DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This description is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will be public information. There fore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

The purpose of this proposal is to foster the scientific development and laboratory skills of *the candidate* in order that *the candidate* may become an independent clinical investigator. The _____center at *the institution* and its _____ will provide *the candidate* with the ideal setting in which to investigate the impact of oral arginine administration on nitric oxide production in sickle cell disease (SCD) at the clinical, biochemical and cellular levels. Through the collaboration with the clinical mentor, _____ and an extensive network of experienced scientific and clinical researchers, *the candidate* will obtain the foundation for the development of an independent academic career.

Vaso-occlusion is responsible for most of the morbidity in SCD. The etiology of vascular obstruction in SCD is multifactorial, but mechanisms regulating vascular tone are likely to include nitric oxide (NO), as NO is one of the most potent vasodilators known. NO metabolites (NOx) are elevated in SCD patients at baseline, but decrease significantly during vaso-occlusive crisis (VOC) and acute chest syndrome (ACS). L-Arginine (L-Arg) is the precursor to NO, and *the candidate* has demonstrated that L-Arg levels are also decreased in SCD patients during VOC and ACS. *The candidate* has also recently demonstrated that _____. L-Arg therefore has the potential to alter the nature of VOC in SCD by increasing NO production. The specific aims of the proposal are: (1) to institute a blinded, placebo control, phase II/III clinical trial in SCD patients hospitalized with VOC to determine if oral L-Arg therapy will decrease the length of hospitalization, and (2) to characterize some of the biochemical and cellular effects of arginine therapy in SCD patients with VOC. The outcome of this proposal may impact both patient care and clinical assessment. It offers greater insight into the pathophysiology of SCD, and is the foundation for future studies. *The candidate* has demonstrated that an L-Arg deficiency may be involved in some of the vaso-occlusive complications of SCD, hence L-Arg supplementation may be a new therapeutic intervention that will improve quality of life for patients with SCD.

PERFORMANCE SITE(S) *(organization, city, state)*

_____ of _____

_____, _____, _____, _____ _____

KEY PERSONNEL. See instructions on Page 11. *Use continuation pages as needed to provide the required information in the format shown below.*

| Name | Organization | Role on Project |
|---|---|---|
| _____ | _____ | Principal Investigator |
| _____ | _____ | Mentor |
| _____ | _____ | Cosponsor |
| _____ | _____ | Cosponsor |
| _____ | _____ | Collaborator |

| | | |
|---|---|---|
| _____ | _____ | Co-Investigator |
| _____ | _____ | Co-Investigator |
| _____ | _____ | Consultant |
| _____ | _____ | Collaborator |

Advisory Committee:

| | | |
|---|---|---|
| _____ | University of _____ | Advisory Committee |
| _____ | University of _____ | Advisory Committee |
| _____ | University of _____ | Advisory Committee |
| _____ | University of _____ | Advisory Committee |

*Use this substitute page for the Table of Contents of Research Career Awards*

*Type the name of candidate at the top of each printed page and continuation page*

# RESEARCH CAREER AWARD

## TABLE OF CONTENTS

## *(Substitute Page)*

*Page Numbers*



*Note:* Type density and size for the entire application must conform to the instructions on page 6 of the general instructions.

*Include these items only when applicable.

CITIZENSHIP

[X] U.S. citizen or noncitizen national

[ ] Permanent resident of U.S.

[ ] If a permanent resident of the U.S., a notarized statement is included with the application

FACILITIES: Specifiy the facilities to be used for the conduct of the proposed research. indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

## RESOURCES

Laboratory: _____. All facilities capabilities and equipment described below are now available at this _____ site. The laboratories at *the institution* are equipped for biochemistry, hematology, cell biology, molecular biology, mass spectrometry, and microscopy (electron and light). All essential equipment is backed by emergency power; critical systems, including freezers, are monitored 24 hours/day. _____ Co-Directs the _____ of *the institution*. This laboratory contains the equipment for the measurement of biophysical and biochemical properties of red cell membranes, density profiles of subpopulations of red cells, and reactive nitric oxide species analysis through the Griess method.

Clinical: Human subjects will be recruited from the _____ patients followed by the _____. The use of human subjects at *the institution* is governed by the _____ under DHHS Assurance No. _____.

Animal: N/A

Computer: _____ computers are available throughout the laboratories and offices, and are linked in a network which includes seven laser printers as well as a Graphics Center. The Graphics Center includes two large-screen _____ computers, a flatbed scanner, slide scanner, slide maker, and color laser printer. Also provided on the internet network are weekly access to current journal titles, and two CD-ROM-based systems for online searching of medical databases and protein/DNA sequences.

Office: All investigators are equipped with private offices on site. Secretarial assistance is available to all investigators. Computers are supported by full-time computer specialists. Staff are available for grants management technology transfer, and facilities support.

Other: **Mass Spectroscopy**: This core facility is a resource for mass spectrometry in *the institution's* hemoglobin and red cell membrane research program. Its services include mass spectroscopy of proteins, peptide mapping by HPLC/MS, and metabolic studies based on stable isotope incorporations into biomolecules.
**Hemoglobin Diagnostics**: This laboratory _____assists in the diagnostic evaluation of variant hemoglobins. Using standard electrophoresis, thin-layer isoelectric focusing, HPLC, mass spectroscopy and molecular diagnostic procedures, a complete molecular and structural analysis of abnormal hemoglobins can be performed.
**Darkrooms**: *The institution's* darkrooms contain a photographic enlarger and equipment for printing, as well as a stand for UV illumination and photography of DNA sequencing gels. It also has a copy stand and high-quality camera for production of publication-quality photographs. An X-ray developer for autoradiographs is also available.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each. The mass spectrometry equipment _____

**Section II: Specialized Information**

**1. Introduction to Revised Application**

**2. Letters of Reference**

**3. The Candidate**

**a. Candidate's Background**

The Candidate:

*The candidate* developed a commitment to research well before medical school, where *the candidate* acquired valuable laboratory experience in the Department of Hematology/Oncology at _____, and in the Department of Immunology at the _____. It was at _____ that *the candidate's* interest in the biochemical and physiological effects of nitric oxide first evolved. *The candidate* was fortunate to have spent time in the laboratory of _____, Hematology Department Chair at _____ and one of the original pioneers in nitric oxide research. Dr. _____ became *the candidate's* first research mentor, and has continued to be a source of advice and encouragement to this day.

*The candidate* completed _____ fellowship training at *the institution* in June of 1999, and is currently an attending physician in the _____ department at the institution. During _____ fellowship, *the candidate* was able to combine _____ research efforts in the department with _____ special interest in hematology. *The candidate's* first project involved investigating the value of empiric chest radiography (CXR) in febrile children with sickle cell disease. *The candidate* found that febrile children with acute chest syndrome demonstrate few clinical signs and symptoms early in their course of illness. Because evaluating physicians often missed the diagnosis, a CXR is now a routine part of the febrile work-up for sickle cell patients at *the institution*. These findings were also reported at_____, as well as _____ and _____ published in _____. With the successful completion of this project, *the candidate* demonstrates the ability to forge a productive collaboration between the Departments of _____ and Hematology.

While engaged in _____ study, *the candidate's* participation at national meetings exposed ___ to the most current advances in sickle cell research. Recent attention to nitric oxide inspired *the candidate's* present research endeavor. Based on *the candidate's* past experiences in nitric oxide research and understanding of NO biochemistry, *the candidate* embarked upon a search to determine if an arginine deficiency could be involved in the low nitric oxide levels that were reported during pain crisis in sickle cell disease. *The candidate* noted that adult sickle cell patients do indeed have a deficit of arginine, although there was no data available on children. From these early observations, *the candidate* formulated the hypothesis and received endorsement from the _____ (_____) in 1998 to proceed with _____ proposal.

*The candidate's* preliminary data demonstrates that there is an association between the vaso-occlusive complications of sickle cell disease and the L-arginine-nitric oxide pathway. It has stimulated much interest within the hematology community _____. The results of the candidate's preliminary research have been submitted as a manuscript _____. *The candidate* is proposing to continue investigations through the research plan outlined in this proposal. This award will give *the candidate* the opportunity to explore the arginine-nitric oxide relationship further, and to develop a clinical trial to determine the potential therapeutic value of supplemental arginine in SCD.

As a Research Career Award (RCA) recipient, *the candidate* will have access to the resources of a major pediatric medical center and research institution. *The candidate's* clinical responsibilities will be carried out in the _____ Department of *the institution*. *The candidate* will devote at least _____ (percent) time to the proposed research in this application, and _____ toward service in the Department. _____, the candidate will continue to identify and enroll patients while carrying out clinical responsibilities.

**b. Career Goals and Objectives (Future Investigations): Scientific Biography**

The results of *the candidate's* preliminary data are very exciting and are the basis for further investigation. It appears that an arginine deficiency plays a role in the pathophysiology of sickle cell disease. As a safe dietary supplement, oral arginine is potentially a new non-narcotic therapy for a disease that is debilitating for both children and adults. This study has the potential to change the current management of sickle cell disease.

Further investigation into the biochemistry of nitric oxide and its metabolism also reveal another realm of potential research from a nutritional perspective. The vitamin and amino acid deficits that exist in the disease process may give some insight into the disease pathophysiology. Further knowledge about superoxide and its interactions with nitric oxide may reveal a significant role for anti-oxidants and superoxide scavengers such as Vitamin C as an adjunct to the present treatment of sickle cell disease.

The outcome of *the candidate's* proposed study could also lead to the development of other therapeutic trials, including the evaluation of the intravenous arginine treatment and the prophylactic role and long-term outcome arginine may play in decreasing the incidence or severity of vaso-occlusive events. As a phase II/III clinical trial, an RCA grant will support a study that will provide the data necessary for a multi-institutional trial of oral arginine therapy in SCD.

*The candidate's* preliminary data also demonstrates utility for the emergency medicine physician. Pain is the most common presenting complaint to emergency departments for patients with SCD. Many hours are spent in the ED on hydration and pain management awaiting improvement before the decision is made to admit to the hospital vs. discharge home, and the evaluation is very subjective. *The candidate* has shown that low arginine levels may be predictive for hospitalization, and that NOx levels are significantly lower than baseline in patients $\geq$10 years of age with VOC who require admission. Neither of these tests are rapidly available. If exhaled $NO_x$ levels correlate with serum $NO_x$ levels, such an analysis may provide an immediate quantitative, objective marker to identify patients more likely to warrant admission. Such a tool would be very helpful to the ED physician, as well as to the patient who may benefit from a more rapid admission process. This question will be answered as a secondary objective in *Specific Aims 2* of this proposal.

