CLINICAL PROTOCOL

PROTOCOL TITLE: Arginine Supplementation in Sickle Cell Anemia: Physiological and Prophylactic Effects

CSCC PROTOCOL NUMBER: Version 6.0

DATE: April 2, 2007

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1 SYNOPSIS

Title of the Protocol: Arginine Supplementation in Sickle Cell Anemia: Physiological and Prophylactic Effects
CSCC Protocol Number: April 2, 2007, Version 6.0
Overview: Nitric oxide is an important inflammatory mediator produced from arginine by nitric oxide synthase. Nitric oxide has a multitude of functions which could impact favorably on vaso-occlusion in sickle cell disease. Oral arginine has been shown to raise levels of nitric oxide. This study will test whether daily oral arginine results in an increase in nitric oxide and other beneficial effects in patients with sickle cell disease. The results of this study will serve as the basis for further clinical trials to determine if daily arginine is a beneficial therapy for patients with sickle cell disease.
CSCC Protocol Chair: Lori Styles, MD
Intervention: Patients will be treated with one dose of oral arginine (0.05 g/kg/day) or placebo for 12 weeks.
IND Holder: Lori Styles, MD
IND Number: 59,995
Objectives: Primary Objective(s): To assess the physiological effects (both beneficial and deleterious) of the administration of oral arginine in patients with SCD. Secondary Objective(s): To evaluate the effect of daily oral arginine on clinical vaso-occlusive events in SCD patients.
Hypotheses/Estimates: The hypothesis of this study is that oral arginine, given daily, will increase nitric oxide production which will, in turn, produce beneficial effects on vascular homeostasis in sickle cell disease.
Criteria for Evaluation: Efficacy: Primary Endpoint: Efficacy will be determined by changes in three laboratory parameters which will serve as surrogates for potential clinical benefit: nitric oxide, Gardos channel activity, and RBC density. Secondary Endpoint: sVCAM, Nitrotyrosine, 8-iso-PGF2a, Ektacytometry, endothelin-1, fetal hemoglobin (Hb F) and echocardiogram results as well as clinical outcomes including hospitalizations, ER visits, and pain medication use. Safety: **SAFETY VARIABLES TO BE COLLECTED IN ADDITION TO ADVERSE EVENTS**
Study Design: Double-blinded, placebo-controlled, phase II trial in which 94 patients will be randomized to receive one dose of arginine or placebo for 12 weeks.
Study Population: Male or female Hb SS patients age 5 or more years **Major Inclusion Criteria:** History of at least one pain event in last 12 months **Major Exclusion Criteria:** Major organ dysfunction, treatment with hydroxyurea or transfusion within 3 months, history of recent priapism or retinopathy, pregnancy.
Sample Size: 94 (grouped into 48 children and 46 adults)
Randomization: One dose of arginine hydrochloride will be evaluated in this trial. Patients in each age group (children and adult) will be treated with one dose of arginine (0.05 g/kg/day) or placebo divided for BID dosing. Sixteen patients will be randomized to each dose level and placebo. Randomization will be stratified by center to preserve balance across treatment groups.
Data Analyses: Within each age group, we will evaluate differences in laboratory measures among the 3 groups (1 arginine group, one placebo group, and the previously collected aginine dose group) using random effects analysis of covariance techniques. Here the change in laboratory measures over each measurement time will act as the dependent variable of interest and the treatment status as the grouping factor. The baseline value of the laboratory measure and the time of the measurement visit will act as the fixed effect covariates and each patient

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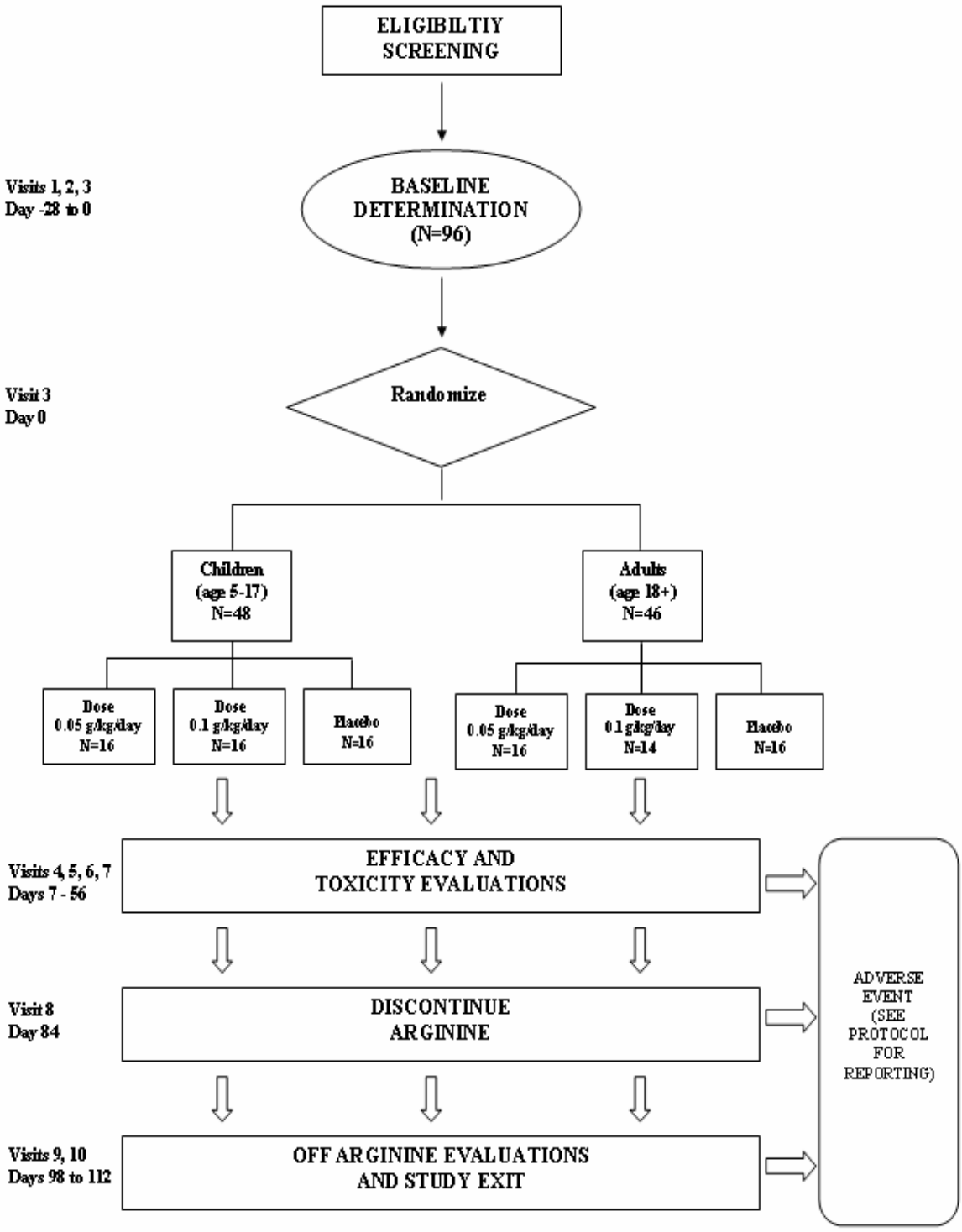
will have a random intercept. Additionally, each laboratory measurement will be summarized by age group (pediatric and adult), treatment arm, and measurement time. No interim analyses are planned for this study. The SDCC will be responsible for data management and analysis.

2 SUBJECT FLOW DIAGRAM



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4 ABBREVIATIONS

AE	Adverse Event
CRF	Case Report Form
CSCC	Comprehensive Sickle Cell Centers
DSMB	Data Safety Monitoring Board
GCP	Good Clinical Practice
Hb F	Fetal hemoglobin
ICH	International Conference on Harmonization
IND	Investigational New Drug
IRB	Institutional Review Board
NHLBI	National Heart, Lung and Blood Institute
NO	Nitric Oxide
NOx	Nitric Oxide metabolites (nitrite and nitrate)
PRC	Protocol Review Committee
RhoFED	Rho Federal Systems Division, Chapel Hill, NC
SAE	Serious Adverse Event
SAP	Statistical Analysis Plan
SCD	Sickle Cell Disease
SDMC	Statistics and Data Management Center (Rho Federal Systems Division, Chapel Hill, NC)
SO	Superoxide
SRBC	Sickle Red Blood Cell
VO	Vaso-occlusion

5 BACKGROUND AND RATIONALE

5.1 Sickle Cell Disease

Sickle cell disease (SCD) is a genetic disease which results from a point mutation at codon 6 which substitutes a valine for glutamine in the β globin protein of hemoglobin. The resulting sickle hemoglobin polymerizes abnormally when deoxygenated forming a rigid fiber. This fiber of polymerized hemoglobin distorts the shape of the cell and causes the characteristic sickle-shaped red cell that gives this disease its name. The clinical manifestations of SCD result from the resulting hemolytic anemia and vaso-occlusion (VO). The anemia of SCD is fairly well tolerated but VO results in most of the morbidity of SCD. As a result of VO, patients with (SCD) suffer from many different clinical manifestations affecting nearly every organ system in the body. The more common vaso-occlusive manifestations that affect these patients include pain episodes, severe pneumonia, overwhelming infections, stroke, and aseptic necrosis of the hip.

5.2 Vaso-Occlusion

Vaso-occlusion is responsible for much of the morbidity and mortality in SCD[1-4]. The pathophysiological processes involved in VO include intravascular sickle RBC sickling and increased adhesion of SRBC to the endothelium [5]. In addition, in situ thrombosis likely plays a role as perturbation of the vascular endothelium results in a highly procoagulant surface[6, 7]. Lastly, cells other than sickle red blood cells (SRBC) such WBC, platelets and the endothelium contribute to VO via secondary activation [7]. Irrespective of the initiating event, VO results from the interaction of SRBC with other cells within the vascular environment. To date, therapy for VO has targeted the sickle RBC but there is now a move toward therapies that target the intravascular environment as well [8-10]. Arginine and nitric oxide are particularly advantageous in that they have beneficial effects on both SRBC and the intravascular environment.

5.3 Nitric Oxide

Nitric oxide (NO) is an important inflammatory mediator with many biological functions that influence vascular homeostasis [11-13]. Cells synthesize NO by the conversion of L-arginine to citrulline via the nitric oxide synthases. Nitric oxide is the most potent vasodilator known but NO also decreases adhesion molecule expression, reverses procoagulant stimuli, and is cytoprotective[14]. Though NO production can be quite beneficial in certain disease states, the potential deleterious effects of NO cannot be overlooked. Nitric oxide is only a weak oxygen radical, but it quickly reacts with superoxide (SO) to form peroxynitrite, a highly reactive oxygen radical that may produce substantial oxidative damage. There have been reports, however, that NO upregulation reduces the production of SO and is actually an antioxidant [14]. The clinical effects of NO likely depend on the physiological milieu in which NO production occurs. In addition, the amount of NO produced is likely to be an important factor as a little NO is

often beneficial (e.g. pulmonary hypertension) but too much NO can clearly be detrimental (e.g. sepsis).

Nitric oxide was initially felt to play a deleterious role in many diseases and early investigations suggested therapies aimed at decreasing NO production could be beneficial. As knowledge of NO increased, it has been recognized that the beneficial effects of NO can outweigh its deleterious effects in many disease states [15-17]. This has resulted in a shift toward the use of NO to ameliorate the pathophysiology of many diseases. In addition to trials with inhaled NO, arginine has also been shown to upregulate NO in many human studies[18, 19]. Nitric oxide (as NOx) and arginine have been documented to be low in SCD patients with VOC and because NO could positively impact on many of the pathophysiological processes of VO, NO becomes an attractive potential therapy for patients with VO [9, 10].

5.4 Nitric Oxide and Arginine Therapy

Nitric oxide and arginine therapy have been used successfully and safely in scores of human trials. Beneficial results in three clinical areas have particular relevance to VO– diseases characterized by endothelial dysfunction, ischemic reperfusion injury, and pulmonary injury.

5.4.1 Endothelial Injury/Dysfunction

Sickle cell disease patients have evidence of ongoing endothelial injury, which worsens with vaso-occlusive episodes [20]. In addition, sickle red blood cells exhibit abnormal adherence to the endothelium [21]. Stabilization of the endothelium and a decrease in adhesion molecule expression should lessen the severity of vaso-occlusion and restore more normal perfusion [22]. The use of NO, and particularly arginine, has been investigated in several studies of patients with endothelial dysfunction. The effectiveness of arginine has been demonstrated in patients with endothelial dysfunction by showing decreases in intimal damage, decreased superoxide production, platelet stabilization, and a reduction in WBC adhesion to the endothelium [23-25]. In a recent double-blind, placebo-controlled, cross-over study, employing supplemental oral arginine (up to 12.6 gm/day) in patients with heart failure, several beneficial effects were demonstrated: significant improvement in functional class, increased forearm blood flow during exercise, and reduced circulating levels of endothelins, which improved arterial compliance [26].

5.4.2 Reperfusion Injury

In reperfusion injury, nitric oxide synthase is stimulated to produce the highly reactive free radical superoxide in lieu of NO [14]. Superoxide production is especially prominent when the NO substrate, arginine, is in low concentration [14, 27]. Superoxide rapidly degrades NO, which normally maintains vascular integrity and counteracts pro-inflammatory forces. In animal models of reperfusion injury, pretreatment with arginine augments NO production and superoxide production is reduced [14, 28]. Nitric oxide

upregulation results in less tissue injury with concomitant decreases in soluble levels of adhesion molecules, lipid peroxidation and neutrophil accumulation. Animal studies also show that in myocardial and cerebral ischemia arginine administration reduces infarct size [28]. However, other recent animal studies indicate that in some instances arginine may increase ischemic injury in brain[29]. In two recent studies, L-arginine has been shown to improve symptoms in patients with peripheral arterial disease [30, 31]. Daily infusions of L-arginine for 7 days increased calf blood flow and transcutaneous oxygen saturation, improved walking distance, shortened the time period for recovery from pain, and reduced platelet aggregation. More recently, a randomized, placebo-controlled study found that L-arginine induced peripheral vasodilation via an NO-dependent mechanism in patients with critical limb ischemia[32]. In this study, intravenous L-arginine increased lower limb blood flow by 42 percent, while the placebo had no effect. Due to periodic and regional vaso-occlusion, reperfusion injury almost certainly contributes to the pathophysiology of VO. Nitric oxide upregulation could therefore impact favorably on the severity of VO.

5.4.3 Pulmonary Injury

Acute pulmonary failure and pulmonary hypertension are two areas in which NO and arginine therapy have attracted particular interest. Inhaled NO has been shown to selectively dilate the pulmonary circulation and to improve oxygenation when ventilation-perfusion inequality exists [33]. Nitric oxide has also been shown to attenuate pulmonary vascular leak in animal models [28]. Arginine administration results in decreases in inflammatory cytokines and tissue factor expression in the setting of acute lung injury [14]. Studies in patients with acute respiratory distress syndrome have, in general, shown that inhaled NO improves oxygenation in these patients although it does not seem to reduce overall mortality [34]. Inhaled NO has also been used successfully and safely in neonates with respiratory distress syndrome and pulmonary hypertension [35, 36].

