCD patients. These studies will enhance our understanding of the genetic basis of the cardiopulmonary and vascular disease which afflicts SCD patients.

3. Methods:

I. Selection of patients

Patients with SCD will be recruited from the sickle cell clinic at the University of Chicago Medical Center. 172 patients will be needed to adequately power the study. Based on prior experience 15% of patients will have inadequate perfusion MRI data quality; therefore, approximately 200 patients will be enrolled into the study. As most of these patients are already enrolled in TRIDOM (Translational Research Initiative in the Department of Medicine), plasma screens for the markers listed earlier will be conducted using the blood already available from this initiative and hence new blood draws will not be needed. For the patients who are not enrolled in TRIDOM yet, we will inform of them of their opportunity to participate in the program (but not require them to participate) while also recruiting them for this study. In addition, 20 control subjects (age, race, and sex distributions similar to those of the SCD patients) will be used to determine baseline or normal values for myocardial blood flow, LV size and function, RV size and function, and myocardial iron content (i.e. T2* values). These normal ranges will be specific for the techniques and equipment used at The University of Chicago Medical Center. Healthy control subjects will not be eligible if they have a history of coronary artery disease or renal insufficiency based on direct

questioning. For all subjects who do not have a blood sample available from TRIDOM, we will collect a blood sample from them as part of this study (about 20 ml or 4 teaspoons).

a) Inclusion criteria
-Patients must be 18+.
-Patients who were diagnosed with SCD confirmed by high-pressure liquid chromatography or hemoglobin electrophoresis will be eligible for the study.
-Only outpatients in stable condition will be included.
-Patients receiving transfusions will not be excluded.

b) *Exclusion criteria*
-Patients with a history of vaso-occlusive crises or an episode of acute chest syndrome within the previous four weeks will be evaluated at a later time.
-Patients with high degree heart block; active, hemodynamically significant, ventricular arrhythmias; unstable coronary syndromes; history of myocardial infarction within 1 month of the study.
-Contraindications to gadolinium-enhanced magnetic resonance examination:
    -severe claustrophobia,
    -Pacemaker, defibrillators, cerebral aneurysm clips, or neurostimulator.
    -GFR<30 mL/min/m^2
-Pregnancy
-Patients with sinus node dysfunction

II. *Data collection*
A prospective study will be performed. Patients will be provided a schedule for their CMR and TTE. Each subject will be assessed and the patient chart will be reviewed by one of the investigators to document the presence of any cardiovascular signs and symptoms as well as a history of cardiovascular events, and all subjects will undergo a routine 12-lead surface electrocardiography.

a) A comprehensive ultrasound study will be performed on all enrolled patients to evaluate the cardiovascular system in patients with SCD. Cardiac measurements will be performed according to the guidelines of the American Society of Echocardiography [23, 24]. Diastolic function will be assessed in all patients using standard Doppler and tissue Doppler measurements of the mitral inflow, pulmonary veins, and mitral annulus. Diastolic dysfunction will be graded as normal, mild, moderate, or severe. Pulmonary hypertension will be quantified using standard Doppler measurements through the tricuspid and pulmonic valves. Both 2D and 3D echocardiography will be used to quantify left and right ventricular systolic function. Color Doppler will be used to identify and quantify the presence of significant valvular disease. Additionally, during their echocardiography session, all patients will undergo an evaluation of their aortic compliance using radial tonometry. This additional examination will take approximately 5 extra minutes. Briefly, a blood pressure measurement will be obtained and then a high fidelity pulse wave contour will be acquired by placing a pencil-like probe that is a pressure sensor over the radial artery. The waveform will provide important information with regards to arterial stiffness and how it may relate to the diastolic dysfunction seen in many SCD patients. Clinically relevant results from the echocardiogram will be dictated and reported to the patient's physician.

b) CMR will be performed on all patients. Patients will be asked to not eat or drink for 6 hours prior to the CMR study. Additionally, beta-blockers, rate-lowering calcium channel blockers, and nitrates will be held on the morning of the study. Upon arrival to the MRI unit, an 18-guage IV will be placed in the upper extremity. EKG leads will be attached to the patient's chest wall and a flexible surface coil will be placed over the thorax. The blood pressure and heart rate will be monitored throughout the study. Steady state free precession imaging will be performed to quantify left and right ventricular size and function and T2* images will be acquired to determine whether myocardial iron overload is present. In order to assess microcirculatory function, first-pass perfusion CMR using Gd-DTPA will be performed under resting and hyperemic conditions. The subject will receive a 0.4mg bolus of intravenous regadenoson to induce hyperemia. Immediately afterwards, 0.01 mmol/ kg of Gd-DTPA will be infused intravenously at a rate of