**c. Career Development Activities during Award Period**

A five-year career training grant will provide *the candidate* with the foundation necessary to transition into an independent clinical investigator. The supportive environment of the _____ (_____) at *the institution* provides an arena of expertise and mentorship ideal for career development. This proposal supports *the candidate's* long-term goal of becoming an independent clinical investigator within emergency medicine by allowing an opportunity for further didactic study, and by providing the building blocks by which _____ can create a career in clinical research.

**4. Statements by Sponsor(s), Consultant(s), and Collaborator(s)**

a. Sponsor/Mentor: _____.
Co-Mentor _____.

b. Consultants:
1) _____
2) _____
3) _____

**5. Environmental and Institutional Commitment to Candidate**

a. Description of Institutional Environment

*The institution* ranks among _____, and houses _____. The emergency department of *the institution* sees more than 40,000 children a year, and provides an excellent arena for clinical research. The

environment at *the institution* provides a unique opportunity for the collaboration of the ED with the Department of Hematology/Oncology, as the emergency department is often the first place that children with sickle cell disease present to with vaso-occlusive complications. During _____ fellowship, *the candidate* developed a commitment to clinical investigation, and was able to incorporate _____ ambitions with _____ interests in sickle cell disease. A Research Career Award will allow *the candidate* to attain the fund of knowledge necessary to become an independent clinical investigator, and provide _____ with the skills necessary to continue _____ research interests in the _____ department.

b. Institutional Commitment to Candidate's Research Career Development

1) Didactic Plan: The *institution*, and Departments of Emergency Medicine and Hematology/Oncology at _____ are committed to *the candidate's* development as an academic _____ and an independent investigator.

The Research Career Award will allow *the candidate* to pursue a didactic phase of study, which is essential to _____ development as an independent investigator. During _____ fellowship, *the candidate* had the opportunity to attend the annual Pediatric Emergency Medicine Fellows' Conference which provided workshops on *grant writing*, *research design in pediatric emergency medicine* and on *research ethics*. *The candidate* also completed the *Clinical Research Workshop* of the *ORACLE* course*, Outcomes Research and Clinical Epidemiology*, at the _____, _____ under the direction of _____ and _____. The course provided *the candidate* with the essential basic skills necessary to plan and conduct a clinical research project. *The candidate* was instructed in study design, sample size and power calculations, data management and data analysis. This class was crucial to the successful completion of _____ first research project that resulted in the manuscript "_____", recently published in the _____. The candidate also had the opportunity to present these results at several national meetings and at Grand Rounds at _____. The candidate's workshop mentor for this course was _____, who holds a position as an _____ in the _____ at the _____, _____, _____. *The mentor* was of great assistance during the course, and was also of significant help in developing the biostatistical plan for *the candidate's* initial arginine proposal to the _____ that resulted in the preliminary results for this grant application, and *the candidate* will continue to work with him closely.

In the first year of the Research Career Award, *the candidate* plans to enroll in the second phase of the _____ course that is offered at _____, _____. This course will provide *the candidate* with an overview of large database manipulation, meta-analysis, risk assessment and decision-making analysis. This will complete the prerequisites for the _____(_____) course, also offered at _____. It is a comprehensive 10 month course created by _____, that covers epidemiology, biostatistics, design methodology, bioethics and the implementation of clinical research projects and clinical trials. This course involves a 20 hour per week combined lecture, lab, and seminar series. Specific topics that are covered by the ATCR course include:

1. *Introduction to Biostatistics*
2. *Biostatistical Methods for Clinical and Epidemiological Research*
3. *Epidemiologic Methods*
4. *Clinical epidemiology*
5. *Clinical Trials*
6. *Conduct and Presentation of Clinical Research*
7. *Ethical Issues in Clinical Research:* Teaches responsible conduct in research
8. *Health Services Research*

In addition to the ethics education *the candidate* will obtain from the _____course, _____ will enroll in an additional course, _____ (_____), offered at _____. Directed by _____, _____, it is a forum for scientists to familiarize themselves with institutional policies/practices and professional standards that define scientific integrity. It gives an overview of ethics in research, authorship, patents, and human interest at the academic-commercial interface, and small group sessions for more extended discourse between students and faculty. Completion of this course fulfills the NIH requirement for instruction in the ethical conduct of research.

During the course of this award, *the candidate* also plans to improve upon _____ statistical background by enrolling in additional biostatistical courses offered at _____:

1. _____, a course on bivariate correlation, simple linear regression, multiple regression, analysis of variance and the interpretation of results
2. _____, a course covering modeling of categorical responses
3. _____, including logistic regression and log-linear models, with a focus on interpretation of results
4. _____, covering statistical methods for analyzing repeated measurement data including classical ANOVA and ANOVA-RM approaches, likelihood approaches and semi-parametric approaches.

In conjunction with _____ coursework, *the candidate* will also attend weekly research conferences within *the institution*, and will participate in quarterly _____ research meetings at which time updates of _____ research progress will be presented and discussed with the senior investigators. *The candidate* will also present her data to her colleagues in the _____ department monthly, at fellows' conferences. *The candidate* will continue to present _____ results to the members of the _____, as well as two national conferences, annually. *The candidate* will also have the opportunity present her work during Grand Rounds at _____.

2) Mentoring: *The candidate's* primary mentor for this project will be _____. *The mentor* is the _____ at *the institution*, and the _____ at *the institution*, and an _____at the _____, _____. *The candidate* will be working closely with the mentor. *The mentor* has been a great source of encouragement and advice during *the candidate's* previous research endeavors, and *the mentor* will continue to guide *the candidate's* career development. *The mentor* has supervised many junior researchers and hematology fellows, and has successfully served as a mentor to former Clinical Associate Physician award recipients. *The mentor* has published extensively on the complications of sickle cell disease, and is well suited to mentor *the candidate*. *The candidate* will have weekly discussions with the mentor concerning the clinical aspects of this project and the relationship between laboratory findings and the clinical outcome of patients with VOC. *The mentor* will closely monitor *the candidate's* progress, and will continue to assist in problem solving, and provide direction through any pitfalls that may arise during the completion of this project.

*The candidate* will also be working closely with _____, who is a _____ at *the institution*, and an expert in red cell membrane biology. _____ will act as a cosponsor for *the candidate*, supervising the laboratory component of this application. His laboratory is one of the few labs in the country that measures red cell deformability through ektacytometry, and he has helped *the candidate* with the successful determination of nitric oxide measurements that are accurate and reproducible. His expertise and laboratory supervision is essential for *the candidate's* project. Under *the cosponsor's* laboratory guidance, *the candidate* will be studying arginine's effect on nitric oxide metabolism and its impact on platelet function. *The candidate* will build upon _____ previous 2 years of laboratory experience, applying _____ skills to the performance of ELISA assays, use of mass spectrometry, and learning new laboratory skills as outlined in this proposal. *The candidate* will also become proficient in the use of the nitric oxide analyzer.

Director of *the institution*, _____ is also the Co-Director of the _____ and the Principal Investigator of the satellite _____ at *the institution*. _____ and _____ have been working together for over 25 years, and have successfully collaborated on many projects. The candidate will benefit from their many years of experience,

as _____ will also serve as a cosponsor. The environment at the institution is supportive of junior investigators and _____ has demonstrated a commitment to supporting *the candidate* in _____ endeavors.

*The candidate* will learn techniques involved in mass spectrometry under the guidance of _____, _____, _____, _____ at *the institution*. As a _____ and a world-renowned expert in mass spectrometry, _____ will be a valuable resource, as *the candidate* explores the potential deleterious effects of nitric oxide activity by measuring oxidative damage.

In addition to this impressive group of clinical and scientific investigators, *the candidate* will be consulting with several other individuals whose expertise is pertinent to _____ project. Dr. _____, from the _____ at _____, has done extensive nitric oxide research with a particular interest in the relationship of $NO_x$ and endothelin-1 in pulmonary disease. With expertise in nitric oxide metabolism, Dr._____ is a valuable resource. Interpretation of pain score data will be done under the guidance of _____, _____ from the _____ at _____. Dr. _____ is an expert in sickle cell pain evaluation, and supervises the pain management program for sickle cell disease at the institution. Dr. _____'s assistance will be helpful in measuring a significant outcome measure for this project. *The candidate* also has the support of _____, _____ at the institution, and a current Clinical Associate Physician Award recipient. Dr. _____ is a successful clinical researcher, and _____will help to identify patients for enrollment, and will serve as an additional consultant within the Department of Hematology. *The candidate* is also collaborating withDr. _____, _____, who is the _____ at the institution. Dr. _____ will oversee the randomization and distribution of oral arginine and placebo in this clinical trial.

The collaborative efforts and talents of these consultants and mentors will promote *the candidate* pursuit to become an independent clinical investigator.

3) Role for the _____: The support from the _____ has enabled *the candidate* to gather _____ preliminary data, and has introduced _____ to the many levels of expertise that are available within this research establishment. The candidate will continue to work closely with the *institution* _____ nurses and staff, as in _____ preliminary study. Once the clinical trial is underway, *the candidate* will rely on the support of members of the _____ on a daily basis. Together they will identify and enroll eligible patients, obtain consent, collect blood samples, administer the arginine or placebo, and utilize the exhaled NO analyzer. *The candidate* will expand _____ study to include outpatient follow-up in order to obtain baseline exhaled $NO_x$, serum $NO_x$ and arginine levels on enrolled patients, which will take place at the _____. *The candidate* has already established a relationship with Dr. _____, _____ at the _____, _____, and will continue to utilize _____ statistical expertise throughout the duration of this grant.

*The candidate* has also had extensive involvement with _____ lab group at *the institution*, as they have been essential to perfecting the assay to measure nitric oxide metabolites. They have also been very supportive of this project, and _____ has been particularly helpful in providing guidance through the very complex biochemistry of nitric oxide as it may pertain to sickle cell disease.
_____ will provide space in _____ laboratory for *the candidate* to perform _____ investigations, and _____ will continue to oversee _____ work, serving as a valuable resource.