5.5 Nitric Oxide and Arginine in Sickle Cell Disease

Studies of NO levels in adults with SCD have generally documented normal or slightly increased NO in the steady state [37, 38]. It is postulated that the slight increase in NO may be in response to sub-clinical vaso-occlusion [10, 39]. While NO is normal or high at baseline, it decreases in patients during a vaso-occlusive pain event [10, 38]. The potential benefit of NO upregulation in many of the manifestations of SCD has prompted several investigators to assess inhaled NO in patients with SCD. The first study of inhaled NO in SCD documented that NO increased oxygen affinity of sickle erythrocytes which could potentially inhibit the polymerization of sickle hemoglobin leading to less sickling[8]. A subsequent study was not able to document this effect but did find that inhaled NO augmented NO transport to the microvasculature where it should improve microvascular perfusion [40]. These studies were done in patients at baseline (no apparent VO) and two other reports have documented the clinical use of inhaled NO in patients with SCD and ACS. Atz and Wessel reported on the use of inhaled NO in two children with SCD and severe ACS[41]. These two children were treated with up to 80

ppm inhaled NO for several days and experienced a dramatic improvement in oxygenation. There were no adverse effects of prolonged NO inhalation therapy and, specifically, no increase in methemoglobin production. A subsequent case report documented the use of NO at 20 ppm for 72 hours with a rapid clinical response and weaning of ventilatory support [42].

These preliminary findings of inhaled NO use in SCD are promising, but the fact that NO gas is not readily available and is difficult to administer, probably limits its use to only the most seriously ill patients. Arginine, on the other hand, is easily administered and has little apparent toxicity especially when given orally. Because SCD patients are known to be arginine deficient and arginine levels drop further during VO pain events, this suggests that arginine could be an effective, non-toxic therapy in SCD patients. The use of oral arginine in sickle cell disease has been limited to trials utilizing arginine butyrate as a fetal hemoglobin modulator. In one study, high doses (2 g/kg/day) of intravenous arginine butyrate were given to sickle cell disease patients for 2-3 weeks in an attempt to raise fetal hemoglobin levels [43]. Despite high doses of arginine, there were no side effects except for a transient rise in blood urea nitrogen (but not creatinine) in two patients. Another case report, using the same dose of arginine butyrate for 10 weeks, documented rapid resolution of recalcitrant leg ulcers implying enhanced peripheral circulation potentially from increased NO production [44]. There have been no studies assessing the long-term use of arginine in preventing VO.

5.6 Pharmacology and Toxicity of Arginine

L-Arginine is the natural L (+) stereoisomer of arginine (2-amino-5 guanidovaleric acid), and is a semi-essential amino acid in humans [17]. No formal dose response studies of arginine in humans have been reported and the minimum amount of arginine required to restore nitric oxide activity is not known. One study investigated the pharmacokinetics of L-arginine during chronic administration to patients with hypercholesterolemia[45]. Because of the spontaneous variation in arginine plasma concentrations seen in a 24-hour period, this study was unable to determine the half-life of arginine. This study was, however, able to compare the effects of intravenous and oral doses of arginine and documented that the bioavailability of arginine approaches 50% after oral administration. This study also documented that there were no changes in peak plasma arginine concentration or in the AUC0-8 with prolonged administration.

Arginine has been used intravenously and orally in a variety of doses, and its efficacy has been tested in hundreds of human and animal trials. It has been shown that high doses of supplemental arginine (30-60 gm/day) are well tolerated in humans. Experience with arginine in the pediatric population is limited. Recently, it was reported that low dose oral arginine can be used safely in otherwise normal children with short stature (age 5-14 years)[46]. This study documented that orally administered arginine is able to enhance basal and growth hormone releasing hormone-induced growth hormone secretion in children with familial short stature.

Side effects attributable to arginine have largely been seen in patients who were given rapid parenteral infusions of large doses of L-arginine (≥30 gm). Nausea, vomiting, flushing and headaches have occurred with rapid intravenous infusion of arginine. In patients with advanced renal or hepatic insufficiency, hyperkalemia may develop with arginine administration [47]. Allergic reactions to arginine are very rare [17]. After oral administration, only gastrointestinal upset and headaches have been reported with long term use. Notably, the potential mild hypotensive effect of high dose intravenous arginine has not been reported in studies using oral arginine.

5.7 Preliminary Studies

5.7.1 Arginine Treatment in Sickle Cell Mice

Plasma arginine levels are low in sickle cell anemia and we found low plasma arginine in our sickle transgenic mouse model that expresses human α, human β^S, and human β^S-Antilles and is homozygous for the mouse β^{major} deletion (S+S-Antilles)[48] . S+S-Antilles mice were supplemented with a four-fold increase in dietary arginine that was maintained for several months. Mean corpuscular hemoglobin concentration (MCHC) decreased and the percent high-density red cells were reduced.

Deoxy K+ efflux is characteristic of red cells in sickle cell disease[49] and contributes to the disease process by increasing the MCHC and rendering the cells more susceptible to polymer formation. The deoxy flux versus the room air flux was reduced in S+S-Antilles red cells from an average value of 1.6 ± 0.3 mmol/L cell x min (FU) in non-supplemented mice to 0.9 ± 0.3 FU (n=4, P<0.02, paired t-test) in supplemented mice. We then demonstrated that the decrease in deoxy K+ efflux was primarily due to reduction of Gardos channel activity by measuring the K+ efflux inhibitable by clotrimazole under room air conditions. In room air, Vmax of the Ca++ activated K+ channel (Gardos channel activity was reduced from 4.1 ± 0.6 FU (off diet) to 2.6 ± 0.4 FU (n=7 and 8, P<0.04, t-test) in arginine supplemented mice versus clotrimazole (Figure 5.1 Panel A). The EC50 was not changed by arginine supplementation (Figure 5.1 Panel B).

We conclude that the major mechanism by which arginine supplementation reduces red cell density (MCHC) in S+S-Antilles mice is by inhibiting the Ca++ activated K+ channel [48]. We speculate that this may be the result of decreased endothelin levels that have been observed in arginine supplementation, since it has been demonstrated that endothelin stimulates the Gardos channel in the mouse[50].

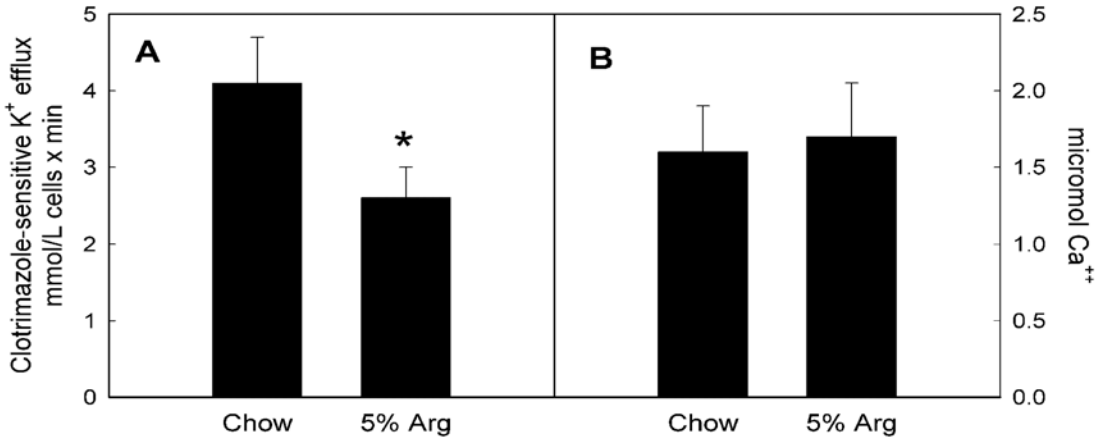

Figure 5.1 Arginine diet inhibits Vmax of the Gardos channel activity in S+S-Antilles mouse red cells but does not change EC50. Gardos channel activity under oxygenated conditions was measured on mice consuming normal chow (Chow) and after 10 weeks of 5% arginine diet (5% Arg). Panel A Vmax, Panel B EC50. Values are the difference between those obtained with and without clotrimazole.

5.7.2 Arginine/ NOx Correlation in Vaso-occlusive Crisis and Acute Chest Syndrome

NO levels cannot be directly measured and presumably vary significantly between different sampling sites (plasma, in endothelial smooth muscle, kidney, urine etc). Therefore, NO is commonly measured using a surrogate measurement--nitrate+nitrite levels (designated as NOx) as an indicator of NO level. To date, our studies and those of other investigators have used this measure.

Studies of NOx levels in adults with SCD have generally documented normal or slightly increased NOx in the steady state [37, 38]. It is postulated that the slight increase in NOx may be in response to sub-clinical vaso-occlusion [10, 39]. While NOx is normal or high at baseline, it decreases in patients during a vaso-occlusive pain event. The decrease in NOx during VOC is paradoxical because other inflammatory conditions are marked by a striking increase in NOx levels. Given that arginine is low in SCD, we postulated that low arginine concentration limits the upregulation of NO that would be expected to occur with vaso-occlusion and that during crisis, arginine levels reach a critically low concentration and NO production falls. To explore this hypothesis further we prospectively followed NOx and arginine in 16 children admitted with VOC.

At presentation for painful crisis, arginine is low compared to SCD patients at baseline (37.4 ± 2.7 μmol/L vs. 53 ± 4.6 μmol/L, respectively, p=.008) (Figure 5.2)[39]. Arginine slowly recovers to baseline levels by the end of hospitalization in VOC patients. In VOC patients who go on to develop ACS, arginine drops further after admission and reaches its lowest levels within 24 hours of developing ACS.

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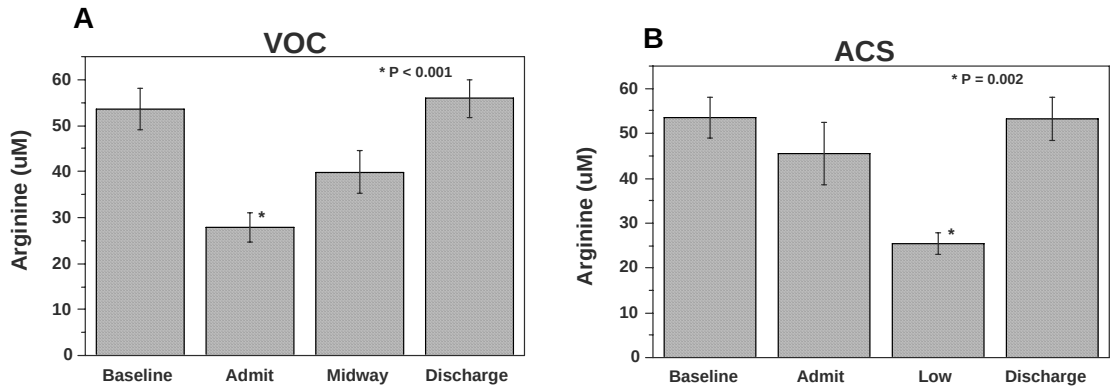

Figure 5.2. Arginine levels in SCD patients who were admitted with VOC. A. Arginine levels in patients with uncomplicated VOC (n= 10). Arginine levels are low at admission but slowly recover to baseline by discharge. B. Arginine levels in patients admitted with VOC but whom subsequently went on to develop ACS (n=4). Arginine levels drop during hospitalization and reach their lowest levels within 24 hours of diagnosis of ACS.

In these same patients, despite low arginine levels, NOx was near baseline levels at admission for VOC but dropped significantly during hospitalization (Figure 5.3). In VOC patients who went on to develop ACS, NOx levels decreased more dramatically during hospitalization and reached their lowest values within 24 hours of diagnosis of ACS, corresponding to changes in arginine.

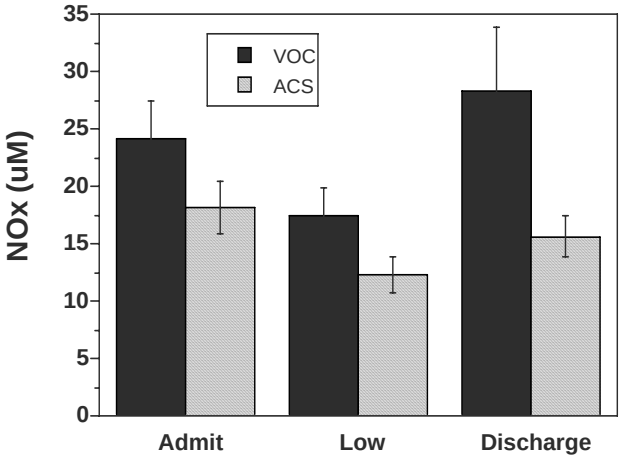

Figure 5.3. Nitric oxide NOx levels in patients with uncomplicated VOC (n=10) and those who went on to develop ACS (n=9). NOx levels were near baseline in VOC patients at admission but dropped during hospitalization. In ACS patients, NOx was lower at admission and decreased even more dramatically during hospitalization[39].

This preliminary data suggests that at the initiation of a vaso-occlusive event, arginine concentration is low but sufficient to maintain NO production. Ongoing demand for arginine to support NO production is quickly overwhelmed, however, as arginine drops further and subsequently, NO production drops. Figure 5.4 documents sequential levels

of arginine and NOx in a single patient and demonstrates how arginine demand and depletion due to increased utilization appear to limit NOx production in ACS.

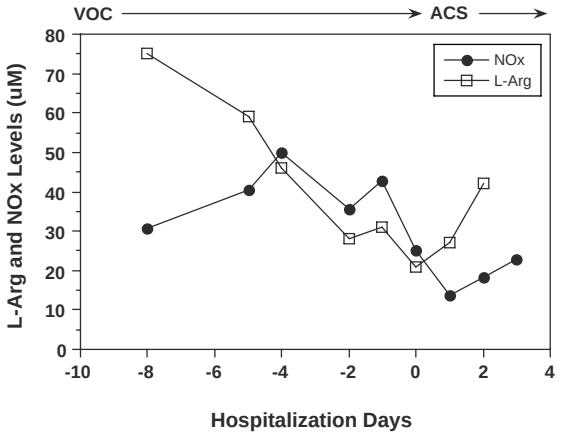

Figure 5.4. Correlation between NOx and arginine in a patient admitted with VOC who then developed ACS (Day 0). At admission, arginine concentration is low but NOx production is maintained. With continued hospitalization, arginine drops and NOx production parallels this drop. Arginine and NOx reach their lowest levels at the time ACS is diagnosed.

5.7.3 Arginine Administration in SCD—Steady State

Because our preliminary data suggested arginine might be a rate limiting substrate in NO production in SCD patients, we initiated a clinical investigation to determine the impact of arginine administration on NO production in SCD patients. We initially treated 6 SCD patients and 4 normal controls at baseline (after 8 hour fast) with a single oral dose of 0.1 g/kg arginine and assessed arginine and NOx levels over a 4-hour period. Both normal controls and the SCD patients demonstrated the same expected rapid rise in arginine concentration (Figure 5.5). This data confirmed that SCD patients metabolize arginine in a similar fashion to normal controls[51] and that absorption is not limiting in SCD.