0.5 ml/ sec followed by a 0.1mmol/ kg infusion of Gd-DTPA at a rate of 5ml/sec. Images will be acquired as the Gd-DTPA transits the left ventricle using a hybrid GRE-EPI pulse sequence [25]. The same perfusion protocol will be repeated under resting conditions. Using time intensity curves extracted from the acquired images, the absolute resting myocardial blood flow, hyperemic myocardial blood flow, and perfusion reserve will be calculated for each of the 16 American Heart Association myocardial segments using a Fermi function deconvolution [26]. A perfusion reserve <1.85 will be considered abnormal. After the perfusion imaging is completed, late gadolinium enhancement using an inversion recovery technique will be used to detect the presence of myocardial scar. The presence or absence of late gadolinium enhancement will be determined for each of the 17 American Heart Association myocardial segments. The presence of any late gadolinum enhancement will be considered abnormal. Clinically relevant results from the cardiac MRI study will be dictated and reported to the patient's physician.

Whole genomic SNP survey- This proposal will utilize the new Affymetrix® Genome-Wide Human SNP Array 6.0 with 1.8 million genetic markers (including more than 906,600 single nucleotide polymorphisms (SNPs) and 946,000 probes for the detection of copy number variation). Given the large numbers of probes, the SNP Array 6.0 will enable the design of this association study to take advantage of larger sample sizes in the proposal, thereby significantly increasing the overall genetic power. Briefly, total genomic DNA (500 ng), obtained from the TRIDOM initiative, will be utilized for the amplification and technical protocol as recommended by the Affymetrix manufacturer. PCR conditions have already been optimized to preferentially amplify fragments. Affymetrix Genome-Wide Human SNP Array 6.0 contains validated and qualified reagents for the most critical steps in the assay. This includes the PCR primer and adaptors, reagents to fragment and label the PCR products and several control reagents. Details of this kit and protocol are all available from the Affymetrix website (http://www.affymetrix.com/products/arrays/specific/genome_wide_snp6/genome_wide_snp_6.affx). The measurements and analyses for all of the SNP, gene, and microRNA studies will be performed at the Functional Genomics facility here at the University of Chicago Medical Center (details of the protocols for DNA, RNA, and microRNA isolation and measurement protocols as well as quality control are described at http://fgf.uchicago.edu/).

Gene and microRNA Expression Profiling- This proposal will utilize the GeneChip® Human Gene 1.0 ST Array by Affymetrix (details at the website) got gene expression profiling, covering 28,869 genes represented on the array by approximately 26 probes spread across the full length of an individual gene. The Human Gene 1.0 ST Array has greater than 99 percent coverage of sequences present in the RefSeq database. To determine microRNA differences, this proposal plans to use the mercury LNA microRNA array offered by Exiqon. The array provides access for examination of over 1300 human microRNAswith as little as 30ng total RNA (obtained from the TRIDOM initiative). Details of this technology are also provided at http://www.exiqon.com.

Soluble protein levels of select biomarkers will be assessed via commercially available enzyme-linked immunoassay kits for VCAM-1, ICAM-1, E-selectin (Invitrogen Corporation), NOx (Cayman Chemical), P-selectin, erythropoietin and VEGF (R&D Systems) according to manufacturer's instructions (blood obtained from the TRIDOM initiative).

e) *Statistical Consideration*
The study will be powered to detect a 10% difference in myocardial blood flow in sickle cell patients with and without pulmonary hypertension. Because myocardial blood flow has the highest variability of all the measurements to be collected in this study, the study will be adequately powered to detect differences in all the other measurements that will be made. Based on my previously published work involving MRI measurements of myocardial blood flow, in order to detect an approximately 10% difference in perfusion reserve between groups, 54 subjects will be needed in each group (alpha 5%, beta 50%). Approximately 40% of sickle cell patients have pulmonary hypertension. In order to recruit enough sickle cell patients with pulmonary hypertension to adequately power the study, approximately 150 subjects in total with sickle cell disease will need to be recruited. Differences in the various left and right ventricular function and size measurements derived from the sickle cell disease population will be compared to age and gender matched controls using t-tests. The sickle cell disease population will be divided into two groups: those with pulmonary hypertension (as determined by Doppler echocardiography) and those without