**6. Research Plan**

**a. Statement of Hypothesis and Specific Aims**

Vaso-occlusion is at the heart of most of the morbidity of patients with sickle cell disease (SCD). Vaso-occlusion was long thought to result from the mechanical obstruction of the vasculature by "sickled" cells but it is now appreciated that many other factors play a role in the pathogenesis of vaso-occlusion. Such factors include the abnormal adherence of sickle red blood cells (SRBC) to the vascular endothelium, changes in vascular tone, and the contributions of other blood elements such as platelets, WBC, and plasma proteins.

Nitric oxide (NO) is a potent vasodilator which has also been shown to decrease platelet activation,

decrease adhesion receptor expression on the vascular endothelium and decreases vascular smooth muscle proliferation[1,2]. All these factors would likely impact favorably on vaso-occlusion in SCD. NO metabolite levels ($NO_x$) are normal or slightly increased in SCD patients at baseline but decrease significantly during vaso-occlusive crisis (VOC) and acute chest syndrome (ACS). Thus, under these circumstances, vasoconstriction is more likely to occur in the micro-vascular system. Arginine is the precursor to NO and arginine levels are low in SCD patients, decreasing further during VOC and ACS. My hypothesis is that arginine, by increasing NO production, has the potential to alter the nature of vaso-occlusion in SCD. My preliminary data documents that the administration oral arginine increases $NO_x$ production in SCD patients with VOC. The specific aims of this proposal are as follows:

1) **To institute a prospective, double-blind, placebo controlled phase II/III trial in SCD patients hospitalized with VOC to determine if oral arginine therapy will decrease the length of hospitalization for VOC.**

2) **To characterize the biochemical and cellular effects of oral arginine administration in SCD patients with VOC.**

**b. Background, Significance, and Rationale**

**Nitric Oxide and Vaso-Occlusion**

Vaso-occlusion is responsible for most of the morbidity in sickle cell disease (SCD), including painful crisis, acute chest syndrome (ACS), stroke, priapism, aseptic necrosis of bone, and leg ulcers. [1,2] The etiology of vascular obstruction that occurs in SCD is multifactorial. Some mechanisms believed to influence vaso-occlusion include abnormal adherence of sickle erythrocytes (SRBC) to the vascular endothelium, cytokine upregulation, increased platelet activation and altered vasomotor tone. [1,2] Factors involved in vaso-regulation are likely to include nitric oxide, as NO is one of the most potent vasodilators known.[3] The actions of NO are not confined to regulation of vascular smooth muscle tone. The diversity of biological functions include regulation of platelet reactivity[4], nonadrenergic, noncholinergic neurotransmission, and the cytotoxic and cytostatic actions of inflammatory cells. It decreases endothelial cell adhesion receptor expression, and is involved in the maintenance of blood pressure,[3,5-10] and release of growth hormone. [11,12]. With these vital functions, NO is crucial in maintaining physiologic homeostasis. An imbalance of NO production has been implicated in several disease processes and inflammatory conditions that can lead to tissue damage. Hence, the biological response of NO, whether beneficial or deleterious is determined by timing, location and amount of NO present under a given circumstance.[4,13]

**L-Arginine and Nitric Oxide**

L-Arginine (L-Arg) is the precursor to nitric oxide, catalyzed by a family of enzymes, the nitric oxide synthetases (NOS) via the L-arginine-nitric oxide pathway. Through this mechanism arginine is converted to citrulline, and nitric oxide is produced[3,5,6,9,14]. Interestingly, patients with SCD have been shown to be significantly deficient in L-Arg despite adequate dietary intake when compared to healthy non-SCD controls.[15,16] Sickle cell disease patients also have a high concentration of plasma citrulline when compared to normal controls.[16] This may be due to increased utilization of arginine and $NO_x$, leading to higher citrulline levels. Alternatively, there may be an abnormal metabolism of arginine in SCD, but decreased urinary excretion of the amino acid compared to normal controls[16] supports the hypothesis of increased utilization. An increase in vaso-constriction may result from conditions that interfere with the L-arginine-nitric oxide pathway, including a deficiency in substrate[17-19]. Such a condition has been demonstrated in several disease processes[17,19,20], including an arginine induced restoration of endothelial-dependent vasodilation in hypercholesterolemic patients [17,21,22] and in patients with coronary artery disease.[23]

**Nitric Oxide in Sickle Cell Disease**

I would like to propose a model for VOC in SCD. Several studies have reported that adults with SCD have increased $NO_x$ levels at baseline as compared to normal controls [24]. Because of potential on-going intermittent vaso-occlusion, SCD patients may be in a constant state of increased demand to initiate a vasodilatory process. This could explain high baseline levels of $NO_x$ in SCD. With a severe crisis, baseline compensatory mechanisms may be overwhelmed. NO can subsequently become depleted, especially in the presence of an L-Arg deficiency. This hypothesis is supported by a recent study that demonstrated significantly reduced levels of serum $NO_x$ during VOC and ACS[25], as well as my own preliminary data showing low serum L-Arg and $NO_x$ levels during the vaso-occlusive complications of SCD in children.[26]

Because most of the morbidity of SCD results from vaso-occlusion, NO production is likely to play an important role in the pathophysiology of SCD. In support of this, plasma NO metabolite levels ($NO_x$, measured as nitrite and nitrate) have recently been correlated with pain scores experienced in SCD patients with vaso-occlusive crisis [27]. Plasma $NO_x$ levels inversely correlate with pain scores with higher $NO_x$ levels associated with lower pain scores. My preliminary data also demonstrates that children $\geq$ 10 years of age with VOC requiring hospitalization had presenting $NO_x$ levels that were significantly lower than baseline . Since adults with SCD are significantly deficient in L-Arg [15,16], and children with VOC demonstrate substantially reduced arginine levels upon initial evaluation [26,28], my hypothesis is that low levels of arginine may contribute to complications in SCD, by becoming the rate-limiting substrate for NO production.

The hypothesis that a state of upregulated NO production as a compensatory mechanism in SCD is similar to what has been reported for asthma. An expression of inducible NO synthase (NOS) has been found in the epithelium of asthmatic patients but not in healthy non-asthmatic patients.[29,30] $NO_x$ can also be measured in exhaled air[31,32], and asthmatics have exhaled air $NO_x$ levels that are 3.5 times higher than non-asthmatics.[33] This initially led to the assumption that NO was associated with bronchoconstriction, but when NO production was blocked by L-Arg analogues, an increase in allergen-induced bronchoconstriction occurred. This suggests a protective role for NO in asthma. Thus, NO may be acting as a feedback against bronchoconstriction[34], just as NO may be acting as a feedback against vasoconstriction in SCD. The involvement of NO in asthma may also be relevant in SCD as many patients also have hyperreactive airways. As with asthma, baseline exhaled $NO_x$ concentrations are also significantly higher in anemic animal models[35]. Since exhaled $NO_x$ has never been monitored sequentially in sickle cell patients, it may represent another means to measure and follow nitric oxide levels.

Nitric oxide therapy by inhalation has been used for years to manage pulmonary hypertension and severe respiratory distress syndrome in newborns. Recent studies have demonstrated that inhaled NO normalizes oxygen $P_{50}$ in SCD[56], and it has also shown promise by improved oxygenation in several children with ACS[57]. Thus, the upregulation of nitric oxide production may be beneficial in SCD.

**Peroxynitrite**

It has recently been demonstrated that patients with SCD have an increase in superoxide production in vitro, compared to normal controls [36,37]. It has also been reported that NOS synthesizes superoxide in lieu of NO under conditions of lower L-Arg concentrations[38]. NO rapidly reacts with superoxide to form peroxynitrite, a potent mediator of cellular injury[38-40]. Thus, low levels of serum $NO_x$ in SCD may reflect a shunting of NO away from nitrite/nitrate and towards the production of peroxynitrite, particularly under conditions of diminished arginine availability. Thus, it is possible that by increasing the serum level of arginine, we may be able to decrease tissue damage secondary to peroxynitrite generation in patients with SCD.

**Endothelin-1 (ET-1)**

A balance between vasoconstrictive and vasodilatory agents is essential to maintaining normal vascular tone. Endothelin-1 (ET-1) is the most potent vasoconstrictor known [41], and has been shown to be elevated in SCD[42-45]. Like nitric oxide, ET-1 is also derived from endothelial cells. Recent studies have indicated that ET-1 is significantly elevated during VOC and ACS. [44]. NO directly inhibits ET-1 production and release [41]. Hence, low $NO_x$ levels found during vaso-occlusive events in SCD[25-27] may also impact vasoconstriction by allowing an upregulation of ET-1. Recent trials of L-Arg for cardiovascular disease have demonstrated that supplemental arginine decreases circulating plasma ET-1 levels [23,46]. Thus, addition benefits of supplemental L-Arg in SCD may result from the upregulation of NO production as well as the potential inhibition of ET-1 release.

**L-Arginine Therapy**

Arginine is a nutritional supplement with very low toxicity and few side effects. There is much accumulated evidence suggesting that high doses of L-Arg (30-60gms/day) are well tolerated in humans [11,12,23,47-51]. Both intravenous (IV) and oral L-Arg have been safely and effectively trialed in CF patients[48], as well as patients with both renal and cardiovascular disease.[14,18,20,21,23] Studies using high doses of arginine butyrate in patients with SCD were well tolerated. [50, 51]

Oral arginine is well absorbed by the GI tract reaching peak plasma concentrations after about 2 hours on an empty stomach. It is metabolized by the liver, and is almost completely reabsorbed once filtered at the glomerulus by the renal tubules. Individual utilization will vary based on levels of stress and activity. High doses of supplemental arginine will competitively inhibit absorption of lysine, which is a theoretical side effect, as lysine is a potent antiviral agent[52]. As sickle cell patients may have liver and renal dysfunction, metabolic studies should be followed.

Recent studies have demonstrated that oral L-Arg significantly increases serum arginine levels and $NO_x$ levels (in both serum and in exhaled air) in healthy non-SCD adults.[53]. L-Arginine has already demonstrated potential for therapeutic utility. In animal studies, inhalation of low doses of L-Arg completely blocks hyper-responsiveness of reactive airways [29,30]. Infusions of L-Arg initiate release of growth hormone due to NO [11,12]. Supplemental dietary arginine accelerates wound healing[47], and as a result, L-Arg is now routinely added to many commercially available enteral and parenteral nutritional formulas. Inhaled L-Arg improves pulmonary functions of cystic fibrosis (CF) patients[48], and there is also growing evidence that arginine has some benefits for diabetes-associated abnormalities[18] and cardiovascular disease[23]. It has been suggested that L-Arg has a stabilizing effect on sickle-hemoglobin[54]. Another interesting finding is that rapid healing of chronic leg ulcers during arginine butyrate therapy was noted in several patients with SCD. Leg ulcer healing occurred in patients treated with arginine butyrate despite little change in fetal hemoglobin,[55] which is thought to be butyrate's mechanism of action.[50, 51] It is possible that it was butyrate's arginine component that may have been the therapeutic agent, by causing increased perfusion to the damaged tissue through NO generation.