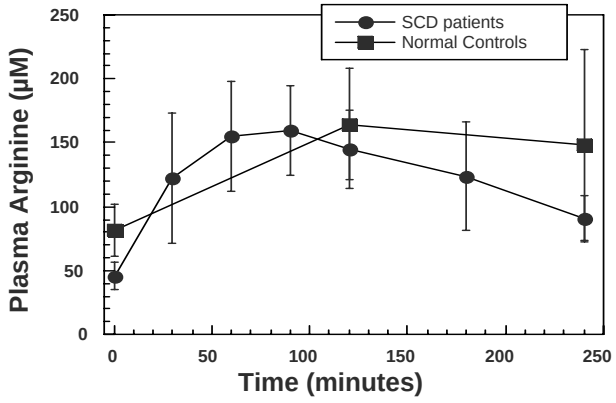

Figure 5.5. Arginine levels in normal controls (n=4) and SCD patients (n=6) at baseline. Both groups of patients were given a single oral dose of 0.1g/kg of arginine and followed with sequential arginine levels for 4 hours.

Despite similar increases in arginine serum concentration, SCD patients had a different pattern of NOx production than normal controls. Normal controls had a small increase in NO (+18.8 ± 68%) after 4 hours but SCD patients had no increase or even a small decrease in NOx production (mean= -16.7 ± 4 %, p=.004). It is unknown why SCD patients did not respond with the expected increase in NOx but it is possible that the response occurred later than 4 hours or that repeated doses of arginine are needed to

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restore the already depleted arginine stores at baseline [51]. This data also suggested that at baseline, with no apparent vaso-occlusion, arginine concentration does not limit the production of NOx. We postulated that the lack of response to exogenous arginine in steady state SCD patients is due to the absence of vaso-occlusion and the accompanying physiological stimuli that result in NO upregulation. We also hypothesized that in SCD patients suffering from a VO pain event, the biological triggers for increased NO production should be present and that SCD patients with VOC may have a different NO response to arginine.

5.7.4 Arginine Administration in SCD—Vaso-Occlusive Pain Crisis

To test the hypothesis that exogenous arginine will upregulate NO production in response to vaso-occlusion, we administered arginine to 7 SCD patients hospitalized with VOC. Four hospitalized SCD patients were given the same oral dose (0.1g/kg) of arginine and followed for 4 hours to see if NOx production could be upregulated. Three of the four SCD patients had an increase in NOx within 4 hours and the mean increase in NOx production for all 4 patients was 77 ± 103 %. These findings imply that, in contrast to SCD patients at baseline, arginine availability is rate limiting in the setting of VOC.

5.7.5 Arginine Administration in SCD—Acute Chest Syndrome

Given this data from patients with VO pain events and the knowledge that NOx and arginine levels are even lower in ACS patients we then initiated a phase II study in SCD patients hospitalized with ACS. Because we have little information on the relative efficacy of different doses in ACS this study is designed to test three different doses of arginine on NOx production. In addition, this study will give us our first information on the effects of repeated doses of oral arginine. We have treated 6 patients with the lowest dose (0.025mg/kg/dose PO TID) of arginine. Despite using a dose that was much lower than in our previous studies we have found that repeated doses of oral arginine upregulate NOx production even at very low doses. By twenty four hours, the mean increase in NOx was 87 ± 49% as compared to ACS patients who did not receive arginine who had a mean decrease in NOx levels of -29 ± 4.8% (p=.06, Figure 5.6).

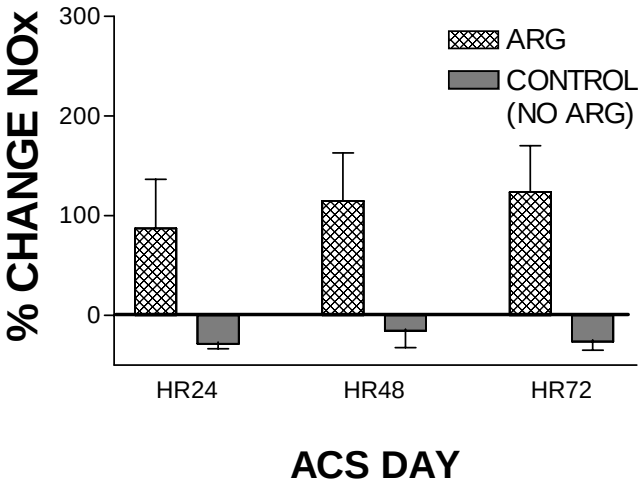

Figure 5.6. Mean changes in NOx in ACS treated with Arginine. Comparison is made to 4 patients who did not receive arginine.

In addition to documenting a substantial increase in NOx production we were also able to demonstrate a significant reduction in sVCAM levels (Figure 5.7). sVCAM levels reflect endothelial dysfunction are known to be elevated in SCD patients[10]. NO upregulation has been shown to decrease endothelial adhesion receptor expression in other disease states. This demonstrates that not only is NO upregulated but it seems to be impacting favorably on endothelial function.

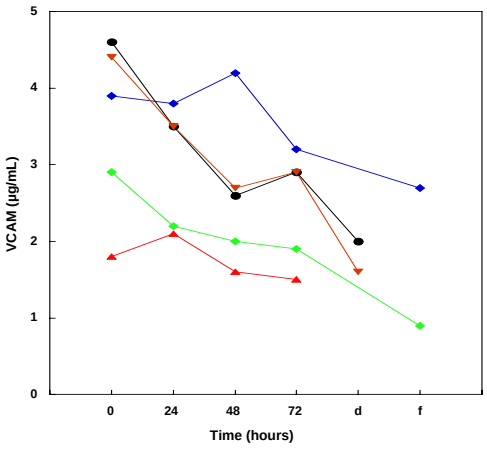

Figure 5.7. sVCAM levels in ACS patients treated with arginine. sVCAM levels consistently decreased while on arginine.

Lastly, 4 of the 6 ACS patients treated with arginine had a favorable increase in cellular hydration as indicated by ektacytometry and technicon. The data agree with the findings of other investigators who documented in sickle cell transgenic mice that a several week course of oral arginine improved sickle cell hydration by altering Gardos channel function [48].

5.7.6 Arginine Administration in SCD-Pulmonary Hypertension

Pulmonary hypertension is a common and life-threatening complication of sickle cell disease. Because NO is a potent vaso-dilator, NO deficiency may play a role in pulmonary hypertension in SCD. Morris et al treated 10 patients with oral arginine and were able to decrease pulmonary artery pressure with only 5 days of therapy (Figure 5.8)[52]. These results provide further evidence that oral arginine can upregulate NO production that affects vascular homeostasis.

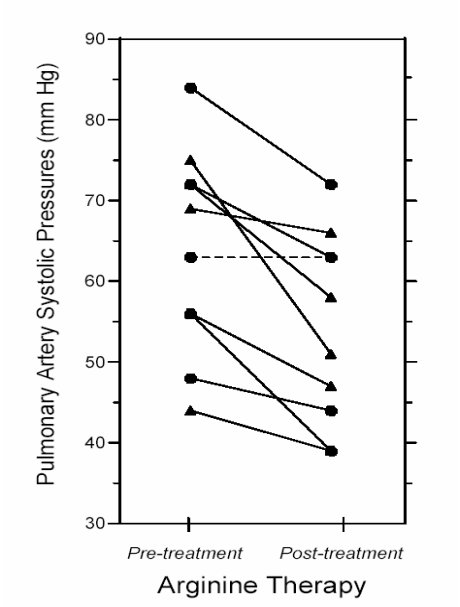

Figure 5.8. Change in pulmonary artery systolic pressure in 10 patients treated with oral arginine (0.1 g/kg TID) for five days.

Taken together, all of our preliminary data support our hypothesis that arginine concentration is critical in determining NO production during periods of vaso-occlusion. In addition, our data shows that exogenous arginine can be used to increase NO production and that NO upregulation results in several changes that could impact favorably on vaso-occlusion in SCD patients. This data suggests that arginine could be used prophylactically to prevent or reduce the severity of vaso-occlusion in SCD patients. By administering arginine daily to maintain arginine supplies, SCD patients would respond early to vaso-occlusion with an appropriate NO response and possibly abort the event. In addition, regular use of arginine could reduce the overall frequency of VO events as the intravascular environment may be improved through decreases in vascular adhesion molecule expression and improvements in SRBC hydration.

Although these preliminary studies are encouraging, several questions remain unanswered. First, our preliminary work evaluates only the effects of 3 days of arginine in ACS patients. It is unknown how sickle cell patients at baseline will respond to long-term daily therapy with arginine. We anticipate that daily therapy with arginine will be beneficial but the magnitude of response to daily arginine is unknown. Secondly, we have only tested one dose level in ACS patients and it is possible that a different dose will be needed in patients at baseline. Third, the physiological consequences of daily arginine administration (both beneficial and deleterious) have not been evaluated comprehensively. This protocol addresses these important questions and will provide additional data on the proper dosing and physiological effects of daily arginine in patients with SCD. This data will be crucial in supporting the need for further Phase III clinical trials of the effects of arginine in SCD patients.

6 STUDY OBJECTIVES AND PURPOSE

The primary and secondary objectives of the study are as follows:

Primary Objectives:

- **To assess the physiological effects (both beneficial and deleterious) of the administration of oral arginine in patients with SCD.**

We will initiate a double blinded, placebo controlled phase II trial where SCD patients will receive placebo or a dose of arginine hydrochloride orally for 12 weeks. We will comprehensively assess the physiological effects of arginine administration throughout this clinical trial. These investigations will focus on three target areas— sickle erythrocyte characterization, free radical biology/damage, and endothelial function.

Secondary Objectives:

- **To evaluate the effect of daily oral arginine on clinical vaso-occlusive events in SCD patients.**

Clinical efficacy will be a secondary endpoint of the clinical trial, but patients will be assessed regularly to determine if arginine treatment results in a reduction in clinical VO events or patient reports of pain.

7 STUDY DESIGN

7.1 Description of Study Design

7.1.1 Description Of Primary And Secondary Endpoints

The primary endpoints for this trial are:
- Nitric Oxide (NOx—nitrite+nitrate)
- Sickle Red Blood Cell Gardos Channel Activity
- Sickle Red Cell Density (Advia)

The secondary endpoints for this trial are:
- Soluble VCAM
- Nitrotyrosine
- 8-iso-PGF2α
- Ektacytometry
- ET-1
- Fetal hemoglobin (Hb F)
- Echocardiogram
- Measured by emergency room visits, hospitalizations, clinical visits, and pain medication use

7.2 Description of Type of Study

The general research design of this proposal is a double blinded, placebo-controlled, Phase II trial of oral arginine in SCD patients. Due to concern over differences in response due to age, two age groups will be tested independently—children under 18 and adults 18 and over. Patients in each age group (children and adult) will be treated with one dose of arginine (0.05 g/kg/day) or placebo divided for BID dosing. Sixteen patients will be randomized to the arginine and placebo arms. Added to this will be the 16 children and 14 adults previously randomized to the high dose arginine arm (0.10 g/kg/day). In total, 94 patients (48 children and 46 adults) will be evaluated as to the effects of arginine.

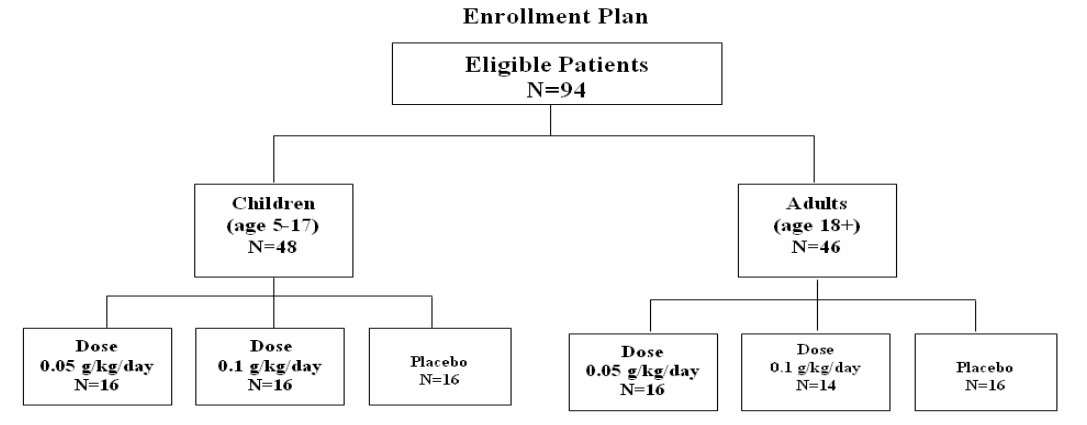

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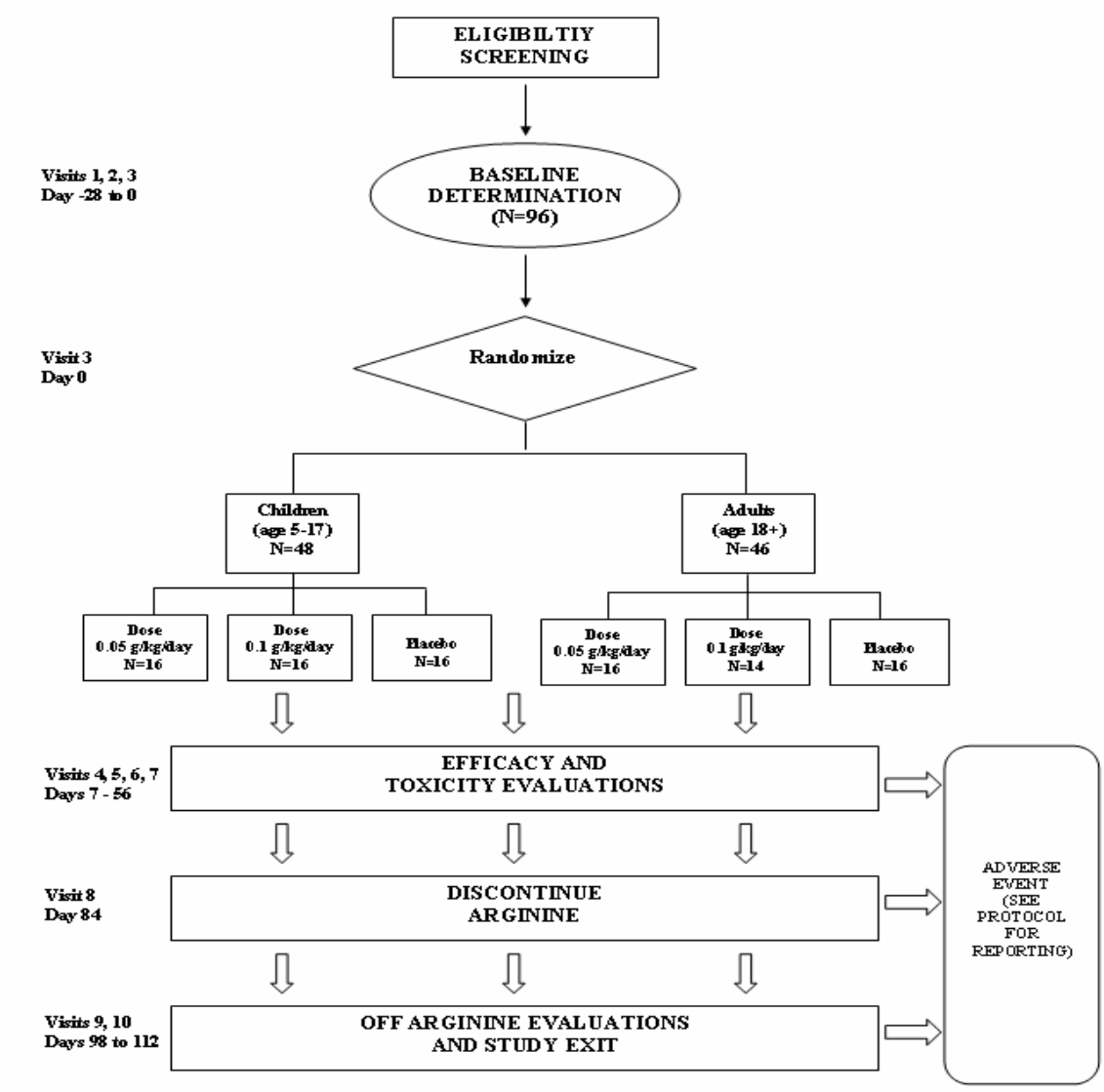
Figure 7.1. Study Design



7.2.1 Arginine Administration

Two doses of arginine hydrochloride will be evaluated in this trial; however, for the remaining portion of the trial, randomization will be to only one of those doses (0.05 mg/kg/day). As stated above, patients in each age group (children and adult) will be treated with one dose of arginine (0.05 g/kg/day) or placebo divided for BID dosing. Sixteen patients will be randomized to this dose level and placebo. Patient dose will be calculated according to the patient's weight. The research pharmacist at each institution will distribute a one month's supply of arginine at each clinic visit. Patients and study

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personnel will be blinded as to whether the patient is receiving arginine or placebo. At each clinic visit, pill counts will be performed to assess overall compliance with the therapy. Unused medication will be returned to the pharmacy after each clinic visit. Patients will receive arginine for 12 weeks.