pulmonary hypertension. Differences in left ventricular and right ventricular systolic function, diastolic function, myocardial perfusion reserve, myocardial scar burden, and myocardial iron content that exist between the two groups will be determined by t-tests. Linear regression analysis will be used to compare the severity of pulmonary hypertension with each of the above mentioned continuous variables. The sickle cell disease population will also be divided into another 2 groups: those with diastolic dysfunction and those without diastolic dysfunction. Once again, the above listed parameters will be compared using t-tests. Patients with diastolic dysfunction will also be classified according to a well established grade of severity, and the relationship between the various continuous variables and the severity of diastolic dysfunction will be investigated through analysis of variance (ANOVA). A p-value <0.05 will be considered statistically significant.

f) *Data Safety*
Included patients will be issued a unique identifying number that will be linked to the collected data. No personal identifiers such as name, medical record number or address will be stored with this data. A separate list linking the patients' unique identifying number and their medical record number will be stored on a different computer. All electronic data will be protected using secure, password protected computer files. Physical charts will be kept in a cabinet under lock and key. This system will ensure that patient information is protected and accessible only to the principal investigators and co-investigators. Data will only be used for the research study.

4. Special precautions to be taken by the researchers *(including dose modifications and whether subjects will be asked to take a pregnancy test before and, as applicable, during the study).*

Any female patient that is enrolled in the study will be tested for pregnancy using a urine test.

5. Exact location where research is to be conducted:

The following locations will be utilized for the proposed study:
-University of Chicago Medical Center Outpatient Clinics
-Dr. Nicole Artz' Sickle Cell Clinic
-University of Chicago Medical Center Echocardiography laboratory
-University of Chicago Medical Center MRI facility
-Outpatient lab collection

6. Duration of protocol:

The duration of the protocol will depend on when all patients that are expected to enroll complete the proposed testing. This will include all of the patients seen in the clinic by Dr. Artz that are eligible for the study. Therefore, the time to enroll such patients will be a gradual and open process given her clinic is scheduled on a per weekly basis for SCD patients. We expect the protocol to be completed within 2 years.

7. Potential risks and benefits to subjects:

The risks to the proposed study are the following:
A. Patient discomfort and time- Patients will undergo a single blood draw which can be transiently painful/uncomfortable. The TTE can also take typically 10-15 minutes per exam. The MRI exam will be estimated to take ~ 45-60 minutes per exam, where patients will have to lie flat and steady for the duration which can lead to discomfort. Additional risks of MRI include intolerance to the noise created by the MRI machine and also feeling uncomfortable due to being in an enclosed space. While there are no known serious hazards (assuming the patient does not have any contra-indications to MRI), MRI is not proven to be safe during pregnancy and hence, we have excluded them from this study.

B. Gadolinium safety- The FDA declared Gadolinium safe for use in MRI in 1988. A few side effects, such as mild headache, nausea and local burning can occur. Very rarely (less than one in a thousand), patients are allergic to Gadolinium. The use of gadolinium has been associated with nephrogenic

systemic fibrosis (NSF) in patients with advanced renal insufficiency. Hence, any patients that with advanced renal insufficiency (GFR<30 mL/min) will be excluded from this study.

C. Regadenoson- Some individuals experience facial flushing, lightheadedness, diaphoresis, or nausea after administration of regadenoson. These symptoms are transitory. Only FDA approved dosing will be used. The subjects blood pressure and heart rate will be monitored throughout the study. If the patient has significant symptoms or changes in hemodynamics, the Regadenoson effect will be reversed by using aminophylline.

8. Description of how the subject's primary physician will be notified of and, as appropriate, involved in the proposed research:

A clinical report of all the tests performed on the subject will be provided to the patient's primary care physician.

9. Description of anticipated coordination between appropriate interdepartmental faculty, and where necessary, inclusion of those faculty as participants:

Given that this proposal requires an interdepartmental level of involvement, we will have monthly meetings with all co-investigators to address the status of the project, any areas of improvement, and the results to that point.

10. References:

Appendixes – if applicable:

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