As the precursor to nitric oxide, supplemental arginine has demonstrated benefits in many disease processes that involve endothelial dysfunction[18,b 23], and may also have therapeutic value SCD.

**c. Preliminary Studies and Results**

I hypothesized that low arginine levels might contribute to some of the complications in SCD, possibly by becoming the rate-limiting substrate for NO production. I prospectively examined L-Arg and $NO_x$ levels in children with SCD at baseline and during vaso-occlusive crisis. Thirty-six SCD patients with 39 episodes of VOC and 10 patients at baseline were evaluated. In contrast to the arginine deficiency found in adults with SCD, baseline L-Arg levels were not lower in children with SCD when compared to normal controls. However, they dropped significantly during VOC (mean±SE: **37.4 ± 2.7 vs. 53.6± 4.6 μmol/L, p=0.008, Fig 1**), with lowest levels found in VOC patients requiring admission.

**Figure 1:** Comparison of mean L-Arginine levels in **Normal Controls** (n = 20), SCD patients at baseline (**SCD Baseline**, n = 10), SCD patients with VOC discharged home from the ED (**VOC Discharged**, n = 18), and SCD patients with VOC admitted to the hospital (**VOC Admitted**, n = 16). All p values reflect comparisons to values of SCD patients at baseline. L-Arg levels are significantly lower then baseline upon presentation for VOC, and may help to identify patients warranting hospitalization. [28]

Low L-Arg levels in patients with VOC returned to baseline during convalescence in the hospital (**Fig 2A**). It is possible that low arginine levels during VOC reflect a poor nutritional state during illness, which resolves with hydration, pain therapy and an improved appetite during the hospitalization. My sequential data on patients who developed ACS during their hospitalization, however, implicates another process independent of diet. Patterns of L-Arg (**Fig 2**) and $NO_x$ (**Fig 3**) appear to be different under the circumstances of VOC when compared to patients who develop ACS. L-Arg levels were near baseline at presentation for patients with VOC who developed ACS, but dropped during hospitalization within 24 hours of the chest radiograph becoming positive (**Fig 2B**), This drastic change in L-Arg concentration occurs despite adequate hydration, pain control and diet. Arginine levels return to baseline as ACS resolves.

**Figure 2** : L-Arginine levels in SCD patients admitted for uncomplicated VOC (**A**, n = 10) and those with VOC who developed ACS (**B**, n = 4) **Midway**= L-Arg levels corresponding to the lowest nitric oxide metabolite level during the admission. **Low**= lowest L-Arg level during admission. All p values reflect comparisons to SCD patients at baseline.

$NO_x$ levels also dropped significantly from steady state during hospitalization in patients admitted for VOC, with lowest levels found in patients developing ACS (**25.5±2.2, 17.4±2.4, and 12.3±1.6 □M,** respectively, $p<0.05$, **Fig 3**). $NO_x$ levels at the time patients presented to the emergency department (ED) with VOC were similar to baseline, yet VOC patients who developed ACS had low presenting $NO_x$ levels compared to steady-state **(18.3±2.3 vs. 25.5±2□M**), despite normal arginine levels.

In contrast to low presenting $NO_x$ levels documented in adults with VOC, $NO_x$ levels at the time of presentation to the ED in children with VOC were near baseline, and there was no significant difference

between mean NOx levels for patients discharged from the ED compared to children requiring admission (**22.5±2.1 vs. 19.8±1.8 µmol/L,** respectively). For patients ≥ 10 years of age, however, presenting NOx levels were significantly lower than baseline (**25.5±2.2 vs. 17.1±2.6 µmol/L, p = 0.025, n = 9**) in VOC patients requiring admission. This suggests that $NO_x$ levels may represent an addition tool in the evaluation of pain crisis in SCD patients ≥ 10 years of age, in that low levels may be predictive of a more severe crisis requiring hospitalization.

**Figure 3**: Serum $NO_x$ levels in SCD patients with uncomplicated VOC (n = 10) and those who developed ACS (n = 9). **Low** = lowest $NO_x$ level during admission.

The drop in $NO_x$ levels during hospitalization in patients developing ACS corresponds to changes in L-Arg levels (**Fig 4**). An initial rise in $NO_x$ is noted as its arginine substrate is utilized. It is possible that as L-Arg becomes depleted, $NO_x$ production decreases, or is shunted towards other reactive nitric oxide species (RNOS).

**Figure 4**: L-Arginine and nitric oxide levels in a representative patient admitted for VOC who subsequently developed ACS. Day 0= day ACS diagnosed. **TXN**=transfusion. Lowest levels of L-Arg and $NO_x$ are demonstrated within 24 hours of the discovery of ACS on chest x-ray.

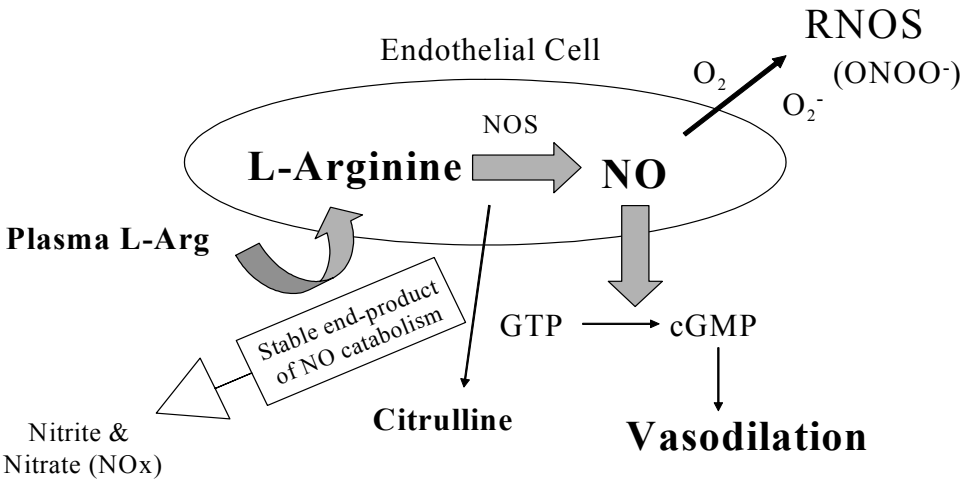
Four patients who developed ACS also had baseline $NO_x$ levels determined. Steady-state $NO_x$ levels were nearly double that of the values obtained during ACS, confirming that $NO_x$ is significantly reduced in ACS (**11.2±1.6 vs. 22.1±1.6 µmol/L, p = 0.003**).

**This data suggests that there may be a relationship between the L-arginine-nitric oxide pathway and vaso-occlusion in SCD**. Arginine is the precursor to nitric oxide; hence arginine stores may become depleted as a result of the increased demands for nitric oxide as a feedback against ongoing vaso-occlusion in SCD. Support for this hypothesis lies in the demonstration of high nitric oxide levels in adult SCD patients at baseline, with low baseline arginine levels in adults, and low-normal arginine levels in children. It is possible that during times of VOC, this protective mechanism of upregulated NO production is overwhelmed, leading to an acute arginine depletion and a subsequent drop in NO levels that resolves as the acute crisis resolves. **These results lead me to hypothesize that low arginine levels during VOC reflect a state of acute substrate depletion, possibly impeding the compensatory increase in NO**

**production that would be expected from vaso-occlusion.** $NO_x$ levels may also correlate with clinical course (**Fig 4**), and rising $NO_x$ levels may reflect improved vascular perfusion during resolution of VOC.

The variation in patterns of L-Arg and $NO_x$ levels during ACS compared to patients with uncomplicated VOC may indicate another mechanism in addition to arginine substrate depletion during the development of ACS. ACS evokes a significant inflammatory process causing the upregulation of many cytokines[25,58,59]. Superoxide production is also increased in SCD patients[36]. Superoxide rapidly reacts with and inactivates NO[38]. Sickle erythrocytes have been shown *in vitro* to interfere with endothelium-dependent vaso-relaxation by inhibiting NO, an effect that is significantly diminished by superoxide dismutase [60]. Increased superoxide production in SCD may contribute to the development of vaso-occlusive events, through the inactivation of nitric oxide. During ACS, it is possible that $NO_x$ levels decrease as a result of reactions that shunt NO towards production of other reactive nitric oxide species (RNOS). In the presence of superoxide, NO will react to form peroxynitrite ($ONOO^-$)[39,40,60-63], a potent oxidant and mediator of cellular injury (**Fig 5**). In particular, peroxynitrite has been implicated in lung injury [64]; thus it may play a destructive role in ACS. Support for this hypothesis would be found in the demonstration of increased peroxynitrite levels in those patients who developed ACS as their $NO_x$ levels dropped. If further studies indicate that increased superoxide production during inflammatory complications of SCD causes increases in peroxynitrite, these patients may benefit from the addition of a superoxide scavenger such as vitamin C to avoid such a shift in NO metabolism.

**Figure 5:** Simplified Biochemistry of NO




In light of studies indicating that L-Arg increases $NO_x$ production in healthy adults, and my data suggesting that an arginine deficiency may impact vaso-occlusion, arginine supplementation may prove beneficial for SCD patients by resulting in an increase in $NO_x$ production. I assessed whether supplemental L-Arg results in an increase in $NO_x$ in SCD patients by administering oral L-Arg (0.1gm/kg) to 6 steady state SCD patients and 4 normal controls. L-Arg and $N0_x$ levels were determined at baseline, and followed sequentially over a 4-hour period. As expected, baseline Arg levels were lower (mean±SD: **45.5̣10.6 vs. 81.2̣20.5□M**) and $NO_x$ levels higher (**24.0̣8.9 vs.15.9̣10.8□M** respectively) in SCD patients compared to normal controls. Serum Arg concentrations rose in both groups, peaking at approximately 2 hours after arginine administration (**144.7̣30.3 in SCD patients**, and **164.5̣43.4 □M in controls, Fig 6**). SCD patients

may utilize their arginine stores more quickly than controls, as L-Arg levels decrease more rapidly in the SCD patients by the end of 4 hours, although this difference did not reach statistical significance.