7.2.2 Patient Monitoring

Patients enrolled in this trial will receive all therapy and monitoring that is considered standard of care for patients with SCD. Patients will be asked about signs and symptoms as they relate to their illness and arginine administration. To assess potential clinical efficacy, study personnel will collect information on emergency room visits and hospitalizations, clinic visits for pain, and pain medication use. This data will be compared with the patient's own history of these events in the 12 months before starting arginine. All clinical information will be collected using standardized study forms. As part of this study patients will have regular blood draws to assess safety and efficacy of arginine. Laboratory monitoring of patients enrolled in the trial will occur as outlined in the table below:

Test	Week -4	Week -2	Study Entry	Week 1	Week 2	Week 4	Week 8	Week 12	Week 14	Week 16
NO—serum	X	X	X	X	X	X	X	X	X	X
Arginine	X	X	X	X	X	X	X	X	X	X
Arginase	X	X	X	X	X	X	X	X	X	X
CBC/retic	X		X	*X*	X	X	X	X	X	X
Chem panel	X		X	X	X	X	X	X		X
Urinalysis	X		X	X		X		X		X
Met Hb	X		X	X		X	X	X		
βHCG	X	X	X	X	X	X	X	X	X	
Echocardiogram***			X					X		
EKG, CPK isoenzymes and troponin levels[#]			X	X	X	X	X	X	X	X
Research Labs**	X	X	X	X	X	X	X	X	X	X

** See Laboratory Assessment below for details

***The second echocardiogram will be performed at Week 12 only if the first echocardiogram is abnormal at Study Entry.

These cardiac enzyme tests need to be done for all subjects who present with chest pain or any event that could be indicative of a cardiac event..

7.2.3 Research Laboratory Assessment

Patients will have ongoing assessment for both primary and secondary outcomes throughout the course of the study. Whenever possible, these assessments will be made when the patient is in the steady state and not in the midst of a complication due to sickle cell disease. The schedule for assessing research lab outcomes is outlined in the table below:

Test	Wk -4	Wk -2	Study Entry	Wk 1	Wk 2	Wk 4	Wk 8	Wk 12	Wk 14	Wk 16
Free Radical Biology										
Nitrotyrosine	X	X	X	X	X	X	X	X		X
8-*iso*-$PGF_{2\alpha}$	X	X	X	X	X	X	X	X		X
Endothelial Function										
ET-1	X	X	X	X		X	X	X		X
sVCAM-1	X	X	X	X		X	X	X		X
Erythrocyte Characterization										
Advia	X	X	X	X	X	X	X	X	X	X
Ektacytometry	X	X	X	X	X	X	X	X	X	X
Gardos Channel	X	X	X	X	X	X	X	X	X	X
Fetal Hemoglobin	X	X	X			X	X	X		X

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8 SELECTION AND WITHDRAWAL OF SUBJECTS

8.1 Inclusion Criteria

Subjects who meet all of the following criteria are eligible for enrollment into the study:

1. Participant has signed the informed consent.
2. Established diagnosis of Hb SS or S-beta thalassemia
3. History of at least one vaso-occlusive pain events in last 12 months
4. Regular compliance with comprehensive care
5. Aged 5 years or greater
6. Patient is in his/her steady state and not in the midst of any acute complication due to sickle cell disease at enrollment

8.2 Exclusion Criteria

Subjects who meet any of the following criteria are disqualified from enrollment in the study:

1. Inability to take or tolerate oral medications
2. Hepatic dysfunction (SGPT $\geq$ 2X normal and albumin $\leq$ 3.2)
3. Renal dysfunction (Creatinine $\geq$ 1.2 for children and $\geq$ 1.4 for adults)
4. Allergy to arginine
5. Pregnancy
6. Transfusion within the last 90 days
7. > 10 hospital admissions for pain in the last 12 months **or** daily use of opiods and unstable pain (that interferes with work or daily routine) requiring > 3 Hospital Admissions **and** > 10 Emergency Department/Day Hospital visits in the last 12 months
8. Treatment with hydroxyurea within the last 90 days
9. Treatment with any investigational drug in last 90 days
10. Previous evidence of ischemic heart disease or heart failure

8.3 Subject Discontinuation

Subjects may decide to discontinue participation at any time during the study. Investigators may discontinue any subject at their discretion, if in their professional opinion, the subject's health, safety, and/or well-being is threatened by continued participation in the study. The following circumstances require discontinuation of subjects:

1. Drop in hemoglobin below 5gm/dL
2. Pulmonary failure requiring intubation
3. Hepatic dysfunction (SGPT $\geq$ 3X normal and albumin $\leq$ 3.0)

4. Renal dysfunction (Creatinine ≥ 1.4 for children and ≥ 1.6 for adults)
5. Focal neurological changes
6. Increase in methemoglobin level to > 2X normal level
7. Apparent allergic reaction to arginine
8. Severe headache
9. Pregnancy

Adverse events caused by participation in the study may necessitate modifications to the level of participation of a subject or discontinuation of subjects from participation in the study. Subjects who discontinue early from the study will be replaced.

Subjects who discontinue prematurely from the study for any reason will be encouraged to complete the lab evaluations required at Visit 10; these include a urinalysis, the chemistry and hematology panels, central lab evaluations, and completion of the interim health history form. These follow-up evaluations will be conducted as soon as the patient is able to come back in for one follow-up visit.

In the event that a local principal investigator wishes to discontinue a subject at his or her discretion (i.e. not due to one of the criteria listed above) then the investigator must contact the study Principal Investigator, Dr. Lori Styles, to formalize discontinuation.

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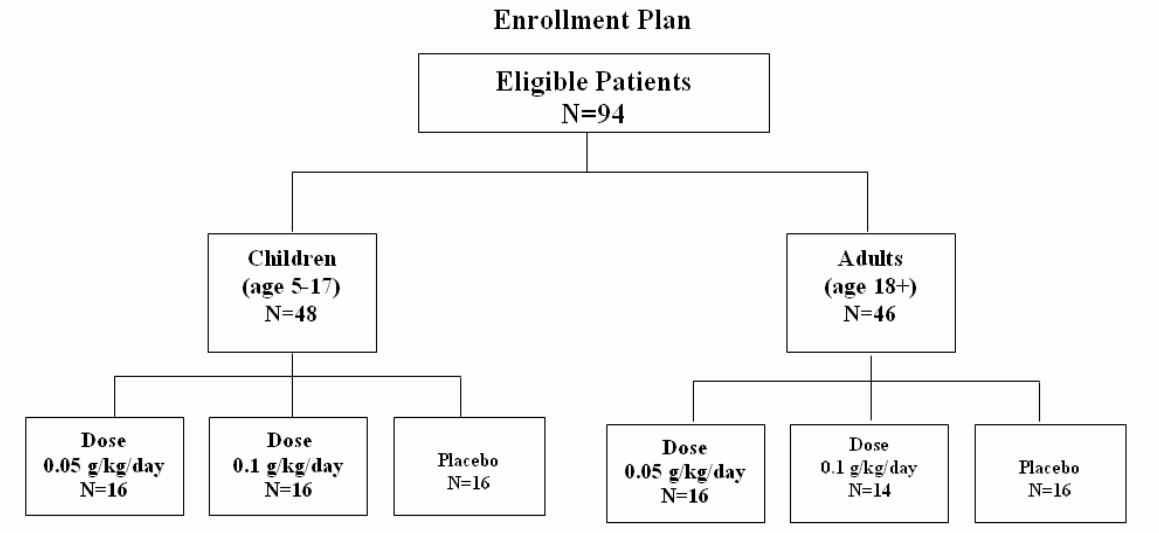
9 TREATMENT OF SUBJECTS

9.1 Treatments

9.1.1 Description of Study Drug and Dosing Regimen

Arginine hydrochloride is a semi-essential amino acid, which is available in purified powder form in bulk from many sources throughout the United States. After shipment to the contract pharmacy, the arginine hydrochloride will not be modified further. It will, however, be encapsulated into 250 and 500mg gelatin capsules by the contract pharmacy (Abbott's Compounding, Oakland, CA). Appropriate control capsules will also be produced by the contract pharmacy.

Two doses of arginine hydrochloride will be evaluated in this trial; however, one dose (0.10 g/kg/day) has been discontinued. Patients in each age group (children and adult) will be treated with one dose of arginine (0.05 g/kg/day) or placebo divided for BID dosing. Sixteen patients will be randomized to each dose level and placebo. Patient dose will be calculated according to the patient's weight. Thus, pediatric subjects may be given 500 mg capsules if they have a high enough weight and adult subjects may be given 250 mg capsules if they have a low enough weight. The same dose will be given each day. The maximum dose will be 10g of arginine per day. Patients unable to ingest the capsules (e.g. children) will be able to open capsules and mix with food or liquid. The research pharmacist at each institution will distribute a one month's supply of arginine at each clinic visit. Patients and study personnel will be blinded as to whether the patient is receiving arginine or placebo. Capsule counts will be performed once a month at Visits 6, 7, and 8 to assess overall compliance with the therapy. Unused medication will be returned to the pharmacy after the capsules have been counted.



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9.1.2 Dose Modification or Interruption of Study Drug

While oral arginine has been shown to have little toxicity when given in the doses outlined in this study, we will closely monitor signs, symptoms, and laboratory findings to assess for unexpected toxicities. Arginine will be discontinued in any patient who experiences the following:

--Drop in hemoglobin below 5gm/dL
--Pulmonary failure requiring intubation
--Hepatic dysfunction (SGPT ≥ 3X normal and albumin ≤ 3.0)
--Renal dysfunction (Creatinine ≥ 1.4 for children and ≥ 1.6 for adults)
--Focal neurological changes
--Increase in methemoglobin level to >2X normal level
--Apparent allergic reaction to arginine
--Severe headache
--Pregnancy

9.1.3 Packaging, Labeling, and Blinding of Study Drug

The U. S. Department of Health and Human Services Supply Services Center will be used to package, label and blind study drug.

9.1.4 Return and Destruction of Study Drug(s)

All unused study drug will be returned to the local pharmacy and destroyed appropriately.

9.2 Randomization and Masking

Randomization will be stratified by center and age group to preserve a balance across treatment groups. No other stratification factors will be used and a block randomization method will be used to ensure balance within the center-age strata. A randomization list for each center and age group will be generated by the SDMC and kept at the SDMC. The pharmacist dispensing study drug at the site will have access to this list. Additionally, emergency unblinding envelopes will be generated and sent to the sites. These will be labeled with the subject's ID on the outside and can be opened to reveal the randomized dose in case of an emergency. After the completion of the trial, all sealed randomization envelopes will be returned to the SDMC for confirmation of maintenance of the mask.

9.3 Prior and Concomitant Therapy

Patients enrolled in this trial will receive all therapy and monitoring that is considered standard of care for patients with SCD.

Table 9. 1 Medications Requiring a Washout Prior to Study Drug Dosing

Medication Requiring a Washout Period Prior to Study Drug Dosing	Washout Period
Hydroxyurea	90 days
Transfusion	90 days
Arginine	90 days

9.4 Subject Compliance

Plasma arginine concentration will be measured throughout the study, as a measure of subject compliance.

9.5 Study Procedures

9.5.1 Visit One

At Visit 1 (Week –4, Study Day –28), the first screening visit will occur. The patient must have a signed consent form on file and the inclusion and exclusion criteria will be checked to make sure that the patient is eligible for the study. Blood will be drawn so that the chemistry and hematology labs can be done locally. These tests include the Chemistry panel, LDH, CBC, Reticulocytes, Met Hb, Urinalysis, and Urine BHCG. Both males and females will complete a urinalysis and females will also be screened for pregnancy if they are of childbearing potential. The amount of blood drawn for the local labs will vary between sites. Blood will also be drawn and sent to two different central labs. Approximately a total of 30 ccs (or 6 tsp) will be drawn from the patient. One sample of blood will be sent to the Boston labs where Advia and Gardos Channel tests are performed. The other samples of blood will be sent to Oakland where the Free Radicals, Nitric Oxide, sVCAM-1, Arginine, Arginase and the Ektacytometry tests are performed. In addition to the blood work, personal information will be collected from the patient, including hospitalization, ER and clinical, demographic, medication history, pain history, and concomitant medication.

9.5.2 Visit Two

At Visit 2 (Week –2, Study Day –14), blood will be collected for the two central labs as in Visit 1. Female patients of childbearing potential will receive a pregnancy test. Medical information will be collected including hospitalization and pain information, concomitant medication information, and AE information. The discontinuation criteria will be checked to make sure that the patient is still eligible for the study.

9.5.3 Visit Three Randomization

At Visit 3 (Week 0, Study Day 0), the Baseline/Study Entry Visit, blood will be drawn for both the central and local labs in the same manner that it was collected during Visit 1. A urinalysis will be performed and female patients of childbearing potential will receive

a pregnancy test. All patients will receive an echocardiogram. Hospitalization, pain, concomitant medication, and AE information will be collected. For all patients presenting with chest pain or cardiac events, an EKG will be done and CPK isoenzymes and troponin levels will be measured. The study coordinator will double-check the discontinuation criteria prior to randomizing the patient and distributing enough of the study drug for one month.

9.5.4 Visit Four

At Visit 4 (Week 1, Study Day 7 ± 1), the patient will have been receiving the study drug for one week. The blood will be drawn for both the local and central labs in the same manner that it was collected for Visit 1 and Visit 3. A urinalysis will be performed and female patients of childbearing potential will receive a pregnancy test. A urine sample will be collected for the urinalysis and a pregnancy test (for female patients of child-bearing potential). Hospitalization, pain, concomitant medication, and AE information will be collected. For all patients presenting with chest pain or cardiac events, an EKG will be done and CPK isoenzymes and troponin levels will be measured. The discontinuation criteria will be checked to make sure the patient is still eligible for the study.

9.5.5 Visit Five

At Visit 5 (Week 2, Study Day 14 ± 1), the patient will have been receiving the study drug for two weeks. The blood collected for the central labs will be done in the same manner as in Visits 1, 2, 3, and 4 (except for ET-1 and sVCAM-1). Enough blood will be collected for the local labs to perform the Chemistry panel, LDH, CNC, and Reticulocytes tests. Female patients of childbearing potential will receive a pregnancy test. Hospitalization, pain, concomitant medication, and AE information will be collected. For all patients presenting with chest pain or cardiac events, an EKG will be done and CPK isoenzymes and troponin levels will be measured. The discontinuation criteria will be checked to confirm the patient is still eligible for the study.

9.5.6 Visit Six

At Visit 6 (Week 4, Study Day 28 ± 3), the patient will have been receiving the study drug for four weeks. The blood will be drawn and distributed for both the local and central labs in the same manner that it was collected for Visits 1, 3, and 4. Hospitalization, pain, concomitant medication, and AE information will be collected. For all patients presenting with chest pain or cardiac events, an EKG will be done and CPK isoenzymes and troponin levels will be measured. The discontinuation criteria will be checked to confirm the patient is still eligible for the study. All unused study drug that was distributed at Visit 3 will be collected, counted, and documented. Enough new study drug for one month will be distributed to the patient.

9.5.7 Visit Seven

At Visit 7 (Week 8, Study Day 56 ± 3), the patient will have been receiving the study drug for eight weeks. The blood will be drawn for the central labs in the same manner that it was collected for Visits 1, 2, 3, 4, and 6. The blood drawn for the local labs will be enough for the Chemistry panel, LDH, CBC, Reticulocytes, and Met Hb tests. Female patients of childbearing potential will receive a pregnancy test. Hospitalization, pain, concomitant medication, and AE information will be collected. For all patients presenting with chest pain or cardiac events, an EKG will be done and CPK isoenzymes and troponin levels will be measured. The discontinuation criteria will be checked to confirm the patient is still eligible for the study. All unused study medication that was distributed at Visit 6 will be collected, counted, and documented. Enough new study drug for one month will be distributed to the patient.

9.5.8 Visit Eight

At Visit 8 (Week 12, Study Day 84 ± 3), the patient will have been receiving study drug for twelve weeks. The blood will be drawn and distributed for both the local and central labs in the same manner that it was collected for Visits 1, 3, 4, 6, and 7. An echocardiogram will be performed only if the echocardiogram from Visit 3 was abnormal. If the first echocardiogram was normal, a second one will not be performed. The purpose of the echocardiogram is to see if arginine has any effect on pulmonary hypertension in sickle cell patients. A urinalysis will be performed on all patients, and female patients of childbearing potential will receive a pregnancy test. Hospitalization, pain, concomitant medication, and AE information will be collected. For all patients presenting with chest pain or cardiac events, an EKG will be done and CPK isoenzymes and troponin levels will be measured. The discontinuation criteria will be checked to confirm the patient is still eligible for the study. All unused study drug that was distributed at Visit 7 will be collected, counted, and documented.

9.5.9 Visit Nine

At Visit 9 (Week 14, Study Day 98 ± 3), the first follow-up visit, the patient will no longer be on the study drug. The blood drawn for the central lab will not include the Free Radical Tests and the Endothelial Function as in Visits 1, 3, 4, 6, 7, and 8. The blood drawn for the local labs will be enough for the CBC and Reticulocytes tests. Female patients of childbearing potential will receive a pregnancy test. Hospitalization, pain, concomitant medication, and AE information will be collected. For all patients presenting with chest pain or cardiac events, an EKG will be done and CPK isoenzymes and troponin levels will be measured. The discontinuation criteria will be checked to confirm the patient is still eligible for the study.

9.5.10 Visit 10

At Visit 10 (Week 16, Study Day 112 ± 3), the second follow-up visit, the patient will be seen for the last time. The blood will be drawn and distributed for the central labs in the

same manner that it was collected for Visit 1, 3, 4, 6, 7, 8, and 9. A urinalysis will be performed at this visit. Hospitalization, pain, concomitant medication, and AE information will be collected. For all patients presenting with chest pain or cardiac events, an EKG will be done and CPK isoenzymes and troponin levels will be measured. The discontinuation criteria will be checked to confirm the patient remained eligible throughout the study.

10 EFFICACY EVALUATIONS

10.1 Efficacy Assessments

The following efficacy assessments will be performed during the study:

Primary—
- Nitric Oxide (as NOx)
- Sickle Red Blood Cell Gardos Channel Activity
- Sickle Red Cell Density (Advia)

Secondary--
- Soluble VCAM
- Nitrotyrosine
- 8-iso-PGF2α
- Ektacytometry
- ET-1
- Hb F
- Echocardiogram

The rationale for these endpoints is as follows:

The potential beneficial and deleterious effects of NO upregulation in SCD are protean and have not been explored for sickle cell disease. This study includes a comprehensive assessment of the physiological effects of arginine administration and NO, upregulation in SCD patients. The physiological effects of arginine and NO will be assessed using a panel of in vitro investigations. The results of the in vitro investigations will be correlated with clinical variables and outcomes. Correlation of the clinical and physiological changes associated with arginine administration are crucial as, ultimately, the balance between beneficial and deleterious effects of arginine will determine whether arginine will be useful for patients with SCD. The in vitro investigations to be performed as part of this trial will focus on three areas—free radical biology/damage, sickle erythrocyte characterization, and endothelial function.

1) Free Radical Biology/Damage

The design of this trial is to increase NO production using exogenous arginine. Because the goal is to raise NO production we will regularly assess NOx concentration in patients treated with arginine (primary outcome). NO (NO_2 and NO_3 designated as NOx) will be measured using the NO analyzer from Sievers (Sievers Instruments, Inc., Denver, CO)[53-55].

In addition to measuring NOx (nitrite+nitrate) we will also obtain a second measurement of NO from plasma amino acids. Arginine, citrulline and ornithine levels will be

determined for all patients. Citrulline and NO are formed in equal amounts from arginine and partially reflect the activity of nitric oxide synthase.

Because NO is a free radical and participates widely in free radical reactions, NO upregulation has the potential to produce significant oxidative damage in the host. In contrast, NO production can also act as an antioxidant when NO and superoxide levels are equivalent. In addition to measuring NOx, we will investigate several other measures to assess potential oxidant damage as a result of NO upregulation.

(a) Nitrotyrosine (secondary outcome)--NO is only a weak oxidant but it reacts readily with superoxide (SO) to form the powerful oxidant peroxynitrite (ONOO). An increase in ONOO formation may be deleterious to the host. Studies in patients with ARDS have shown an increase in peroxynitrite formation after the use of inhaled NO. ONOO is difficult to measure due to its short half-life but ONOO formation results in the nitration of tyrosine residues and nitrotyrosine formation is a reliable index of peroxynitrite formation [14]. We will measure nitrotyrosine changes in blood with arginine administration as a means to assess a direct increase or decrease in oxidative damage from NO upregulation. Nitrotyrosine will be measured using an enzyme immunoassay kit from Cayman chemical (Cayman, Ann Arbor, MI).

(b) 8-iso-PGF2α (secondary outcome)—Due to the myriad of compounds that NO and other oxidants can react with, it is valuable to have an overall measure of oxidative stress in addition to the specific marker nitrotyrosine. 8-iso-PGF2α has been shown to be a reliable measure of lipid peroxidation and oxidative damage in vivo. We will measure changes in 8-iso-PGF2α throughout treatment using an enzyme immunoassay kit from Cayman chemical (Cayman, Ann Arbor, MI), according to the instruction provided by the manufacturer.

2) Sickle Erythrocyte Characterization

Sickle hemoglobin within the erythrocyte results in several changes in the erythrocyte including membrane/deformability changes and cellular dehydration. Nitric oxide is known to bind avidly to hemoglobin to form S-nitrosohemoglobin. The effects of inhaled nitric oxide on sickle hemoglobin have been assessed in two studies with conflicting results. One study showed that inhaled NO increased the O2 affinity of sickle hemoglobin thereby potentially inhibiting polymerization[8]. A more recent and careful study of inhaled nitric oxide was unable to reproduce these findings but found that increased nitrosohemoglobin from NO inhalation likely results in increased delivery of NO to the microvasculature[40]. While the impact of inhaled NO on sickle hemoglobin has been investigated, there have been no studies to assess changes in membrane structure/function from NO upregulation. For this proposal we will use several assays that are well established to detect erythrocyte changes due to NO upregulation.

(a) Gardos Channel activity (primary outcome measure)-- Recent studies in sickle cell transgenic mice demonstrated that oral arginine reduced Gardos

channel activity with improved cellular hydration as a result. We will assess Gardos Channel activity in SCD patients treated with arginine[48].

(b) Advia (primary outcome measure)—Sickle cell disease is characterized by a subset of cells that are dehydrated with an increased mean cell hemoglobin concentration (MCHC)[56-59]. The importance of this dehydration is substantial as the propensity of sickle hemoglobin to polymerize is exquisitely dependent on the concentration of hemoglobin within the cell [60]. The Advia 120 Hematology System is a modified flow cytometer that allows one to assess individual cells to generate a histogram of MHC's for a given patient. Reduction of Gardos channel activity will decrease MCHC. A reduction in cellular dehydration would be expected to reduce sickling and benefit the patient. The technicon will also be used to centrally measure reticulocyte count.

(c) Ektacytometry (secondary outcome measure)—Sickle red cells have abnormal deformability due to dehydration and alterations in their membrane (reference). An increase in deformability should reduce vaso-occlusion by increasing the ability of the sickle erythrocyte to pass through the microvasculature. NO could alter deformability via its interactions with hemoglobin. On the other hand, deformability can also be affected by increased oxidative stress. Nitric oxide could negatively impact deformability by the production of free radicals (see above). Ektacytometry will be measured on all patients treated with arginine to determine the overall impact of NO upregulation on red cell deformability. Osmotic gradient ektacytometry indicates cellular characteristics based on surface area to volume ratio, state of hydration and changes in mechanical properties of the membrane[61].

(d) Fetal Hemoglobin (secondary outcome measure)- Hemoglobin F (Hb F) ameliorates the course of sickle cell disease by interfering with polymer formation. The positive effects of elevated Hb F have been documented for end organ damage[62] and red cell survival[63] and form a part of the rationale for treatment with hydroxyurea. A primary endpoint for the proposed trial is the effect of arginine supplementation on red cell density (MCHC). Reduced MCHC will result in a reduced extent and rate of polymer formation. Since both HbF and arginine supplementation are thought to act through an impact on polymer formation, patients with high levels of Hb F may not receive the same benefit from arginine supplementation. We also speculate that arginine supplementation may have an impact on Hb F levels by one or both of two different mechanisms: 1) F-cells are enriched in peripheral circulation due to preferential survival over non-F-cells. If the survival of non-F-cells is improved with arginine treatment due to lower MCHC, we would expect less enrichment and a decrease in peripheral Hb F. 2) Increased Hb F has been observed in human erythroid progenitor cells exposed to NO donors in vitro[64]. If this effect is observed in vivo, arginine supplementation may lead actually lead to an increase in HbF in peripheral blood. To determine the net impact of arginine supplementation on Hb

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F levels, we will measure Hb F at the beginning and end of the arginine supplementation period.

3) Endothelial Function

The contribution of the endothelium to vaso-occlusion in SCD has been demonstrated in several studies. Not only does the endothelium control vascular tone crucial for vasodilation but it also impacts on vascular perfusion via the expression of adhesion cell molecules. Nitric oxide has many potential effects on the endothelium and vascular homeostasis, and NO regulates the production and/or function of several other vasoactive compounds produced by the endothelium. In addition, NO has been shown to reduce endothelial cell expression of adhesion molecules [14]. Vasodilatory changes, as well as adhesion molecule expression, could significantly impact the process of vaso-occlusion. In our investigation we will use several assays to measure changes in vasoactive compounds and adhesion molecule expression.

(**a) Vascular Cell Adhesion Molecule-1 (VCAM-1)(secondary outcome measure)—**Nitric oxide has been shown to decrease the expression of adhesion molecules on the surface of endothelial cells. Sickle erythrocytes have abnormal adhesion to the endothelium and this adhesion is felt to be an important initiator of vaso-occlusion. One mechanism though to be responsible for the increased adherence of sickle erythrocytes is the abnormal expression of the integrin $\alpha 4\beta 1$ on sickle reticulocytes. $\alpha 4\beta 1$ is the ligand of VCAM-1 and NO upregulation, therefore, could reduce vaso-occlusion by decreasing VCAM-1 on endothelial cells. Concentrations of soluble VCAM-1 (sVCAM-1) reflect the extent of VCAM-1 expression on endothelial cells. If arginine decreases VCAM-1 expression on the vascular endothelium then circulating levels would be expected to decrease [10]. We will measure sVACM in the serum of patients treated with arginine and correlate changes in sVCAM with NO production. sVCAM-1 will be measured using an enzyme Immunoassay kit from BioSource (Camarillo, CA), according to the instruction provided by the manufacturer.

(b) Endothelin-1 (ET-1)(secondary outcome measure)—Endothelin-1 is a potent vasoconstrictor and pro-inflammatory agent which is elevated in SCD patients at baseline and rises dramatically with the development of ACS [65-67]. In vitro studies have also demonstrated that sickled erythrocytes directly upregulate the expression of ET-1 in endothelial cell monolayers [68]. NO upregulation results in suppression of the synthesis of ET-1 thereby reducing vasoconstrictor forces. NO has the potential, through its inhibition of ET-1 production, to ameliorate the severity of vaso-occlusion in SCD. We will measure changes in ET-1 production throughout treatment with arginine. Endothelin-1 will be measured using an enzyme Immunoassay kit from Cayman chemical (Cayman, Ann Arbor, MI). Correlation of changes in ET-1 with NO and PGI2 levels will allow us to ascertain the impact of arginine on vasomotor status within the host.

4) Clinical Endpoint

Clinical efficacy data will be collected on all patients at all visits. Specifically, patients will be asked for information on emergency room visits and hospitalizations, clinic visits for pain, and pain medication use since their last visit.

10.1.1 Efficacy Assessments by Study Visit

These assessments will be performed according to the schedule outlined in the table below:

Test	Wk -4	Wk -2	Study Entry	Wk 1	Wk 2	Wk 4	Wk 8	Wk 12	Wk 14	Wk 16
Free Radical Biology										
Nitrotyrosine	X	X	X	X	X	X	X	X		X
8-*iso*-$PGF_{2\alpha}$	X	X	X	X	X	X	X	X		X
Endothelial Function										
ET-1	X	X	X	X		X	X	X		X
sVCAM-1	X	X	X	X		X	X	X		X
Erythrocyte Characterization										
Advia	X	X	X	X	X	X	X	X	X	X
Ektacytometry	X	X	X	X	X	X	X	X	X	X
Gardos Channel	X	X	X	X	X	X	X	X	X	X
Hb F	X	X	X			X	X	X		X
Clincal Assessment	X	X	X	X	X	X	X	X	X	X

10.1.2 Quality Control Procedures for Central Laboratories

All efficacy assessments will be performed in the laboratories of Dr. Frans Kuypers (Children's Hospital and Research Center Oakland) and Dr. Mary Fabry (Albert Einstein College of Medicine). All efficacy samples will be shipped to the two central laboratories at the time of each study visit. Because the efficacy assessments outlined above are not routinely performed in a hospital clinical lab each lab maintains its own strict quality control for each individual laboratory parameter. The quality control measures for each lab parameter is outlined below.