**Figure 6:** Rise in plasma L-Arg levels in SCD patients and normal controls after oral arginine (0.1gm/kg)

Control patients had a median increase in endogenous $NO_x$ levels of **+26.4%**. Remarkably, $NO_x$ levels decrease in all sickle cell patients, with a median % change of **-18%** (**Fig 7**). This data demonstrates that SCD patients at baseline do not respond to L-Arg supplementation with the same increase in $NO_x$ as was seen in normal controls.

**Figure 7**: Percent change of $NO_x$ levels from baseline (Time 0) in normal non-SCD **controls** (n=4) vs. **SCD patients** at steady-state (n=6) over 4 hours after oral arginine administration. $NO_x$ levels begin to rise immediately in normal controls, but decrease consistently in all SCD patients at steady-state.

In order to determine if this unique response to L-Arg in SCD patients at baseline was dose-dependent, I also administered a higher oral L-Arg dose (0.25g/kg) to SCD patients at steady-state (**Fig 8**).

Similar $N0_x$ responses were found after both low and high doses of L-Arg in patients at baseline, despite differences in peak L-Arg levels (mean peak L-Arg levels±SD: **170.7±41.2 vs**. **278.0±116.7μM**), indicating that L-Arg concentration is not a rate limiting factor for $NO_x$ production in SCD patients at baseline.

**Figure 8:** Percent change of serum $NO_x$ levels from baseline (Time 0) in SCD patients at steady-state after oral arginine administered at **low dose** (0.01g/kg, n=6) vs.**high dose** (0.25g/kg, n=3), vs.normal non-SCD **controls given low dose** (0.01g/kg, n=4).

In crisis, however, L-Arg may become rate limiting, as we have previously reported low L-Arg and $NO_x$ levels in SCD patients with VOC. Low dose L-Arg (0.1g/kg) was administered to 4 SCD patients with VOC (**Fig 9**). Unlike SCD patients at baseline, patients with VOC had a dramatic **increase** in $NO_x$ production after oral L-Arg (median % change: **–18.0% at baseline vs. +65.5% during VOC**). This response was even greater than in non-SCD normal controls, (median % change: **+65.5% in VOC patients vs. +26.4% in controls**).

**Figure 9**: Percent change of serum $NO_x$ levels from baseline (Time 0) after oral arginine administration in SCD patients with **VOC vs. steady-state**. VOC patients were given low dose L-Arg (0.1g/kg, n=4), compared to SCD patients at steady-state, who received both low dose (0.1g/kg, n=6) and high dose (0.25g/kg, n=3) L-Arg supplementation. Significant increases in $NO_x$ levels in SCD patients during VOC suggests that L-Arg concentration becomes the rate limiting factor in VOC.

Interestingly, peak L-Arg levels were significantly lower in the VOC group when compared to baseline patients receiving the same low L-Arg dose (mean±SD: **93.8±26.6** vs. **170.7±41.8μM**, **p=.01**), suggesting an

accelerated metabolism of arginine under the conditions of VOC. This data also suggests that, in contrast to SCD patients at baseline, L-Arg *is* the rate-limiting substrate for NO production in patients with VOC. Subsequently, L-Arg may become depleted as a result of increased utilization of the amino acid in order to meet a greater demand for NO, leading to an acute arginine deficiency.

**Pharmacokinetics of L-Arginine**

No dose-response studies have been reported as yet that determine the minimum amount of L-Arg required to restore NO activity [20], and the half-life of L-arginine is unknown. A recent study investigated the pharmacokinetics of L-Arg during chronic administration to patients with hypercholesterolemia, and was unable to determine the half life and volume distribution of this amino acid because of the spontaneous variation in L-Arg plasma concentrations over 24 hours[65]. Dietary amino acids are usually metabolized within 4-6 hours, suggesting that multiple doses are needed. My preliminary data demonstrates that the elevation in arginine in SCD patients lasts for at least 4 hours but the length of $NO_x$ elevation remains to be determined. Individual utilization of arginine will vary, and it is influenced by stress, activity level and nitrogen balance. The catabolic state found in SCD may also impact arginine metabolism.

My results have demonstrated that SCD patients at baseline do not respond to oral arginine supplementation at a dose of 0.1g/kg with the same increase in $NO_x$ production as found in normal controls. I have also determined that the same 0.1g/kg dose of L-Arg results in a significant increase in $NO_x$ production during VOC, during which time the amino acid appears to be more rapidly metabolized. In order to determine if upregulation of $NO_x$ production during VOC is dose-dependent, I have preliminary data on several L-Arg doses (0.05, 0.1, and 0.25g/kg) administered to SCD patients with pain. High dose L-Arg supplementation (0.25g/kg, n=1) produced the expected increase in $NO_x$ production, with a maximum increase of **80%**, 4 hours after L-Arg administration. Although this is data from only one patient, the response to high dose L-Arg is similar to responses from the lower oral dose of 0.1g/kg, suggesting that a higher dose of arginine does not necessarily translate into a greater effect on $NO_x$ production. Lowering the dose of L-Arg further (0.05g/kg), however, does not appear to provide sufficient enough substrate to maintain the increase in $NO_x$ production, and ultimately results in a significant decrease in endogenous $NO_x$ by nearly **–50%** in all patients. (**Fig 10**)

**Figure 10**: Maximum percent change in $NO_x$ production in SCD patients with VOC after oral L-Arg **low dose** (0.05g/kg) vs. **intermediate dose** (0.1g/kg).

Although there is significant variability between patients, data I have gathered on one representative patient strongly supports the use of the intermediate L-Arg dose (0.1g/kg). At steady-state, the patient experiences a paradoxical **decrease** in $NO_x$ levels after oral arginine (0.1g/kg) that is sustained throughout the 4 hours

without much fluctuation, (**Fig 11**). The same dose of oral L-Arg produces a significant **increase** in $NO_x$ production during VOC that persists with continued acceleration 2 hours after L-Arg administration. Interestingly, a paralleling **decrease** in endogenous $NO_x$ occurs during a separate VOC event after supplementation of oral L-Arg at the lower dose (0.05g/kg).

**Figure 11**: Percent change in $NO_x$ levels From baseline (Time 0) in a representative SCD patient after oral L-Arg administration at steady-state (0.1g/kg), and during VOC (0.05g/kg and 0.1g/kg).

The cause of decreasing endogenous $NO_x$ levels after low dose oral L-Arg is uncertain. My data reveals that peak L-Arg levels are significantly lower in VOC patients when compared to SCD patients at steady-state receiving the same dose of arginine, suggesting an accelerated metabolism of the amino acid during VOC. It is possible that supplementation with a lower L-Arg dose may provide enough substrate to activate nitric oxide synthase, (NOS), but is insufficient to sustain increases in $NO_x$ production. Previous investigations have shown that NOS will synthesize superoxide in lieu of NO under conditions of lower L-Arg concentrations[38]. It is possible that superoxide plays a role in this paradox, but regardless of the mechanism, low-dose L-Arg appears to be subtherapeutic in SCD under conditions of VOC.

In summary, my preliminary findings lead me to hypothesize that an acute arginine deficiency plays a role in VOC, possibly by affecting NO production. Arginine supplementation, a therapy with low toxicity even at high doses, may prove beneficial for SCD patients by resulting in an increase in $NO_x$ production during VOC. I propose a prospective, blinded, placebo control trial of supplemental arginine in SCD patients with VOC designed to test this hypothesis.

**Significance**

Vaso-occlusive crisis is responsible for most of the morbidity in sickle cell disease. My data suggests that an arginine deficiency may be involved in some of the vaso-occlusive complications of this disease process. As a safe, nontoxic dietary supplement, oral arginine may prove beneficial for SCD patients by resulting in an increase in nitric oxide production, and is potentially a new therapy for the treatment of vaso-occlusive crisis. The outcome of this study has the potential to change the current management of sickle cell disease. It also lends itself to the development of future investigations. As a phase II/III clinical trial, it has the potential to lead to a multi-institutional trial in the future, and supplemental arginine may also prove to have prophylactic benefits warranting an outpatient clinical trial. Information gathered from Specific Aim 2 will add to the growing understanding of the biochemical aberrations that occur in SCD. This may also lead to new therapeutic treatments that target these specific abnormalities.

Nitric oxide research is a rapidly growing area of interest. This study will also determine whether a correlation exists between plasma and exhaled nitric oxide metabolites in patients with SCD. If similar patterns are demonstrated, exhaled $NO_x$ may represent a rapidly available, objective clinical marker for the ED physician evaluating sickle cell pain. Such a tool does not presently exist. Patients with VOC would then benefit from a more expedient admission process, rather than the long ED stays that are routinely experienced before any disposition decisions are made.

The results of this proposal may impact both patient care and clinical assessment. It offers greater insight

into the pathophysiology of SCD, and it is also the foundation for future studies. It also holds the potential to improve quality of life for patients with sickle cell disease.

**d. Research Design and Methods**

The overall research plan is to institute a phase II/III trial to determine if oral administration of L-Arg increases $NO_x$ production, and if this has a beneficial effect on the clinical events associated with VOC. As part of this plan, several biochemical and cellular effects of L-Arg administration resulting in increased nitric oxide production will be monitored.

**SPECIFIC AIM #1: To institute a prospective, double-blind, placebo controlled trial in SCD patients hospitalized with VOC to determine if oral arginine therapy will decrease the length of hospitalization for VOC, and if this occurs by virtue of its effect on NO.**

***Primary Outcome Measure:*** *The primary outcome measure to be compared between those patients randomized to arginine and those to placebo will be the* ***lengths of hospitalization****. Arginine will be said to be an effective therapy in SCD patients with VOC if the length of hospitalization (in days) can be reduced by 35%.*

***Secondary Outcome Measures:***
*1. Length of parenteral narcotic use (in hours)*
*2. Amount of parenteral narcotic use*
*3. Pain scores*

***General Study Design***

I propose a double-blinded, placebo controlled trial of the effects of oral arginine on SCD patients hospitalized with VOC. A total of 56 patients will be enrolled (see *statistical analysis* at the end of this section). Patients will be randomized to receive either placebo or arginine. Physicians and staff involved in patient care will be unaware of who receives placebo vs. arginine. Patients will be treated for 5 days or until discharge, whichever is shorter. In light of my preliminary data, oral arginine will be administered to SCD patients with VOC at dose of 0.1g/kg TID, a dose and schedule that is consistent with recent clinical trials of oral arginine [14,18,20,21,23].