Albert Eistein College of Medicine Central Laboratory (Mary Fabry PhD, Director)

1) Advia (Red Cell Density):

The Advia 120 Hematology System is a fully automated diagnostic instrument. The Sample Shear Valve divides the sample into 5 aliquots for the different types of tests. The reagents and sample aliquots are delivered to their respective reaction chambers for mixing and reaction.
The reported parameters are:
Hgb (Hemoglobin)
RBC (Red Blood Cell Count)
MCV (Mean of RBC Volume histogram)
Hct (Hematocrit)
MCH (Mean Corpuscular Hemoglobin)
MCHC (Mean Corpuscular Hemoglobin Concentration)
CHCM (Corpuscular Hemoglobin Concentration Mean)
CH (Corpuscular Hemoglobin Content)
RDW (Red cell volume Distribution)
HDW (Hemoglobin concentration Distribution Width)
Platelet Count, Volume and Distribution Width
Reticulocyte % and Hemoglobin content (Chr)
WBC count and differential

Quality control is maintained using the following mechanisms:
1) Raw Data is stored in secure files on the hard drive and will be backed-up daily on an iOmega zip drive. Hard copies will be printed out daily. 2) The analyzer is calibrated using Advia Setpoint Calibrator. The calibrator contains human red and white blood cells and simulated platelets in a preservative medium. Prior to each calibration the gains are checked and adjusted as needed. 3) A primer sample is run daily when the analyzer is idle and controls are run thereafter. Advia Testpoint Hematology Controls are hematology reference materials for monitoring the precision and accuracy of the system. Low, Medium and High controls will be run daily. 4) Patients samples will be run on the Human species set-up on the Multi Species application software. 5) The primary operator of the Advia has had the multi-species software training by Bayer. 6) A service contract with Bayer will be maintained. The service covers an annual maintenance visit and daily trouble shooting as needed. 7) Routine daily, weekly and monthly maintenance will be performed. 8) All reagents and controls will be provided by Bayer and used prior to the expiration date.

Patient sample controls: In addition, we will submit in triplicate blinded samples from three patients not enrolled in the study once every four months.

2) HbF HPLC:

The globin composition will be determined by HPLC using a denaturing solvent that separates the globin chains and a Vydac large-pore (300 Å) C_4 column, 4.6 x 250 mm

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(Separations Group, Hesperia, CA) with a modified acetonitrile/H_2O/trifluoroacetic acid gradient similar to that used by Schroeder et al for separating human chains. Two buffers are used A (0.18% TFA in 36% acetonitrile) and B (0.18% TFA in 46% acetonitrile). Starting with 38% B, the percent B is increased by 0.583%/b min until all of the globin-chains are eluted.

The quality control process will include testing at four month intervals of quality control samples along with patient samples. The quality control (QC) material will be prepared with known HbF concentrations and will be tested blinded, in triplicate, in the same manner as patient samples. The concentration of HbF in the QC samples will be prepared according to published range of HbF levels. We will evaluate a high HbF value (abnormally high), normal and a low level. The acceptable values for each point will be evaluated by analyzing the mean value and standard deviation of the determination. An assay is considered within control range, when all the QC values are within ± 2 standard deviations. Sample QC values outside ± 2 SD will be considered in error and the sample results will be rejected. A table with the QC data will be prepared each time QC is evaluated.

3) Gardos Channel

The transport reaction is initiated under dim light by adding the cells to pre-warmed 37^{o}C media. Initial rates of K+ loss are used to calculate K+ efflux in RBCs by sampling the efflux media in duplicates at 1, 2, 4, and 6 minutes in the presence or absence of clotrimazole. The samples are pipetted into 1.5 mL ice-cold Eppendorf tubes containing 0.4 mL of dibutylphthalate and centrifuged for 10 seconds in a microcentrifuge. The supernatants are removed for K+ measurements by atomic absorption spectrophotometry. K+ efflux is calculated from the slope of the regression line of K+ concentration versus time taking into account the volume of red cells used. The activity of the channel can then be estimated from the difference between the fluxes in the presence or absence of clotrimazole as a function of extracellular calcium. From these data we can estimate using non-linear regression analysis the Vmax and K0.5 of the clotrimazole-sensitive and calcium-dependent potassium efflux also known as the Gardos channel. Records will be kept of water bath temperatures before and after each assay day. We will maintain our Pipette calibrated to standards that meet AACC, ISO, GLP/GMP, NCLLS and CAP guidelines. Logs will be kept on these calibrations for each pipette used in the assay. Atomic absorption calibration is performed routinely on the day of sample measurement by reading 3 known standards from which a standard curve is calculated. If this curve yields an $r^2$is less than 0.0980 machine and calibration reinitiated. Records of calibration for each patient sample studied will be kept. Patient sample controls: We will submit triplicate unmarked samples from three patients not enrolled in the study once every four months to validate reproducibility of the Gardos channel assay. From this we will estimate the coefficient of variation between the samples in order to determine any potential systemic or random errors. Records of these as well as all patient material will be kept guarded as per HIPAA regulations and Hospital stipulations. A table with the QC data will be prepared each time QC is evaluated.

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Children's Hospital and Research Center Oakland Central Laboratory (Frans Kuypers, PhD, Director)

1) Nitric Oxide

Nitrate and Nitrite (NOx) will be determined using a dedicated instrument, the NOA 280 (Sievers Boulder, Co). The protocol and quality control measurements will be followed as indicated by the manufacturer. A standard curve of nitrate concentrations will be generated prior to the measurement of samples. The Cadmium oxide solution used to generate NO from NO2 and NO3 will be made freshly every day, or if more than 50 samples (including standards) have been analyzed. At the time of replacement of the reducing solution, a new standard curve will be generated prior to the measurement of samples. Particular quality control measures will include the following: Samples will be collected in pyrogen/endotoxin free tubes. Samples will be frozen shortly after collection (1-2 hours) in 500 µl aliquots and stored at –20°C to –80°C until further processing, and send on dry ice to the laboratory at CHORI where they will be stored at –80°C, until analysis. Multiple freeze-thaw cycles of frozen samples will be avoided. The sample will be thawed completely and mixed well prior to analysis. When possible, badly hemolyzed or lipemic serum will be avoided. When large amounts of particulate matter are present, samples will be centrifuged or filtered prior to freezing, and a note will be added to indicate this treatment. All standards, controls, and samples will be run in duplicate. In-house controls will be run with every set of assays. If control or standard values fall outside pre-established response of the instrument, the accuracy of the assay is suspect, and will be repeated after appropriate maintenance procedures.

2) Soluble VCAM

The ELISA kit to measure sVCAM will be purchased from BiopSource international (Camarillo, CA). The kit consists of a well plate strips to a total of 96 wells, buffers and standard solution. The protocol to measure sVCAM in human serum or plasma will be strictly followed as provided with this commercial kit. All reagents needed for the assay are provided by the company and will be stored and used according to their recommendations. Particular quality control measures will include the following: When not in use, kit components will be refrigerated. All reagents will be warmed to room temperature before use. Microtiter plates will be allowed to come to room temperature before opening the foil bag. Once the desired number of strips has been removed, the bag will be resealed immediately and stored at 2 – 8°C to maintain plate integrity. Samples will be collected in pyrogen/endotoxin free tubes. Samples will be frozen shortly after collection (1-2 hours) in 500 µl aliquots and stored at –20°C to –80°C until further processing, and send on dry ice to the laboratory at CHORI where they will be stored at –80°C, until analysis. Multiple freeze-thaw cycles of frozen samples will be avoided. The sample will be thawed completely and mixed well prior to analysis. When possible, badly hemolyzed or lipemic serum will be avoided. When large amounts of particulate matter are present, samples will be centrifuged or filtered prior to freezing, and a note will be added to

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indicate this treatment. All standards, controls and samples will be run in duplicate. Samples that are >75 ng/mL will be diluted with Standard Diluent Buffer. When pipetting reagents, a consistent order of addition will be maintained from well to well. This ensures equal incubation times for all wells. All reagents will be capped when not in use. Reagents from lots from various kit lots will not be interchanged or mixed. Reagents will not be used after the kit expiration date. Within 2 hours of assay completion, absorbance will be read. In-house controls will be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect, and will be repeated. All residual wash liquid will be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never will absorbent paper be inserted directy into the well. Because stabilized Chromogen is light sensitive, prolonged exposure to light will be avoided.

3) Nitrotyrosine

The ELISA kit to measure Nitrotyrosine will be purchased from Cayman chemical (Cayman, Ann Arbor, MI). The kit consists of a 96 well plate, buffers and standard solution. The protocol to measure Nitrotyrosine in human serum or plasma will be strictly followed as provided with this commercial kit. All reagents needed for the assay are provided by the company and will be stored and used according to their recommendations. Particular quality control measures will include the following: When not in use, kit components will be refrigerated. All reagents will be warmed to room temperature before use. Microtiter plates will be allowed to come to room temperature before opening the foil bag. Once the desired number of strips has been removed, the bag will be resealed immediately and stored at 2 – 8°C to maintain plate integrity. Samples will be collected in pyrogen/endotoxin free tubes. Samples will be frozen shortly after collection (1-2 hours) in 500 µl aliquots and stored at –20°C to –80°C until further processing, and send on dry ice to the laboratory at CHORI where they will be stored at –80°C, until analysis. Multiple freeze-thaw cycles of frozen samples will be avoided. The sample will be thawed completely and mixed well prior to analysis. When possible, badly hemolyzed or lipemic serum will be avoided. When large amounts of particulate matter are present, samples will be centrifuged or filtered prior to freezing, and a note will be added to indicate this treatment. All Standards, controls and samples will be run in duplicate. When pipetting reagents, a consistent order of addition will be maintained from well to well. This ensures equal incubation times for all wells. All reagents will be capped when not in use. Reagents from lots from various kit lots will not be interchanged or mixed. Reagents will not be used after the kit expiration date. Within 2 hours of assay completion, absorbance will be read. In-house controls will be run with every assay. If control values fall outside pre~stablished ranges, the accuracy of the assay is suspect, and will be repeated. All residual wash liquid will be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never will absorbent paper be inserted directy into the well. Because stabilized Chromogen is light sensitive, prolonged exposure to light will be avoided.

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4) 8-iso-PGF2α

The ELISA kit to measure 8-iso-PGF2α will be purchased from Cayman chemical (Cayman, Ann Arbor, MI). The kit consists of a 96 well plate, buffers and standard solution. The protocol to measure 8-iso-PGF2α in human serum or plasma will be strictly followed as provided with this commercial kit. All reagents needed for the assay are provided by the company and will be stored and used according to their recommendations. As serum of plasma may contain compounds that interfere with the assay, the samples will be purified according a standard protocol, using 8-isoprostane affinity column solid phase extraction purchased from Cayman chemical (Cayman, Ann Arbor, MI). Samples and standards will be subjected to the solid phase extraction protocol as included with the kit, using radiolabeled 8-iso-PGF2α. Particular quality control measures will include the following: When not in use, kit components will be refrigerated. All reagents will be warmed to room temperature before use. Microtiter plates will be allowed to come to room temperature before opening the foil bag. Once the desired number of strips has been removed, the bag will be resealed immediately and stored at 2 – 8°C to maintain plate integrity. Samples will be collected in pyrogen/endotoxin free tubes. Samples will be frozen shortly after collection (1-2 hours) in 500 µl aliquots and stored at –20°C to –80°C until further processing, and send on dry ice to the laboratory at CHORI where they will be stored at –80°C, until analysis. Multiple freeze-thaw cycles of frozen samples will be avoided. The sample will be thawed completely and mixed well prior to analysis. When possible, badly hemolyzed or lipemic serum will be avoided. When large amounts of particulate matter are present, samples will be centrifuged or filtered prior to freezing, and a note will be added to indicate this treatment. All Standards, controls and samples will be run in duplicate. Samples that are out of the optimal range (10-500 pg/ml) will be diluted with Standard Diluent Buffer.
When pipetting reagents, a consistent order of addition will be maintained from well to well. This ensures equal incubation times for all wells. All reagents will be capped when not in use. Reagents from lots from various kit lots will not be interchanged or mixed. Reagents will not be used after the kit expiration date. Within 2 hours of assay completion, absorbance will be read. In-house controls will be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect, and will be repeated. All residual wash liquid will be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never will absorbent paper be inserted directly into the well. Because stabilized chromogen is light sensitive, prolonged exposure to light will be avoided.

5) EKTAcytometry

Osmotic gradient ektacytometry will be performed using a custom build ektacytometer using a National Instrument interface to a Macintosh computer for instrument control and data analysis using Igor software (Wave metrics, Lake Osewego, Or). The osmotic gradient curve will reflect cellular characteristics based

on surface area to volume ratio, state of hydration and changes in mechanical properties of the membrane. In our experience, RBC samples from normal individuals will render virtually identical results over time. All results will be compared to a standard curve which is a compilation of more than 1000 samples collected over time. Weekly a known sample from a laboratory volunteer will be analyzed and compared to the standard curve as well as their previous results available. A shift of more than 5% in the curve indicates the need for calibration. After calibration, the measurement of is repeated to ensure proper alignment. All samples will be accompanied by travel controls, to ensure proper handling by the carrier. When the travel control falls outside of the normal average range, a laboratory control will be run to ensure proper instrument calibration. All results of patient samples will be stored together with travel controls. Specific quality control measures include: Samples (including travel controls) will be collected in ACD tubes according to protocols provided to the collecting sites, stored at 4°C, and send that same day on wet ice to the laboratory at CHORI by ovenight transport. At CHORI the samples will be stored at 4°C, and analyzed as soon as possible. All standards, controls and samples will be run in series.

6) Endothelin-1 (ET-1)

The ELISA kit to measure ET-1 will be purchased from Cayman chemical (Cayman, Ann Arbor, MI). The kit consists of a 96 well plate, buffers and standard solution. The protocol to measure ET-1 in human serum or plasma will be strictly followed as provided with this commercial kit. All reagents needed for the assay are provided by the company and will be stored and used according to their recommendations, since serum or plasma may contain compounds that interfere with the assay. In particular samples that contain less than 50 pg/ml are prone to render inaccurate results. Hence, the samples will be purified according a standard protocol, using a C-18 reverse phase cartridge (Sep-Pak). Samples and standards will be subjected to the extraction protocol as included with the kit, using standard amounts of ET-1 to spike the samples in the range of 90% with a variance of 20%. Particular quality control measures will include the following: When not in use, kit components will be refrigerated. All reagents will be warmed to room temperature before use. Microtiter plates will be allowed to come to room temperature before opening the foil bag. Once the desired number of strips has been removed, the bag will be resealed immediately and stored at 2 – 8°C to maintain plate integrity. Samples will be collected in pyrogen/endotoxin free tubes. Samples will be frozen shortly after collection (1-2 hours) in 500 µl aliquots and stored at –20°C to –80°C until further processing, and send on dry ice to the laboratory at CHORI where they will be stored at –80°C, until analysis. Multiple freeze-thaw cycles of frozen samples will be avoided. The sample will be thawed completely and mixed well prior to analysis. When possible, badly hemolyzed or lipemic serum will be avoided. When large amounts of particulate matter are present, samples will be centrifuged or filtered prior to freezing, and a note will be added to indicate this treatment. All Standards, controls and samples will be run in duplicate. Samples that are out of the optimal range (10-500 pg/ml) will be diluted with standard diluent buffer. When pipetting reagents, a consistent order of addition will be maintained from well to well. This ensures equal incubation times for all wells. All

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reagents will be capped when not in use. Reagents from lots from various kit lots will not be interchanged or mixed. Reagents will not be used after the kit expiration date. Within 2 hours of assay completion, absorbance will be read. In-house controls will be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect, and will be repeated. All residual wash liquid will be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never will absorbent paper be inserted directly into the well. Because stabilized chromogen is light sensitive, prolonged exposure to light will be avoided.

10.2 Drug Concentration Measurements

Plasma arginine levels will be measured throughout the study as outlined in the tables above.

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11 DATA AND SAFETY MONITORING PLAN

11.1 Safety Assessments

11.1.1 Safety Monitoring

While no toxicities from oral arginine are expected, we will comprehensively monitor patients to assess for unexpected toxicity. Patients enrolled in this trial will receive all therapy and monitoring that is considered standard of care for patients with SCD. Patients will be asked about signs and symptoms as they relate to their illness and arginine administration. For all patients presenting with chest pain or cardiac events, an EKG will be done and CPK isoenzymes and troponin levels will be measured. To assess potential clinical efficacy, study personnel will collect information on emergency room visits and hospitalizations, clinic visits for pain, and pain medication use. All clinical information will be collected using standardized study forms. As part of this study patients will have regular blood draws to assess safety of arginine. The potential deleterious effects of increased NO production, such as oxidative damage, will be monitored using CBC, chemistry panel, urinalysis, and met Hemoglobin level. Evaluation of these labs will allow for assessment of ongoing effects on organ function and hematopoeisis.

11.1.2 Safety Assessments by Study Visit

The laboratory monitoring by study visit is outlined in the table below:

Test	Week -4	Week -2	Study Entry	Week 1	Week 2	Week 4	Week 8	Week 12	Week 14	Week 16
CBC/retic	X		X	*X*	X	X	X	X	X	X
Chem panel*	X		X	X	X	X	X	X		X
Urinalysis	X		X	X		X		X		X
Met Hb	X		X	X		X	X	X		
βHCG	X	X	X	X	X	X	X	X	X	
ECHO***			X					X		
EKG, CPK isoenzymes and troponin levels[#]			X	X	X	X	X	X	X	X

* Includes Na,K,Cl,CO2,Ca,BUN,Cr,gluc,total protein,alb,total bili,ALT,alk phos,LDH
***The second echocardiogram will be performed at week 12 only if the first echocardiogram is abnormal at Study Entry.
#These additional tests need to be done for all prospective chest pain events for patients in this study.

11.2 Adverse Events

11.2.1 Definitions of Adverse Events and Serious Adverse Events

An adverse event (AE) is defined for this study as any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product that does not necessarily have a causal relationship with this treatment. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal (investigational) product, whether or not related to the medicinal (investigational) product. AE data are recorded on the CRF form.

A serious adverse event (SAE) is defined as any untoward medical occurrence that at any dose:

- Results in death;
- Is life-threatening (i.e. an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe).
- Requires inpatient hospitalization or prolongation of existing hospitalization.
- Results in persistent or significant disability/incapacity.
- Results in a congenital anomaly/birth defect.
- In the opinion of the investigator, important medical events that may not be immediately life-threatening or result in death or hospitalization but may jeopardize the subject or may require intervention to prevent one of the other outcomes listed in the definition above, may be considered serious. Examples of such events are intensive treatment in an emergency room or at home for allergic bronchospasm; blood dyscrasias or convulsions that do not result in hospitalization; or development of drug dependency or drug abuse.

An *expected SAE* for subjects in this study is an adverse event that is listed in Table 11.1 and is a serious adverse event, as defined above. An *unexpected SAE* for subjects in this study is any AE that is **not** listed in Table 11.1 and is a serious adverse event, as defined above.

Table 11.1. List of Expected Adverse Events in This Study

Expected AE	Expected AE	Expected AE
Acute chest syndrome	Pyelonephritis	Hypoxemia (PO2 < 65mm Hg)
Anemia	Renal failure	Infection, pneumococcal
Aplastic crisis	Renal insufficiency/albuminuria	Jaundice
Aplastic crisis/anemia	Renal papillary necrosis	Leukocytosis
Arthralgia	Reticulocytosis (∃10%–20%)	Meningitis
Avascular necrosis of hip/shoulder	Retinal disease	Rhabdomyolysis
Avascular necrosis of the femoral head	Retinal hemorrhage	Sepsis
Bone infarction	Elevated urinary urobilinogen	Skin ulcers
Cardiomegaly	Fever	Splenic sequestration
Cerebrovascular accident	Hand-foot syndrome	Pain, joint
Cholecystitis, hepatic sequestration	Hematuria	Pain, long bone
Cranial verve palsy	Hemiplegia	Pain, severe abdominal
Decreased kidney function	Hemolysis	Priapism
Decreased lung function	Hepatosplenomegaly	Pulmonary embolism
Delayed growth/puberty	Hyperplastic bone marrow	Pulmonary hypertension
Depressed ESR	Hyposthenuria	Pulmonary parenchymal infiltrates on chest x-ray

Table 11.2. Overview of Definitions of Safety Measures

Word or Phrase and (Abbreviation)	Definition
Adverse event (AE)	Any untoward clinical or medical occurrence.
Serious adverse event (SAE)	Any adverse event that results in any of the following outcomes: Death, a life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant disability/incapacity, or a congenital anomaly/birth defect.
Expected Serious Adverse Event (Expected SAE)	An *expected SAE* for subjects in this study is an adverse event that is listed in Table 11.1 and is a serious adverse event, as defined above.
Unexpected Serious Adverse Event (Unexpected SAE)	An *unexpected SAE* for subjects in this study is any AE that is **not** listed in Table 11.1 and is a serious adverse event, as defined above.
Adverse Clinical	Any safety laboratory measurement that is measured beyond the

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Laboratory Results	acceptable limits (either high or low) for the population being studied, as described by the investigator.

11.2.2 Assessment of Adverse Event Severity and Relationship to Treatment

The following scale will be used to "grade" the severity of all adverse events.

1 Mild. Awareness of sign, symptom, or event, but easily tolerated; does not interfere with usual daily activities or tasks.
2 Moderate. Discomfort enough to cause interference with usual daily activity; may warrant therapeutic intervention.
3 Severe. Incapacitating; inability to perform usual activities and daily tasks; significantly affects clinical status; requires therapeutic intervention.
4 Life-threatening. Adverse event is life-threatening.
5 Death. Adverse event causes death.

The standard nomenclature for defining the causal relationship between an AE and the study drug used by CSCC is listed in the following table. The category that overall best "fits" the relationship between the adverse event and the study drug should be chosen.

Unrelated	• No temporal association to study product. • An alternate etiology has been established. • The event does not follow the known pattern of response to study product. • The event does not reappear or worsen with re-challenge.
Probably not related / remote	• No temporal association to study product. • Event could readily be produced by clinical state, environmental or other interventions. • The event does not follow the known pattern of response to study product. • The event does not reappear or worsen with re-challenge.
Possibly related	• Reasonable temporal relationship to study product. • The event is not readily produced by clinical state, environmental, or other interventions. • The event follows a known pattern of response to the study product or as yet unknown pattern of response.
Probably related	• There is a reasonable temporal association with the study

	product. • The event is not readily produced by clinical state, environmental, or other interventions. • The event follows a known pattern of response to the study product. • The event decreases with de-challenge.
Definitely related	• There is a reasonable temporal relationship to the study product. • The event is not readily produced by clinical state, environmental, or other interventions. • The event follows a known pattern of response to the study product. • The event decreases with de-challenge and recurs with re-challenge.

11.2.3 Outcome of Adverse Events

Every AE must be followed to a satisfactory outcome or stabilization of the event, even when this requires a time period beyond the scope of the study (this is particularly applicable to serious adverse events (SAEs)). Outcome is defined as

Outcome: Information on recovery and any sequelae; what specific tests and/or treatment may have been required and their results. For a fatal outcome, cause of death and a comment on its possible relationship to the suspected reaction should be provided.

The terms used to define outcome are as follows (outcome of reaction/event at the time of last observation):

- Ongoing
- Resolved without sequelae
- Resolved with sequelae
- Death

Actions taken in response to an AE and follow-up results must be recorded in the subject's medical record (this includes follow-up laboratory results). Any treatment administered for the adverse event must be recorded in the subject's CRF. When subjects are discontinued from the study due to an AE, relevant clinical assessments and laboratory tests will be repeated as necessary until final resolution or stabilization occurs.

11.2.4 Reporting of Adverse Events

Serious safety issues that arise in this study will be brought to the attention of the CSCC Data and Safety Monitoring Board (the "DSMB"), which will make recommendations to the NHLBI regarding possible suspension of the study. The NHLBI will consider the DSMB's recommendations, determine an appropriate action and notify the Principal Investigator and the CSCC Statistics and Data Management Center (SDMC). The Principal Investigator will notify all participating investigators, who will implement the actions directed by NHLBI. This section defines "serious safety issues" and describes procedures for bringing them to the attention of the DSMB.

This study is being conducted under an Investigational New Drug Application ("IND"). Consequently, U.S. Government regulations require that all "unexpected serious adverse events" (defined below) must be reported to the Food and Drug Administration (FDA) within either 7 calendar days (fatal or life-threatening events) or 15 calendar days (non-fatal, non-life-threatening). These Suspension Guidelines require that unexpected serious adverse events also be reported to the DSMB within the same timeframe. The FDA has the authority to direct the investigators to suspend or terminate the study, or take other actions.

These Guidelines specify the following types of reports that can alert the DSMB to a potential safety issue:

- Ad hoc reports of unexpected serious adverse events that are made within 7 or 14 calendar days, as specified in subsequent paragraphs.
- Reports of quarterly statistical analyses of all serious adverse events. The SDMC makes such analyses quarterly, but files a report to the DSMB only when analyses indicate that a safety issue has arisen, as defined by the "alert" criteria.

Semi-annual DSMB reports of analyses of adverse events and of clinical laboratory results. These reports will highlight any safety issues revealed by the analyses that meet the "alert" criteria.

11.2.5 Reporting of Unexpected Serious Adverse Events

Within 8 hours of the realization that an unexpected SAE has occurred to a study subject, a study investigator must make an initial report:

- To the Principal Investigator.
- To the CSCC Statistics and Data Management Center SAE Regulatory Specialist.

The investigator must also report the unexpected SAE to the site's IRB in accordance with that IRB's regulations or procedures.

The site investigator, the Principal Investigator, and the SDMC SAE Regulatory Specialist will collaborate to prepare a report of the unexpected SAE using the current version of FDA's SAE reporting forms. The report should describe the event as fully as possible. The initial SAE reports received from the site should include the following minimum information: an identifiable subject; study product; an identifiable reporting source; and an event or outcome that can be identified as serious. Supporting documentation (CRF pages, lab reports, summary notes, autopsy reports) should accompany the report.

A fatal or life-threatening unexpected SAE will be reported to the DSMB [and FDA] within 7 calendar days of the receipt of the initial report by the SDMC SAE Regulatory Specialist. A non-fatal, non-life-threatening unexpected SAE will be reported to the DSMB [and FDA] within 15 calendar days of the receipt of the initial report by the SDMC SAE Regulatory Specialist.

The Principal Investigator will submit the DSMB report to the Chair of the DSMB Subcommittee appointed to monitor this study and to the NHLBI Project Officer. The Principal Investigator will submit the unexpected SAE report to all study investigators. Each study investigator will submit the unexpected SAE report to the local IRB and other local authorities in accordance with the institution's regulations.

All serious adverse events, regardless of expected status, are also recorded in the Adverse Events section of the study's case report form.

The site investigator will follow the progress of a subject who experiences an unexpected SAE until the SAE is resolved. When the unexpected SAE has not resolved by the report deadline, the site investigator will make followup reports in accordance with directions from the DSMB, the FDA, and/or the site's IRB.

11.2.6 Reporting of Expected Serious Adverse Events

Within 3 days of the realization that an expected SAE has occurred to a study subject, a study investigator must make an initial report:

- To the Principal Investigator
- To the CSCC Statistics and Data Management Center SAE Regulatory Specialist

The investigator must also report the expected SAE to the site's IRB in accordance with the IRB's regulations or procedures.

Three months after the first subject is enrolled in the study, and at the end of each 3 months thereafter, if any SAEs (expected or unexpected) have been reported in the study during the preceding 3 months, the SDMC will:

- Use the current version of the MedDRA dictionary to code all AEs (serious or not) that have been recorded on study AE forms.
- Make a "snapshot" copy of the adverse events data, including MedDRA codes.
- Create frequency tables of treatment x occurrence (yes or no, since inception of the study) of all patients. One table will be created for each highest level MedDRA term for which SAEs have been reported. The counting units are patients, not events.
- Compute Fisher's Exact Test (FET) statistic to test the alternative hypothesis that occurrence of SAEs is not independent of treatment group. The FET p-value is not adjusted for multiplicity.
- If the FET p-value is less than the critical value shown in Table 3 and the active treatment group has a higher AE rate, the SDMC will conduct further statistical analyses as indicated by the circumstances and report the results to the Chair of the DSMB subcommittee monitoring this study, the Principal Investigator, and the NHLBI Project Officer.
- The SDMC will not file a report of expected SAEs if none of the FET p-values is less than the critical value shown in Table 3 or if the relative risk is less than 1.

11.2.7 Reporting of All Other Adverse Events

For any and all adverse events, study coordinators and/or Principal Investigators will use the standard procedure of documenting the event using the Adverse Events Case Report Form.

Six months after the first subject is enrolled in the study, and at the end of each 6 months thereafter, the SDMC will:

- Use the current version of the MedDRA dictionary to code all AEs that have been recorded on study AE forms.
- Make a "snapshot" copy of the adverse events data, including MedDRA codes.
- Create frequency tables of treatment x occurrence (yes or no, since inception of the study) of all patients. One table will be created for each highest level MedDRA term for which AEs have been reported. The counting units are patients, not events.
- Compute Fisher's Exact Test (FET) statistic to test the alternative hypothesis that occurrence of AEs is not independent of treatment group. The FET p-value is not adjusted for multiplicity.
- If the FET p-value is less than the critical value shown in Table 3 and the active treatment group has a higher AE rate, the SDMC will conduct further statistical analyses as indicated by the circumstances and alert the DSMB to this finding in the semi-annual DSMB report.
- Collaborate with the Principal Investigator to incorporate the results into the study's semi-annual report to the DSMB and the NHLBI Project Officer.