Sickle cell patients admitted to the hospital with vaso-occlusive crisis will be approached about enrollment into the trial according to the following criteria:

***Inclusion Criteria***

- Established diagnosis of sickle cell disease (Hb SS)
- $\geq$ 8 years of age, $\leq$ 21 years of age
- Pain requiring hospitalization for parenteral narcotics (Morphine or Demerol), not attributable to non-sickle cell causes.

***Exclusion Criteria***

- Hemoglobin less than 5 gm/dL or immediate need for red cell transfusion
- Evidence of acute chest syndrome
- Hepatic dysfunction: increase in SGPT to > 2X normal value
- Renal dysfunction: increase in creatinine to >2X normal value or >1.5
- Mental status or neurological changes
- Pregnancy

- >10 hospitalizations per year or history of dependence to narcotics
- Inability to take oral medications or allergy to arginine
- Inability to use a PCA device

For patients meeting the above criteria, average length of hospitalization will be calculated for the last 5 admissions for VOC. Only patients whose mean length of hospitalization for uncomplicated VOC is 6 $\pm$ 3 days and who have not had a hospitalization for uncomplicated VOC greater than 14 days will be eligible for enrollment. This will help ensure the enrollment of a population of VOC patients whose history of VOC is similar and who do not have a history of prolonged admissions. Patients meeting these criteria and who agree to participate will be enrolled and receive their first dose of arginine or placebo within 24 hours of being admitted.

***Randomization and Arginine/Placebo Administration***

Arginine/Placebo will be administered in a blinded fashion. The pharmacist will perform the randomization and will allocate 5 days of arginine/placebo to the patient. The patient, nurses, and physicians caring for the patient will be blinded as to which medication the patient is receiving.

***Administration of Analgesics to Study Patients***

Patient Controlled Analgesia (PCA) devices will be used in all patients. Intravenous ketorolac or oral NSAIDS may be used in addition to narcotics delivered by the PCA. Dosing of the PCA will follow a standard protocol of pain management already in place at _____. Patients are placed on PCA immediately upon admission to the hospital. Dosage changes in the first 12 hours are made to ensure adequate pain control without excessive somnolence. Thereafter, no changes are made in PCA dosing for 48 hours unless the pain score falls below 4, rises above 8, or there is an acute change in the patient's condition (e.g. development of ACS). After 48 hours of adequate pain control, the one hour maximum of narcotics is weaned 20% per day. When the PCA one hour maximum dose reaches 0.05mg/kg/hour, or at the request of the patient, the patient is switched to oral acetaminophen with codeine and the PCA is discontinued. If the switch to oral narcotics is tolerated, the patient is discharged within 24 hours of starting oral narcotics.

***Monitoring During Study***

Patients will be examined daily and will undergo routine vital signs including blood pressure every 4 hours. A pain score questionnaire will be completed by the patient every shift. A symptom questionnaire to assess for side effects of arginine will be completed by the physician after questioning of the patient (see appendix). Use of parenteral narcotics will be documented every shift and will include narcotics delivered by the PCA device as well as any additional intravenous and oral narcotics used (see appendix).

***Pain Scores***

Pain scores are routinely done on SCD patients admitted for VOC, using the Adolescent Pediatric Pain Tool (APPT)[66]. It is a one page, two sided instrument that is a validated and reliable means to assess pain in the children 8 - 21 years of age. It utilizes a front and back body outline, a 100mm word-graphic-rating scale, and a pain descriptor list. Time for APPT completion ranges from 3.2 to 6.4 minutes. Each of the three components of the APPT is scored separately (see appendix).

Laboratory monitoring will be undertaken according to the schedule below (Table 3):

| | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5* | F/U |
|---|---|---|---|---|---|---|
| CBC | X | X | X | X | X | X |
| CHEM20 | X | | X | | X | X |
| $NO_x$ (Serum and Exhaled) | X | X | X | X | X | X |
| AMINO ACIDS: ARG/CIT/LYS | X | X | X | X | X | X |
| U/A | X | | X | | X | X |
| URINE ONOO- & $NO_x$ | X | X | X | X | X | X |
| HB ELECTRO | X | | | | X | |
| BHCG | X | | | | | |
| RESEARCH LABS | X | X | X | X | X | X |

*Or through day of discharge

*Justification of laboratory tests*

*CBC*: Following possible changes in Hb levels, also following NO effects on platelet count.

*Chem 20*: Toxicity monitoring of potential changes in BUN/creatinine as a result of a high protein load and following liver function tests as arginine is metabolized by the liver.

*Urine analysis*: Following potential development of protein in the urine. Urine will also be used to determine presence of peroxynitrite (ONOO-) and $NO_x$.

> *Urinary ONOO- (peroxynitrite*): Nitrotyrosine is also excreted in the urine and will be correlated with serum nitrotyrosine levels as a marker for potential toxicity.
>
> *Urinary $NO_x$:* Nitrites and Nitrates are also excreted in the urine. Such information, together with serum $NO_x$, and exhaled $NO_x$ will help to determine total body $NO_x$.

*Hb Electrophoresis*: Confirm SCD status, to determine %S and %fetal hemoglobin before/after arginine supplementation

*Research Labs*: See Specific Aim 3

*Patient Removal from Study/Toxicity Monitoring*

Patients will be removed from the study if they meet the following criteria:

1) Development of acute chest syndrome
2) Neurological dysfunction
3) Increase in SGPT to > 2X normal value
4) Increase in creatinine to >2X value at study entry or >1.5
5) Decrease in hemoglobin to < 5 gm/dL or need for transfusion
6) Increase in met-hemoglobin > 2X normal value
7) Development of priapism
8) Refusal or inability to take study medication (arginine/placebo)
9) Patient's request

Data from patients removed from the study will be collected and analyzed for potential side effects or toxicities of arginine. Blood analysis and exhaled $NO_x$ measurements will continue per protocol, but arginine/placebo supplementation will be terminated.

*Sample Size and Statistical Analysis*

Over a 1-year period (1998), 209 children $\geq$ 8 years old were seen in the ED for VOC. Of these, 137 (66%) were admitted to the hospital for treatment of their pain crisis. The average length of stay was $6 \pm 3$ days. Approximately 10% of these patients would be excluded due to histories of prolonged hospitalization. Nearly 120 patients/year with SCD admitted for VOC will be eligible for this study.

Discharge information on 150 recent patients admitted to _____ for VOC was used to develop a power calculation. Five thousand datasets were simulated from the distribution of length of stay for VOC. A placebo group was simulated from the historical data. A $NO_x$ group was simulated with the same shape but with a median stay decreased by 35%. Using the Wilcoxon rank-sum test to compare the groups, a sample size of 28 subjects per group had 82% power to detect a treatment difference on a two-sided 0.05 level (Wilcoxon) test.

**SPECIFIC AIM #2: To characterize the biochemical and cellular effects of oral arginine administration in SCD patients with VOC.**

***Primary Objective:*** *The purpose of Specific Aim #1 is to conduct a clinical trial to determine if oral arginine can favorably alter the clinical course of vaso-occlusive crisis. Specific aim #2, however, entails an attempt to delineate the biochemical and cellular effects of the upregulation of nitric oxide production resulting from oral arginine administration.*

***Secondary Objectives****:*
*1. Follow patterns of plasma amino acids (arginine, citrulline, and lysine) and serum NOx during the course of hospitalization*
*2. Observe effects of oral arginine on exhaled $NO_x$ during VOC, and determine correlation to serum $NO_x$ and clinical course*
*3. Monitor for toxicity/side effects*

The studies outlined under this specific aim will be undertaken throughout the first specific aim. Blood for the biochemical investigations will be drawn at the time of the clinical labs as indicated in the tables above ("RESEARCH LABS"). Namely, preliminary investigations into the physiologic effects of increased nitric oxide production will be assessed. Subsequently, changes seen with arginine administration in VOC patients will be compared with the changes seen in the patients who receive placebo. Investigations into the biochemical and cellular effects of arginine administration and subsequent nitric oxide upregulation will center on four areas:

1. *Oxidative Balance and Damage*
2. *Vasoregulatory Changes*
3. *RBC Effects*
4. *Platelet Function*

1*. Oxidative Balance and Damage*

Nitric oxide can have _____ favorable or deleterious effects, depending on the environment in which it is produced. It becomes crucial, therefore, to measure other components which play an active role in maintaining oxidative balance.

- serum nitric oxide metabolites: nitrite and nitrate ($NO_x$)
- exhaled nitric oxide
- peroxynitrite (nitrotyrosine)
- 8-*iso*-$PGF_{2\alpha}$

***Serum and Urine Nitric Oxide Metabolites***: Although measurements of serum $NO_x$ do not necessarily represent biologically active nitric oxide, this assay is routinely used to measure estimated nitric oxide levels in human studies[9,67]. $NO_x$ can also be measured in urine[68]. The combination or serum, exhaled and urinary $NO_x$ may give us a better idea of total body nitric oxide metabolism.

My preliminary serum $NO_x$ data was obtained using the Griess reaction method. Measurements of serum and urine $NO_x$ by the Sievers NOAnalyzer offers a significant advantage over the Griess reaction by immediate results. My preliminary data demonstrates that low arginine levels may be predictive for need of hospitalization during VOC [28]. I have also demonstrated that children $\geq$ 10 years old with VOC had $NO_x$ levels that were significantly lower than baseline upon presentation to the ED. Such information is useful to the emergency medicine physician only if it is available rapidly enough to impact the evaluation and treatment plan. The availability of real time serum $NO_x$ levels may create a role for the measurement of serum $NO_x$ levels in older children or adults with VOC in the future.

***Exhaled Nitric Oxide***: Nitric oxide is easily measured in exhaled air, using microprocessor-based chemiluminescent NOx analytical instrumentation. Such technology has been studied in animal models[35] as well as in human studies[32,33,53,69], most frequently in asthma research[31,70]. It has been successfully studied in children,[31,70] and is easier to perform by children then spirometry.[80]

Studies have indicated that oral L-Arg increases serum and exhaled $NO_x$ levels in non-SCD adults[53]. In this study, increases in exhaled $NO_x$ persisted proportionally to increasing doses of oral arginine, while the rise in serum NOx maximized at an arginine dose of 0.1gm/kg. An exhaled NOx analyzer may allow for the measurement of changes in NO metabolism that are not detected by serum $NO_x$ levels alone, and may add significant information to my study.

Other _____ have documented that anemic animals have elevated exhaled $NO_x$ levels at baseline[35]. Patients with SCD have high serum NOx levels at baseline. Although exhaled $NO_x$ has never been evaluated in SCD patients, no one has yet attempted to correlate serum $NO_x$ levels with exhaled $NO_x$ levels. My preliminary studies demonstrate that $NO_x$ levels drop during VOC, with lowest levels found in ACS patients within 24 hour of the ACS diagnosis, yet serum $NO_x$ levels are not instantaneously available. Possessing the ability to follow exhaled $NO_x$ levels would create another tool to monitor changes in $NO_x$ more globally, and as an immediate measure, may be helpful if it corresponds to clinical course. Thus, an exhaled $NO_x$ analyzer also has the potential to become a helpful tool in the ED for the evaluation of VOC.

***Peroxynitrite*:** To be followed as a marker for possible toxicity, as a product of nitric oxide metabolism in the presence of superoxide. High levels may support a role for superoxide scavengers such as Vitamin C in SCD in future studies.

***8-iso-$PGF_{2\alpha}$:*** To be followed as a quantitative marker of oxidative stress in vivo, [71,72] as increased NO production with its many possible pathways may have unforeseen oxidative side effects that need to be considered.

*Potential limitations:* While I recognize that this is not an exhaustive determination of the fate of nitric oxide, it will enable me to make some preliminary statements about how an increase in nitric oxide will impact the production of potentially harmful oxidants. There is much controversy surrounding measurement of nitric oxide metabolites. It is unknown what percentage of a measured level of $NO_x$ is biologically active, thus the

meaning of individual levels may be questioned. I will be measuring $NO_x$ in serum, urine and in exhaled air in order to determine if there is any correlation of levels between mediums, and to clinical course. Serum $NO_x$ levels are effected by a patient's hydration status and renal clearance. It is also possible that urinary and exhaled $NO_x$ may represent nitric oxide activity specific to the kidneys or airway _____ than reflecting the molecules involved in vascular tone, and measurements may be effected by changes in renal function or illness. Exhaled $NO_x$ measurements may also be skewed by upper airway contamination, and reliable analysis may be difficult for young children. Evaluating patients greater than 8 years of age will help to limit this problem. These variables will be taken into consideration when interpreting the data. A correlation of this data to changes in platelet function may also allow me to determine whether any of these $NO_x$ levels reflect bioactivity. My primary outcome measure for this clinical trial is *length of hospitalization*, hence it will not be affected by these limitations. Yet information regarding the impact of oral arginine on the patterns of nitric oxide metabolites during VOC may still give some insight into the relationship of nitric oxide to sickle cell pain crisis.

2. *Vasoregulatory Changes*

**Endothelin-1 (ET-1):** In addition to following changes in $NO_x$ levels, I will attempt to determine the effects of oral arginine on levels of the potent vasoconstrictor ET-1. The relationship of $NO_x$ to ET-1 regulation has been previously discussed. An addition benefit of supplemental L-Arg in SCD may result from the potential inhibition of ET-1 release.

3*. RBC Effects*

Nitric oxide is known to affect many cellular elements. Since NO directly binds to hemoglobin, changes in NO concentration could conceivably affect the sickle RBC in an unpredictable manner. We will assess for changes in RBC membrane, hydration, and hemoglobin using the following:

- P50—measures changes in oxygen affinity
- Met Hemoglobin production
- Ektacytometry--measures red cell deformability
- H3 Technicon—measures red cell hydration

**P50:** Recent studies have shown that inhaled NO normalized P50 in SCD patients. If changes in NO levels have an impact on oxygen affinity, changes in P50 may indicate biologically active nitric oxide production, and is a potential benefit of arginine therapy

**Met hemoglobin:** Although we have seen no changes in met hemoglobin levels, its formation is a theoretical toxicity of increased NO production, and should be followed.

**Ektacytometry and H3 Technicon:** The effects of NO on the RBC membrane and hydration status by these methods have never been investigated, but oxidative stress can lead to decreased red cell deformability, as well as water loss. These tests will be preliminarily preformed on the first 20 patients enrolled in the protocol. If effects are detected, I will continue to perform these tests on the remainder of the patients enrolled in the clinical trial. If no differences are noted, these tests will be dropped from the protocol.

4*. Platelet Function*

Nitric oxide effects nearly every aspect of platelet function including activation, adhesion, and aggregation.[14] Aberrant platelet function found in SCD may be related to alterations in NO production. The impact of nitric oxide on platelet function could help ameliorate vaso-occlusion via its platelet effects. In addition, platelet function changes will likely decrease thrombin generation which is increased in sickle cell disease and

upregulates the expression of adhesion receptors on the vascular endothelium. Alternatively, increased nitric oxide production could be deleterious in that it may lead to a higher risk of bleeding with sustained increases, a risk that has been demonstrated in neonates receiving inhaled NO therapy [4].

The current diagnostic tests used to assess platelet function are the bleeding time and platelet aggregometry tests. Several investigators have used platelet aggregation tests to measure NO activity [36,73]. As serum $NO_x$ levels do not necessary reflect biologically active NO, the determination of changes in platelet aggregation represents a method to correlate $NO_x$ levels with NO activity. Both bleeding time measurements and platelet aggregation tests have drawbacks: the bleeding-time test is time-consuming and can cause considerable patient discomfort, and many experts question its reliability. Aggregometry is time-consuming, labor intensive, and costly. The PFA-100, is a new instrument which is simple to use and could be helpful in initial determinations of the impact of increased nitric oxide production on platelet function. The PFA system is an *in vitro* test system capable of detecting platelet dysfunction in a small whole blood sample. The system comprised of the PFA-100, instrument and disposable test cartridges emulates a damaged blood vessel to analyze the function of platelets in primary hemostasis. The PFA test cartridges used with the system are available with collagen/epinephrine for distinguishing normal from abnormal platelet function or with collagen/ADP to subsequently identify aspirin use. The system is easy to use, and allows for the automated assessment of platelet function. It measures the time to closure of a defined aperature under both ADP and epinephrine stimulation and has been shown to reflect platelet function in a variety of diseases. Results of the PFA-100 correlate with results of aggregometry and bleeding time tests.

Determination of the impact of supplemental L-Arg on platelet function is an important component to this investigation, as it will reflect effects from biologically active NO, independent of measured levels. Changes in platelet aggregation are potentially a significant mechanism of action by which arginine may benefit SCD patients with VOC, and should be included in the evaluation of cellular effect of upregulated nitric oxide production.

**Specific Laboratory Methods**

I. *Amino Acid Levels*

**Arginine, citrulline, and lysine:** Quantitative plasma amino acids are routinely done on patients being evaluated for metabolic disorders. L-Arginine, citrulline, and lysine are 3 of a total of 20 amino acids that are often evaluated. Plasma levels will be measured in μmol/L through ion exchange chromatography, done by Quest Diagnostics, Nichols Institute Biochemical Genetics Laboratory, San Juan Capistrano, CA. Amino acid reference ranges are available on normal children, run by Quest Diagnostics.

II. *Oxidative Balance and Damage*

**$NO_x$ :** In my preliminary data, the formation of nitric oxide has been measured in triplicate by determination of its stable end products in serum; nitrite ($NO_2$) and nitrate ($NO_3$) in μmol/L. Serum protein is precipitated by adding 100 μl of 30% zinc sulfate to 1 ml of serum. To 0.5 ml of supernatant, 0.5 ml of a 100 mM sodium tetrabotrate buffer, pH9.0 is added. Nitrate ($NO_3$) is reduced to nitrite ($NO_2$) using cadmium powder [74] in one aliquot. The amount of $NO_2$ is measured in the reduced and non reduced aliquots by determination of color development at 550nm after adding Griess reagent [75], the difference being $NO_3$. Aqueous solutions of sodium nitrate and nitrite are used as standards. The total amount of $NO_2$ and $NO_3$ in the serum samples is defined as $NO_x$. When the Sievers NOAnalyzer becomes available, I will measure $NO_x$ by the method describe below:

**NOAnalyzer:** $NO_x$ can be measured in serum, plasma or urine according to manufacturer's instructions, using Sievers NOAnalysis software for liquid sampling (Sievers Instruments, Inc., Denver, CO), as previously described.[81-83] Briefly, serum nitrite is measured by acidifying serum to a pH <2.0 to convert nitrite to NO. Serum nitrate is measured by incubating serum with Aspergillus nitrate reductase (Boehringer, Mannheim) to reduce nitrate into nitrite and then convert nitrite into NO by the addition of hydrochloric acid. The NO produced is then injected into the NO analyzer (Sievers, Inc), and the NO content of the sample is determined by measuring the luminescence generated in the presence of ozone. The luminescence measured is directly proportional to the amount of NO injected and, in turn, to the nitrite and nitrate content of the samples. Serum samples can be run immediately, or frozen for later analysis.

**Exhaled Nitric Oxide:** Exhaled nitric oxide will be measured in exhaled air, using microprocessor-based chemiluminescent $NO_x$ analytical instrumentation, manufactured by Sievers Instruments, Inc. (Denver, CO). The test is easily preformed and has been successfully used in many clinical trials [53,70,76]. Subjects inhale to total lung capacity from a reservoir bag through a one-way valve (Hans Rudolph, Kansas City, MO) with incoming NO-free air to ensure the absence of environmental NO. Next, the subjects exhale to residual volume into the Teflon tube, which enters into the NO analyzer. The subjects exhale at a pressure of +20 mmHg into the tubing connected to the analyzer. Exhalation at this expiratory pressure without a nose clip is a maneuver that closes the velum of the posterior nasopharynx and excludes contamination by nasal NO.[84] Results are immediately available. I will perform the tests, and the results will not be revealed to the patient or the medical staff providing care for the patient. The cost for this equipment has been placed in the initial budget period of this grant proposal.