11.2.8 Reporting of Safety Laboratory Measures

Six months after the first subject is enrolled in the study, and at the end of each 6 months thereafter, the SDMC will:

- Make a "snapshot" copy of the study's clinical laboratory data.
- Perform an appropriate statistical analysis of clinical laboratory change-from-baseline data for each clinical laboratory evaluation obtained in this study.
- Perform an appropriate statistical test of: H_o: The mean (or median, or proportion, as appropriate) change-from-baseline of the clinical laboratory values for the active treatment group is the same as for the placebo group, vs. H_a: The mean (or median, or proportion) change-from-baseline in the active treatment group is "worse" than for the placebo group. The meaning of "worse" depends upon the specific clinical lab measurement. The test statistic p-value is not adjusted for multiplicity.
- If the hypothesis test p-value is less than the critical value shown in Table 3, the SDMC will conduct further statistical analyses as indicated by the circumstances and highlight this finding in the semi-annual DSMB report.
- Collaborate with the Principal Investigator to incorporate the results into the study's semi-annual report to the DSMB and the NHLBI Project Officer.

Table 11.3. Summary of Procedures and Timing for Alerting the DSMB and NHLBI Project Officer of Possible Serious Safety Issues

Situation or Event	Summary of Procedure (See text for details.)	Critical Value for DSMB "Alert"
Unexpected SAE	1. Site investigator notifies SDMC SAE Regulatory Specialist and Principal Investigator within 8 hours.	Alert all cases.
	2. Site investigator, SDMC SAE Regulatory Specialist and Principal Investigator prepare report using FDA forms and submit report to DSMB, NHLBI Project Officer, IRBs, study investigators. Report: 1. Fatal or life-threatening: within 7 calendar days. 2. Otherwise: within 14 calendar days.	Alert all cases.
Expected SAE	SDMC performs quarterly analyses of MedDRA-coded SAEs, tabulates patients with SAEs classified by highest level MedDRA term. Report only when p < critical value and active treatment group has higher AE rate.	$p < 0.01$ p not adjusted for multiplicity
Adverse Events (all)	SDMC performs semi-annual analyses of MedDRA-coded AEs, tabulates patients with AEs classified by highest level MedDRA term. Report every 6 months. Alert only when FET p < critical value and active treatment group has higher AE rate.	$p < 0.01$ p not adjusted for multiplicity
Adverse	SDMC performs semi-annual analyses of clinical lab	$p < 0.005$

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Clinical Lab Results	change-from baseline using analyses appropriate for the data type. Report every 6 months. Alert only when p < critical value and change is in "adverse" direction.	p not adjusted for multiplicity

11.2.9 Subject Discontinuation due to Adverse Event(s)

The following criteria will be used to determine whether or not specific subjects exhibit toxicities of the study drug(s) sufficient to require discontinuation from the study.

While oral arginine has been shown to have little toxicity when given in the doses outlined in this study, we will closely monitor signs, symptoms, and laboratory findings to assess for unexpected toxicities. Arginine will be discontinued in any patient who experiences the following:

--Drop in hemoglobin below 5gm/dL
--Pulmonary failure requiring intubation
--Hepatic dysfunction (SGPT ≥ 3X normal and albumin ≤ 3.0)
--Renal dysfunction (Creatinine ≥ 1.4 for children and ≥ 1.6 for adults)
--Focal neurological changes
--Increase in methemoglobin level to >2X normal level
--Apparent allergic reaction to arginine
--Severe headache
--Pregnancy

In addition, patients will also have arginine discontinued if they become unable to orally ingest arginine or at the request of the patient for any reason. In the event that arginine is stopped, patients will continue to be followed by study personnel to assess for potential side effects of arginine administration.

11.2.10 Pregnancy Testing

Arginine is not expected to have any significant toxicity in pregnant patients, however, it is unknown what the effect of pregnancy on NO and vascular biology will be. For this reason, pregnant patients will be excluded from the study. Female subjects who are pubertal will be regularly assessed for pregnancy throughout the study. In the event of a test indicating the study subject is pregnant they will be informed of this result and will immediately have arginine discontinued.

11.3 Data Collection and Data Monitoring

11.3.1 CRF and Source Documentation

The site study coordinator will complete a case report form (CRF) for each subject. A CRF manual will be provided to each site to assist in correct CRF completion. Study

participants must not be identified by name on any study documents. Subjects will be assigned and identified by CSCC subject numbers assigned at enrollment.

11.3.2 Data Management

The SDMC will provide statistical and data management support for the study. Validation rules will be applied at several points in the data management process. An error correction procedure will be applied to correct data values that fail validation rules.

11.3.3 Data Monitoring

After study initiation, individual sites will monitor CRFs and source documents for accuracy, protocol compliance, subject safety, and adherence to guidelines outlined in the CSCC Manual of Procedures. A member of the SDMC will visit the site to assess data quality at least once annually.

11.3.4 Reporting Protocol Violations

All protocol violations and deviations will be reported as soon as possible using the SDMC Deviation/Violation Form. This form will ask for a description of the event and what corrective action is planned.

12 STATISTICAL ANALYSIS

12.1 Sample Size

A total of 94 patients will be enrolled into this trial over an 18-month period (48 children and 46 adults). It is estimated that 6 to 8 clinical centers (12-16 patients per center) will be required to enroll sufficient patients in this period.

Initially, subjects were randomized to one of three treatment groups: placebo, low dose arginine and high dose arginine. With this amendment, subjects are now only randomized to placebo and low dose arginine. Subjects will be stratified by age group (5-17 years, ≥18 years) and since there could be drastic differences between these groups, they will be analyzed separately. Thus, all sample size calculations will be for each of these two groups.

We have identified three measurements that are of primary interest: Nitric Oxide, Gardos Channel Activity, and erythrocyte density (MCHC >41). We will measure our panel of investigative laboratory measures at various time points between baseline and sixteen weeks (up to eight timepoints). In order to avoid making more assumptions than necessary for our sample size calculations, we will use a simpler model that reflects a single timepoint. Specifically, we will use a one-way ANOVA with three groups (one for each treatment arm) modelling the change from baseline in each of our three measurements. We will additionally assume a two-sided alpha of 0.05. The hypothesis tested from this model will be any difference in mean change from baseline between the three treatment groups.

The literature suggests that the average NOx level in stable sickle cell patients is 25.5, with a standard deviation of 2.2. We assume that both groups start out with this mean and that the placebo group maintains this level across the length of the study. Based on preliminary findings, we expect a minimum increase of 1.75 for the high dose in the mean change from baseline NOx and the low dose to have a related 1.375 increase. If we assume that the variance is equivalent for the pre and post measurements, we can calculate the variance of the difference as the sum of the pre and post variances minus twice the covariance between pre and post measurements. Thus, our variance estimate depends greatly on the correlation between the pre and post measurements. Assuming a range of correlations (0.3, 0.5, and 0.7), we can then estimate the sample size we will need to detect the differences of 1.375 and 1.75 at 80% and 90% power. (See table below.)

Total sample sizes (for each age group) by power and correlation for NOx assuming a 1:1:1 randomization.

	Power		
		80%	90%
Correlation	0.7	27	33
	0.5	42	54

	0.3	57	72

Given that the goal of this trial is exploratory, not confirmatory, we do not need to achieve a significant value on any specific hypothesis test. Additionally, a much larger treatment effect is anticipated, potentially giving us more power. Thus, a reasonable sample size to achieve our goals and provide us with a reasonable estimate, with the potential to see a significant effect is in the range of 48 total subjects in each age group. With the elimination of one dose of Arginine, the sample size of 16 adults per remaining treatment group will be used.

Insufficient data is available in the literature at this time to sufficiently estimate the expected within subject variability (at steady state of SCD) for the Gardos Channel Activity and erythrocyte density (MCHC >41) as measured by Advia. Sample size calculations would therefore be based on unfounded assumptions and are therefore not provided.

Since the rate of dropout cannot be estimated at this time, completion of the study will be monitored and dropouts will be replaced as they occur. This should pose no problem due to the relatively short duration of the trial. All available data on dropouts will be used in the analyses.

Although clinical events, such as vaso-occlusive pain episodes will be analyzed secondarily in this trial, it is believed that enough information on these endpoints will be gathered to plan for an appropriately sized Phase III trial that would use a clinical endpoint in the primary hypothesis. Since all subjects must have had at least one vaso-occlusive pain event in the past year, it is estimated that for each age group, at least 24 events would occur. Although probably not enough to provide a confirmatory test of the effect of arginine on clinical outcomes, it should certainly be enough to provide planning estimates for the confirmatory trial.

12.2 Analysis Populations

Patients who receive any clinical trial material (any dose of arginine or placebo) will be included in the intent-to-treat analysis. All analyses will be performed on the intent-to treat population.

12.3 Statistical Considerations

12.3.1 Covariates

Baseline laboratory measurements will be controlled when analyzing laboratory outcomes. Other covariates may be considered in the analysis of clinical, laboratory, pharmacokinetic, and safety outcomes as needed.

12.3.2 Multi-center Studies

Randomization will be stratified by center to preserve a balance across treatment groups. Center effects will be examined as permitted by sample size.

12.3.3 Multiple Comparisons and Multiplicity

Since this is an exploratory Phase II study, analyses should be considered descriptive and no adjustments for multiple comparisons will be made.

12.3.4 Examination of Subgroups

All analyses will be stratified by age group children under 18 and adults 18 and over.

12.3.5 Missing Data

Data from patients who are lost to follow up will be used as much as possible since the analyses allow for missing data. If deemed necessary, a comparison of completers versus non-completers will be performed.

12.4 Statistical Methods

12.4.1 Study Population

Patient disposition, populations, demographic and baseline information will be summarized by age strata and treatment arm.

12.4.2 Efficacy

The goal of this study is to obtain laboratory and clinical information to justify a phase III clinical trial and to provide data to generate accurate power calculations for a subsequent phase III clinical trial. The two age groups (children and adults) will be analyzed separately to assess for differences in response to arginine therapy.

The laboratory variables NO, Gardos Channel activity, sickle red cell density (% of cells >41, as measured by Advia) are of primary interest. Additionally, sVCAM, H3 Technicon, Nitrotyrosine, 8-iso-PGF2α, Ektacytometry, ET-1, and ECHO will be measured and analyzed as secondary efficacy variables. Since the goal of this protocol is to gain insight into the effect of arginine on multiple physiologic measures (e.g. erythrocyte characterization, endothelial function and free radical biology), we will model the change from baseline for all of the collected measurements over time using random effects longitudinal mixed models, with treatment and baseline measurement as fixed effects. This class of model allows us to control for the correlation induced by having multiple measurements on an individual. We will focus on estimating the relationship between each of the laboratory measures and treatment group over time.

Additionally, by judiciously choosing the structure of the fixed effects over time, we can investigate non-linear dose relationships as well.

Each laboratory measurement will be summarized by age group (pediatric and adult), treatment arm, and measurement time.

Clinical efficacy is a secondary endpoint for this trial. Clinical efficacy data will be collected on all patients. Specifically, patients will be asked for information on emergency room visits and hospitalizations, clinic visits for pain, and pain medication use. All clinical efficacy measurements will be summarized by age group, treatment arm, and measurement time (where applicable). Treatment differences may be examined using appropriate methods as the data permits.

All efficacy data will be analyzed descriptively to provide information for future trials. All analyses will be performed on the intent-to-treat population. Model assumptions will be confirmed and if broken, appropriate methods will be investigated.

12.4.3 Safety Data

The assessment of safety data will be based mainly on the tabulation of adverse events (AE), by age group and treatment arm. Additionally, AEs will be tabulated by severity and relationship to drug. Serious AEs will be described in detail. Additionally, blood chemistry, hematology, and urinalysis laboratory measurements will be tabulated by age group, treatment group, and measurement time. Other specific laboratory measurements, such as Met Hb, will also be collected and tabulated by age group, treatment group, and measurement time.

12.4.4 Other Data

In addition to the efficacy and safety laboratory measurements, blood levels of arginine and arginase will be measured. These measurements will be summarized by age group, treatment arm, and measurement time. Additionally, graphic displays may be produced to examine trends in absorption over time. A dose response analysis may be performed if the data warrants. Echocardiograms will also be collected baseline and at the end of treatment with arginine to determine if pulmonary artery pressure is reduced by therapy with arginine.

12.4.5 Unmasked Interim Analysis and Masked Interim Data Monitoring

No interim analyses will be performed in this study.

13 HUMAN SUBJECTS PROTECTION

13.1 Discontinuation of Study

The National Heart Lung and Blood Institute (NHLBI) reserves the right to discontinue the study at any time for administrative reasons. A data safety and monitoring board will monitor the trial by periodically examining the unblinded safety data. The DSMB can recommend discontinuation of the study due to safety concerns at any time. Investigators will be reimbursed for reasonable expenses incurred to the date of discontinuation on the basis of completed subjects.

There are few known risks associated with taking arginine and the safety of the subjects will be monitored by an independent data and safety review board. Subjects can withdraw from the study at any time. Discontinuation of any part of the protocol does not interfere with the patient receiving their routine medical care.

The benefits of improving our understanding the mechanisms and impact of taking arginine in patients with SCD strongly outweighs the minimal risks associated with this therapy. If efficacious, this therapy could be used at little cost to improve the disease progression in other SCD patients.

All data collected for the purposes of this study will be kept as confidential as other medical records. Data will be stored in one location in a locked filing cabinet to be accessed only by study personnel with the direct permission of the principal investigator. No names will appear on the data; however, subject materials will be identifiable through a unique code number assigned to the subject at the beginning of the study. This master list with the subject's name and his/her identification number will be locked in a separate filing cabinet to further ensure confidentiality. Any published findings as a result of the study will not identify participants by name.

13.3 Disclosure of Data

The investigator, his or her staff and associates, and the appropriate regulatory agencies may use the information included in this protocol as necessary for the conduct of the trial and the safety of subjects. Data from the trial are confidential and may not be disclosed without the written permission of the NHLBI.

13.4 Publication of Research Findings

Manuscript(s) and abstract(s) prepared from the data collected during this trial will be prepared by the study investigators and the SDMC.

14 SUBJECT COMPENSATION

Patients will receive $500 for full participation in the study. Partial compensation will be given on a pro-rated basis based on number of study visits completed.

15 PROTOCOL SIGNATURE PAGE

I, _____, MD agree to conduct:

Arginine Supplementation in Sickle Cell Anemia: Physiological and Prophylactic Effects

I understand that no deviations from this protocol, _____(Version 6.0, April 2, 2007) may be made without the written permission of the NHLBI CSCC Protocol Chair, except where necessary to eliminate immediate hazard(s) to trial subjects, or when the change(s) involve only logistical or administrative aspects of the trial.

Signature: _____

Date: _____

This protocol is sponsored by
the National Heart, Lung, and Blood Institute.

16 LIST OF INVESTIGATOR(S) AND CLINICAL LABORATORY(S)

CENTER NUMBER	INVESTIGATOR	ADDRESS AND TELEPHONE OF STUDY CENTER
	TO BE DETERMINED	

RESEARCH LABORATORY	ADDRESS AND TELEPHONE
Children's Hospital and Research Center Oakland (Frans Kuypers' lab) Albert Einstein College of Medicine (Mary Fabry's lab) Dr. Jose R. Romero Endocrine, Diabetes & Hypertension Division, EBRC-201 Brigham and Women's Hospital	5700 Martin Luther King Jr. Blvd, Oakland, CA 510-450-7620 1300 Morris Park Ave, Aullman, Rm #921, NY (718) 430-2186 221 Longwood Avenue Boston, MA 02115 Tel: 617 732-5978

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