**Peroxynitrite:** Peroxynitrite (ONOO-) formation will be assessed in plasma by the presence of the stable end product of its interaction with cellular tyrosine residues, 3-nitrotyrosine, via ELISA per the manufacturer's protocol (Upstate Biotechnology). Incubated samples, controls and standards are diluted in 100nM carbonate-bicarbonate coating buffer for 1 hour at 37° Celsius. Plates are washed three times with phosphate buffered saline (PBS). After a 1 hour incubation with the blocking agent, 1% bovine serum albumin (BSA), samples are incubated with polyclonal rabbit anti-nitrotyrosine antibody for 90 minutes, followed by a goat anti-rabbit IgG antibody coupled with the color development solution, pNitrophenyl phosphate in 10% diethanolamine buffer. Nitrotyrosine is measured spectrophotometrically at 405nm. [77]

**8-*iso*-$PGF_{2\alpha}$:** Urinary 8-*iso*-$PGF_{2\alpha}$ will be assayed by gas chromatography/mass spectrometry as previously described [71,72]. Briefly, urine samples will be collected in polyethylene bottles containing 0.1% of the antioxidant butylated hydroxyanisole, and stored at -20° Celsius. A known amount of the internal standard ($^{18}O_2$)-epi-$PGF_{2\alpha}$ will be added to each sample. After solid-phase extraction, the sample will be applied to a silica gel TLC plate in 25$\mu$L methanol and developed with a mobile phase of 90% ethyl acetate, 10% methanol, and 0.1% acetic acid. Next, the pentafluorobenzyl ester derivative is applied to a second TLC plate in 25$\mu$L ethyl acetate and developed with a mobile phase of 100% ethyl acetate. After incubation of the N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide derivative at 50° Celsius for 24 hours, each sample is run on a gas chromatography/mass spectrometer. Quantification is performed with peak area ratio. Urinary creatinines are determined by a standardized automated colorimetric assay with a Beckman Synchron CX system. Samples are expressed as picomoles per millimole of creatinine.

III. *Vasoactive Molecules*

**Endothelin-1:** ET-1 levels will be determined in triplicate in serum by enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's instructions (Amersham, UK). Briefly, each kit uses a quantitative immunometric sandwich ELISA performed in a 96 well plate coated with

monoclonal antibody against the ET-1 peptide and is incubated overnight at 4 degrees Celsius. A monospecific antibody conjugated to horseradish peroxidase is added. During an additional incubation of 30 minutes, the conjugate antibody binds to the affixed antibody/peptide. Upon addition of the substrate, a peroxidase-dependent color reaction occurs that is proportional to the amount of bound ET-1. The sample can be read at 630 nm, or the samples can undergo a 30 minute acidification by the addition of sulphuric acid into each well (preferred method, and the one I will employ), and the optical density can be read at 450 nm.

IV. *RBC Effects*

**P50:** . P50 is the partial pressure of oxygen at which hemoglobin is 50% saturated with oxygen, and is a test routinely done using a Hemox-Analyzer (TCS Medical Products, PA), to measure the oxygen dissociation curve. [78]

**Met-hemoglobin production:** Met-Hb levels are routinely run on venous blood using a CO-Oximeter in the pulmonary function laboratory at CHO by trained respiratory therapists.

**Ektacytometry:** _____ is one of the few laboratories in the country performing this analysis. Intact red blood cells (RBCs) are studied using osmotic gradient ektacytometry by a previously described method. [79] RBC deformability is measured as a function of osmolality of the suspending medium. For each sample, the degree of maximal deformability ($DI_{max}$), the hypotonic osmolality at which minimal deformability is found and hypertonic osmolality at which the DI = ½ $Di_{max}$ is recorded

**H3 Technicon:** H3 Technicon measurements will be done following manufacturer's protocol. Briefly, whole blood will be collected and stored refrigerated. Samples will be run within 72 hours of collection. Samples and controls are mixed with an oxazine Working Dye solution, which stains cellular RNA. They are then incubated at room temperature for 15 minutes. Samples are run on the H3 analyzer (Technicon, NY), and analyzed using a special computer program available at CHORI. This instrument will identify changes in red cell hydration status.

V. *Platelet function*

**PFA-100:** Platelet function will be measured with the PFA-100. Whole blood is collected into a buffered sodium citrate tube and may be stored at room temperature for up to 4 hours. The method is followed per manufacturer's instruction (Dade Behring Inc). 800μl of blood are placed onto the test cartridge, after which the cartridge is placed in the cassette and snapped closed. The cassette with the test cartridge is then placed into the incubation wells of the PFA-100 instrument, which then performs the test. Results are available in 5 minutes. The cost for this equipment has been placed in the initial budget period of this grant proposal.

**Research Timetable:** The didactic portion of this grant application will be fulfilled throughout the 5 years of the Research Career Award as described in the career development section. Specific Aim 1, the clinical trial, will take place throughout years 1-5. By the fifth year, the clinical trial should come to completion, and data analysis will be undertaken during the remaining 6-12 months of the award.

| | Year 1 | Year 2 | Year 3 | Year 4 | Year 5 |
|---|---|---|---|---|---|
| **Didactic Phase** | XXX | XXX | X | X | X |
| **Aim 1: Clinical trial** *Start-up* | XXXX | | | | |
| *Patient Enrollment* | X | XXXX | XXXX | XXXX | X |
| *Close-out* | | | | | XX |
| *Data Analysis* | | | | | XXX |
| **Aim 2: Research labs** *Lab evaluation* | X | XX | XXXX | XXXX | |
| *Data Analysis* | | | | XX | XXXXX |

**e. Human Subjects**

Human subjects, specifically children and young adults 8 -21 years old with SCD will be included in this study. Patients will be recruited from patients followed regularly at the _____. Nationally, SCD is a disease seen almost entirely in the African-American population and affects both male and female patients equally. The population of SCD at the _____ is overwhelmingly African-American and equally represented by both genders. There is no reason to assume differences in outcomes according to gender or ethnicity. Although equal recruitment of both males and females will be attempted, all data will be analyzed together. Also, non-African American SCD patients who meet eligibility criteria will be included in the study and every attempt will be made to recruit these patients.

1. Peripheral blood will be required from study patients for serum, plasma and whole blood studies. This blood will generally be obtained at the time of other routine phlebotomies so the patient should experience no additional discomfort. Patients enrolled for Specific Aims 1 of this proposal will, however, need additional samples that would not routinely be drawn.

2. Patients with SCD at baseline and those with vaso-occlusive crisis meeting entry criteria will be approached concerning entrance into the clinical trial. The principal investigator will explain the study to the patients and their families and will obtain consent on all patients. Patients and/or parents will sign an IRB-approved consent form. Patients are free to exit the study at any point.

3. Blood samples for this study will be drawn at the time of other phlebotomies whenever possible to limit discomfort to the patient. For patients enrolled in the Specific Aims 1 portion of this proposal, discomfort from an venipuncture during placement of the intravenous catheter will be experienced, and bruising from the blood draws are a potential risk to the patient. For patients enrolled in the clinical trial, the risks of the study are confined to those associated with the oral arginine administration. As with any food or medication, the risk of an allergic reaction is a small but possible complication. Arginine is a nutritional supplement with a low toxicity and has been safely used in many human studies. Mild stomach discomfort may be experienced at higher doses. Patients with compromised liver or kidney functions may not tolerate the high protein load, and are therefore excluded from participation. Priapism is also a theoretical toxicity of increased nitric oxide production in male participants, as nitric oxide is an important component in erection physiology. All patients enrolled in the study will also receive the standard treatment for vaso-occlusive crisis offered at _____.

4. Parents will be informed that they and their child may decline to participate in this study, and that their decision will have no effect on their child's care. Alternative treatments include the standard treatment for vaso-occlusive crisis without arginine or placebo.

5. Patient confidentiality will be protected as much as possible during the on-going clinical trial, and strictly for inclusion in grant proposals, abstracts and publications. Samples will be assigned a code that is known only to the investigator, _____.

6. The risks of this study are reasonable because of the significant benefits it may provide for patients with sickle cell disease. As a safe, nontoxic dietary supplement, oral arginine may prove beneficial for SCD patients by resulting in an increase in nitric oxide production, and is potentially a new therapy for the treatment of vaso-occlusive crisis. This trial could result in a new treatment that shortens hospital days, and decreases pain during vaso-occlusive events. It may be the foundation for future studies expanding arginine therapy as a prophylactic measure. The benefits of this study may impact significantly on the SCD population as a whole.

**GENDER AND MINORITY INCLUSION TABLES-- SICKLE CELL DISEASE**

A. National Demographics for Disease

| | American Indian or Alaskan Native | Asian or Pacific Islander | Black, not of Hispanic origin | Hispanic | White, not of Hispanic origin | Other or Unknown | TOTAL |
|---|---|---|---|---|---|---|---|
| Female | 0 | 0 | 36,375 | 375 | 375 | 375 | 37,500 |
| Male | 0 | 0 | 36,375 | 375 | 375 | 375 | 37,500 |
| Total | 0 | 0 | 72,750 | 750 | 750 | 750 | 75,000 |

B. _____ Patient Population Available (%)

| | American Indian or Alaskan Native | Asian or Pacific Islander | Black, not of Hispanic origin | Hispanic | White, not of Hispanic origin | Other or Unknown | TOTAL |
|---|---|---|---|---|---|---|---|
| Female | 0 | 0 | # | # | # | # | # |
| Male | 0 | 0 | # | # | # | # | # |
| Total | 0 | 0 | # | # | # | # | # |

There is no difference with respect to ethnicity or gender between the national and _____ populations.

C. Planned Enrollment

| | American Indian or Alaskan Native | Asian or Pacific Islander | Black, not of Hispanic origin | Hispanic | White, not of Hispanic origin | Other or Unknown | TOTAL |
|---|---|---|---|---|---|---|---|
| Female | 0 | 0 | 28 | <1 | <1 | <1 | 28 |
| Male | 0 | 0 | 28 | <1 | <1 | <1 | 28 |
| Total | 0 | 0 | 56 | <1 | <1 | <1 | 56 |

**Participation of Children**

There will be no exclusion of children from the clinical trial included in this application. Children comprise _____% of the sickle cell disease patients followed by the _____. Because this is the same population which will be recruited for entry into the clinical trial included in this grant application, and patients age 8 – 21 years will be enrolled, it is anticipated that children will represent the vast majority of study subjects. _____ is a board certified pediatrician, and _____ mentor is a board certified pediatric hematologist/oncologist. They therefore have expertise to care for children of all ages. _____ is a tertiary care center for children.

**f. Vertebrate Animals**

Not applicable.

**g. Literature Cited**

**h. Consortium/Contractual Arrangements**

Not applicable.

**i. Consultants**

**7. Appendix**